Bioaccumulation of microcystins in terrestrial food plants and the development of novel sorbents for monitoring microcystins in irrigation water

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Declaration

I Pindihama Glynn Kuziva hereby declare that the thesis for the Doctor of Philosophy in Environmental Sciences research project titled “**Bioaccumulation of microcystins in terrestrial food plants and the development of novel sorbents for monitoring microcystins in irrigation water”** at the University of Venda, hereby submitted by me, has not been submitted previously for an award of any degree at this or any other University, that it is my work in design, execution and writing, and that and that all reference materials contained therein have been duly acknowledged.

Pindihama Glynn Kuziva

Signature: Pindihama Date: 11/04/2023

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Above all, praises to the Almighty God, with whom all things are possible.

Dedication

This work is dedicated to my mother, Ms. Miriam Nyadzai Chabikwa and my late grandmother Ms. Cecilia Chabikwa (May her soul continue to rest in eternal peace).

Abstract

In the past few decades, South Africa's freshwater resources have faced a severe problem of eutrophication and frequent occurrence of cyanobacterial blooms. This is of great concern since some of the cyanobacteria species have the potential to produce cyanotoxins that pose health risks to livestock and humans. Recently, there have been evidence of the toxic effects of microcystins (MCs) and cylindrospermopsin (CYN) on terrestrial plants including plants used for food. Ever since, the use of surface waters contaminated with cyanobacteria and cyanotoxins for agricultural purposes has been receiving growing attention. In hypereutrophic waters, such as commonly found in impoundments around major metros in South Africa, MCs co-exist with other pollutants such as metals and Linear Alkylbenzene Sulfonates (LAS), and these can have synergistic effects on the crops irrigated by such waters and exacerbate the human health risks posed by MCs. The current study investigated the bioaccumulation and effects of MCs on food plants, developed and evaluated the use of crosslinked chitosan (sorbents) passive samplers to monitor the bioavailability of MCs in water intended for irrigation. The findings are presented in six major sections.

The first section detailed literature on the impacts of cyanotoxins on food plants with emphasis on the South African context. The reviewed literature demonstrated the potential of exposure of irrigated plants to multiple stressors such as MCs, LAS, metals, endocrine disrupting chemicals (EDCs) and high levels of dissolved salts around South Africa’s urban provinces. The literature also showed that South Africa lacks research on human exposure to cyanotoxins via irrigated crops and regulations to manage emerging pollutants such as MCs in irrigation water. Such lack of data and policies thus prompts an urgent need for local evidence-based research to guide policies and guidelines on cyanotoxins in irrigation water, food plants and, water used for livestock.

In the second part of the thesis, the bioaccumulation of MCs in parts of the plants *Brassica oleracea* (cabbage) and *Solanum tuberosum* (common potato) was investigated in pot-culture experiments. Water used to irrigate the plants was collected from Roodeplaat Dam and had total MCs ranging from 0.12–2.84 µg L^{-1}. The pH for the water was slightly alkaline (pH 7.29±0.71 to 10.03±0.29) whilst the Electrical Conductivity (EC) ranged from 296.67±13.87 to 878.67±42.44 µS cm^{-1}. The findings showed that the two plants can bioaccumulate MCs to concerning levels when irrigated with water derived from Roodeplaat Dam. MCs accumulation levels in the two tested plants ranged from 0.00142 to 0.136 mg kg^{-1} DW for individual MC congeners. These findings demonstrated that terrestrial food crops can bioaccumulate MCs to levels that can pose human-health risks.

The third part of the thesis investigated the uptake and accumulation of metals Al, Mn and Sr in distinct parts of the plants *Brassica oleracea* and *Solanum tuberosum* in the presence of the anionic surfactant LAS. Pot-culture experiments were conducted to assess the effect of LAS on the accumulation of the three metals by watering the plant with Roodeplaat dam water containing 3.48 mg L^{-1} of LAS and Mn (0.257mgL^{-1}), Al (0.6mgL^{-1}) and Sr (0.16mgL^{-1}). Findings indicated that the presence of LAS, in the irrigation water, did not enhance the uptake of Sr, Mn and Al by the two plants as demonstrated by the comparable levels of the metals in plants exposed to the metals in presence and absence of LAS.

The fourth section investigated the uptake and the accumulation of MCs in distinct parts of the plants *B. oleracea* and *S. tuberosum* in the presence of the anionic surfactant LAS. Pot-culture experiments were conducted with the two plant species watered Roodeplaat dam water containing 3.48 mg L^{-1} of LAS and MCs with mean concentrations of: MC-LR: 10.47±3.879; MC-RR: 6.158± 4.127 and MC-YR: 8.160 ± 2.544 µg L^{-1}. Findings indicated that the presence of LAS in the irrigation water at the levels investigated did not enhance the uptake of MCs by the two plants, even though in some cases the levels of MCs bio-accumulated by the two plants exceeded WHO-recommended tolerable daily intake (TDI). These findings imply that, the tested levels of LAS and MCs did not have any synergic effects on the two plant species.

The fifth part of the thesis focused on the synthesis of a composite of glutaraldehyde-crosslinked chitosan and multiwalled carbon nanotubes (ChMWCNT) for application in the Solid Phase Adsorption Toxin Tracking (SPATT) for monitoring MCs in freshwaters. Batch experiments were conducted to evaluate the adsorption efficiency of the composite. The composite was found to be efficient in adsorbing MC-LR showing 97% removal and maximum adsorption capacity of 4.639 µg g^{-1} under optimized conditions of 5 µg L^{-1} of MC-LR, adsorbent dose of 0.03g 5 mL^{-1}, and contact time of 30 mins. For desorption, 100% methanol was most effective, with efficiency of 84.71 %. When applied for the adsorption of MCs in raw dam water, the composite was saturated within two days of exposure and effectively adsorbed and desorbed three congeners tested in the order MC-LR>MC-RR>MC-YR.

The last part of the thesis evaluated the field applicability of the newly synthesised glutaraldehyde crosslinked chitosan hydrogel (ChGLA) and the composite of glutaraldehyde-crosslinked chitosan and multiwalled carbon nanotubes (ChMWCNT) in a SPATT bag format and compare them to the commonly used DIAION HP20 resin. The constructed SPATT samplers were deployed in canals and farm dams around Roodeplaat and Hartbeespoort Dam sites. The findings elucidated a good correlation between the MCs detected by the two newly synthesized sorbents (ChGLA and ChMWCNT) and grab samples. The detected total MCs for the ChGLA samplers were: 0.003 and 1.742 (µg g^{-1}) and for the ChMWCNT samplers 0.006 and 3.300 (µg g^{-1}) (lowest and highest respectively).

In conclusion, the two plants were found to accumulate MCs to levels that can pose human-health risks when water derived from the Roodeplaat Dam was used for irrigation. The effects of other pollutants such LAS found in hypereutrophic water bodies on the uptake and accumulation of MCs in food plants was for the first times tested on terrestrial food plants. The findings showed that the presence of LAS had no impact on the uptake of MCs and heavy metals such as Al, Mn and Sr. For the first time, chitosan-based sorbents were synthesized and applied in SPATT for MCs. The use of the chitosan sorbents demonstrated a lot of promise for the monitoring of MCs in water and can possibly be used as an early warning sign for the presence of MCs in irrigation water derived from eutrophicated impoundments in South Africa.

Keywords: Microcystins, LAS, SPATT, chitosan, hypereutrophic, *Brassica oleracea*, *Solanum tuberosum*

Academic Output

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Conference presentations/papers

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Other scientific output

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List of Acronyms & Abbreviations

ANOVA:	Analysis of Variance
CYN:	Cylindrospermopsin
DWAF:	Department of Water Affairs and Forestry
EC:	Electrical Conductivity
ELISA:	Enzyme-Linked Immunosorbent Assay
GAC:	Granulated Activated Carbon
GST:	Glutathione S-transferase
HAB:	Harmful Algal Blooms
HLB:	Hydrophilic–Lipophilic Balance
HPLC:	High-Performance Liquid Chromatography
IARC:	International Agency for Research on Cancer
ISO:	International Organization for Standardization
LCMS:	Liquid Chromatography Mass Spectrometry (LCMS)
LAS:	Linear Alkylbenzene Sulfonates
MC:	Microcystins
MC-LR :	Microcystin-LR (Leucine-Arginine)
MC-RR :	Microcystin-RR (Arginine-Arginine)
MC-YR:	Microcystin-YR (Tyrosine-Arginine)
PAC:	Powdered Activated Carbon
PDA:	Photodiode Array
PVC:	Polyvinyl Chloride
RO:	Reverse Osmosis
SABS:	South African Bureau of Standards
SDS:	Sodium Dodecylbenzene Sulfonate
SANS:	South African National Standards
SOP:	Standard Operating Procedures
SPATT:	Solid Phase Adsorption Toxin Tracking
SPE:	Solid Phase Extraction
TDS:	Total Dissolved Solids
TFA:	Trifluoroacetic Acid
TWQR:	Target Water Quality Ranges
UNECE:	United Nations Economic Commission for Europe
USA:	United States of America
USEPA:	the United States Environmental Protection Agency
UV:	Ultraviolet
WHO:	World Health Organisation
WTP:	Wastewater Treatment Plants

CHAPTER 1: INTRODUCTION

1.1 Background

Eutrophication can be defined as ‘the enrichment of water by nutrients causing an accelerated growth of algae and higher forms of plant life to produce an undesirable disturbance to the balance of organisms present in the water and to the quality of the water concerned’ (Adams et al., 2020). The negative impacts of eutrophic and hypereutrophic waters include blooms of phytoplankton, dominance of cyanobacteria, depleted dissolved oxygen (anoxia), elevated fish deaths, biodiversity loss and loss of aesthetic value (Matthews & Bernard, 2015 ; Adams et al., 2020).

The challenges of harmful algal blooms (HABs) have been on a rapid increase globally due to the human induced climate change and the increased loads of nutrients into the aquatic environment as a result of anthropogenic activities (Nwankwegu et al., 2019). The impacts of the intensifying HABs have had detrimental impacts on a global scale affecting sectors like fisheries, tourism, public health, water monitoring and management. In Africa, the consequences and management challenges of eutrophication on lakes are not different from other regions and continents (Harding, 2015).

South Africa is known for having scarce and extremely limited water resources and depends mainly on surface water resources for most of its urban, industrial, and irrigation requirements. The importance of water as a critical resource to drive economic growth and development in the country cannot be overemphasised. Water is needed in sufficient quantities and quality to meet human needs, requirements for industries, agriculture and other industries such as tourism. However, South Africa’s scanty freshwater resources are susceptible to pollution and more so to eutrophication (Matthews & Bernard, 2015). In addition to the challenges of eutrophication, the country’s surface water resources are also threatened by acidic effluents, endocrine disrupting compound (EDCs), heavy metals and pesticides among other pollutants (Harding, 2015). This is particularly so in catchments where wastewater effluents and urban runoff contribute a relatively significant component of the water.

Agriculture plays a significant role in South Africa’s economy and the country largely depends on water stored in man-made reservoirs for the sustained supply of raw potable and irrigation water. In South Africa, irrigation is a common agricultural practice involving the use of water from public supply reservoirs, rivers, and ponds to irrigate crops. Unfortunately, these surface water sources are sometimes contaminated with cyanobacteria and cyanotoxins and recent studies have indicated that these may be taken up and bioaccumulated in plants tissues. This, makes the consumption of crops and vegetables irrigated with contaminated water a potentially dangerous route of human exposure to different cyanotoxins, including microcystins (MCs) in the country.

1.2 Statement of the problem

In South Africa, eutrophic conditions have been reported in 18 out of the 25 major catchments and about 28% of surface water was reported to be hypereutrophic and 33% to be eutrophic (Matthews & Bernard, 2015). Toxic cyanobacteria such as *Anabaena* and *Microcystis* species have also been reported in all the major impoundments and frequent poisonings of wildlife and livestock have been reported throughout the country (Matthews & Bernard, 2015), with MCs have been identified as the fifth most important toxin in areas around Mpumalanga and Gauteng (Rastogi et al., 2014).

Cyanotoxins have been shown to inhibit plant growth and development (Purkayastha et al., 2010) and recently there have been concerns of indirect exposure to the toxins via consumption of crop plants irrigated with contaminated water. In South Africa, Turton (2016) warned of a possible human health risk when the food is grown on farms near dams and rivers polluted with cyanotoxins. Besides such concerns, cyanotoxin contaminants in water utilized to irrigate food crops have not yet been considered within any official monitoring program on water quality in the country and very few studies have been conducted to assess the impacts of cyanotoxins on crops locally.

Linear Alkylbenzene Sulfonates (LAS), is a group of anionic surfactants commonly used in several important applications, most noticeably in detergents and laundry. Large quantities of laundry detergent ingredients enter the environment continuously, either through wastewater streams or as a direct result of detergent product use in, or near to, surface water bodies. Most reservoirs in the South Africa’s urban metros (as is the case in the Crocodile (West) and Marico catchment) are heavily impacted by discharge of improperly treated domestic water, industrial wastewater and mine discharges. The combined pollution of cyanotoxins, toxic metals and LAS in such eutrophic dams is highly likely since the LAS degradation process is reduced under anoxic conditions such as those found in hypereutrophic reservoirs (Wang et al., 2012). LAS is known to alter membrane permeability and in turn affect the toxicity and accumulation of other toxins such as cyanotoxins in organisms (Wang et al., 2012). However, the risk of multiple classes of cyanotoxins in combination with other stressors such as LAS and metal pollutants on terrestrial food plants has not been previously investigated in South Africa.

Despite the predicted increase in incidents of harmful cyanobacterial blooms and the toxins they produce, strategies for monitoring and managing them in South Africa tend to be reactionary and there is still a lack of initiative-taking early warning capabilities. Traditional monitoring programs of cyanotoxins are based on the collection of individual samples at specific single time points. These traditional sampling techniques have several drawbacks, such as the need for large volumes to recover sufficient mass of toxin or time and labor-consuming clean-up before instrumental analyses. Further, cyanotoxin concentrations may vary over time, and episodic peaks of high concentrations may be missed in the traditional monitoring schemes (Kohoutek et al., 2010).

To overcome the drawbacks of grab sampling, MacKenzie et al. (2004) came up with the solid-phase adsorption toxin tracking (SPATT) technology, for possible use in the detection and early warning of the presence of cyanotoxins. SPATT involves suspending small bags containing adsorbent which accumulate toxins in the water body. The toxins can then be extracted and measured, providing information on extracellular toxins over an extended period. Different sorbents have been used for SPATT (including the polymeric adsorbents DIAION HP20 to SEPABEADS type resins) for the accumulation of cyanobacterial toxins of different polarities (Wood et al., 2011). Although passive sampling has been successfully used several times to monitor cyanotoxins using different bulk polymeric sorbents (Wood et al., 2011), most of these sorbents are synthetic and relatively costly to buy. Studies on the characterization and mechanism of SPATT resins for the adsorption of lipophilic and hydrophilic cyanotoxins are also limited (Zhao et al., 2013).

1.3 Significance of the study

Human health risk assessment with regards to exposures to cyanotoxins requires the identification of common exposure routes, among which consumption of contaminated food has been recognized (Bittencourt-Oliveira et al., 2016). To prevent any potential risks, guideline values for cyanotoxins need to be reviewed in light of more recent research and publications particularly on potential exposure through food, and implemented into national regulations taking local exposure patterns into account (Drobac et al., 2013). The current study was motivated by the need to fill in this gap by evaluating the threats posed by using eutrophic waters for irrigating food crops and contribute to the development of policies on the use of such water for irrigation and the acceptability of such plants for human consumption.

The study developed chitosan-based sorbents for use as passive samplers of MCs in solid phase adsorption toxin tracking technology (SPATT). This given the drawbacks of grab-sampling and the relatively high costs of the commercial resins which in most cases are not made from environmental friendly materials. Chitosan is a low cost, natural and abundant polysaccharide which can be derived from a variety of sources such as seafood waste. Recent studies, for example Gomez-Maldonado et al. (2022) and Tran et al. (2022) have demonstrated that chitosan can be modified and be applied for the adsorption of MC-LR in water purification, but no studies have evaluated its use as a sorbent in the passive sampling of MCs in SPATT.

The thesis contributed to the understanding of the bioaccumulation of MCs by food plants under local conditions, the impact of multiple stressors such MCs, LAS and metals on irrigated crops and the possible human health impacts thereof. The thesis also contributed to the understanding of the possible applications of chitosan-based sorbents in the passive sampling of freshwater, to be used as an early warning tool for the presence of MCs in irrigation water.

1.2 Objectives

1.2.1 Overall objective

The study aimed at investigating the bioaccumulation and effects of MCs on food plants and to develop and evaluate the use of crosslinked chitosan (sorbents) passive samplers to monitor the bioavailability of MCs in water intended for irrigation.

1.2.2 Specific Objectives

The following were the specific objectives of the study:

- To conduct a literature survey on the impacts of cyanotoxins and other stressors in irrigation water on food plants.

- To investigate the bioaccumulation of MCs in edible plant organs in cabbage (*Brassica oleracea*) and cultivated potato (*Solanum tuberosum*).
- To investigate the impact of multiple stressors such as metals, LAS and MCs by examining the accumulation of MCs in the presence of the anionic surfactant (LAS) in cabbage (*Brassica oleracea*) and cultivated potato (*Solanum tuberosum*).
- To investigate the impact of multiple stressors by examining the accumulation of metals (Manganese, Aluminum & Strontium) in the presence of the anionic surfactant, LAS in cabbage (*Brassica oleracea*) and cultivated potato (*Solanum tuberosum*).
- To develop a crosslinked chitosan-based solid phase adsorption toxin tracking technology (SPATT) sorbent suitable for use in the monitoring of bioavailable microcystins in aquatic environments.
- To evaluate the field applicability of the developed crosslinked chitosan-based sorbent in SPATT samplers for the passive sampling of microcystins in water intended for farming and irrigation.

1.3 Hypothesis

- The levels of MCs in the water used for irrigating crops in the Crocodile (West) and Marico catchment area are high enough for MCs to bioaccumulate in the irrigated crops to exceed the World Health Organization (WHO) set tolerable daily intake (TDI) of (0.04 µg kg^{-1}).
- The co-existence of cyanobacteria and other pollutants such as LAS, enhances MCs uptake by plants.
- Chitosan has numerous amino ligands and hydroxyl groups on its surface (Sanford et al., 2012), which are active sites for the sorption of MCs.
- Crosslinking chitosan with glutaraldehyde reduces its disadvantage of swelling in aqueous solutions and the wide presence of hydroxyl groups (-OH) and primary amine ($-NH_2$) (adsorption sites) in chitosan are active sites for the sorption of MCs, making it an efficient adsorbent.
- Crosslinking chitosan and then insertion of multiwalled carbon nanotubes (CNTs), followed by freeze-drying increases its surface area and pore sizes, thus giving it better stability in aqueous solutions and improving its adsorption and desorption of MCs.

1.4 Underlying assumptions

Due to prevailing hypereutrophic conditions of the impoundments in the Crocodile (West) and Marico catchment area, the underlying assumptions for the study were:

- The water from Roodeplaat, Hartbeespoort, Rietvlei, and Bospoort dams was being used for irrigation purposes.
- There was a co-occurrence of multiple microcystins, at several locations in the study area.
- Together with microcystins, the irrigation water in the study area had elevated levels of LAS and toxic metals species.
- Soils and other environmental factors where pot-culture experiments were conducted resemble conditions in the Crocodile (West) and Marico catchment area.

1.5 Study area

The Crocodile (West) and Marico catchment is one of the most advanced and economically active Water Management Areas (WMAs) in South Africa. The Crocodile (West) and Marico WMA is found in the North-West Province and includes the northern part of Gauteng as well as the southwestern corner of Limpopo Province towards the northwest border with Botswana. The two main rivers in the WMA are the Crocodile and the Marico rivers which flow northwards to join the Limpopo River at their confluence (Stevens, 2007; DWA, 2012). Figures 1.1 and 1.2 show the location of the study area.

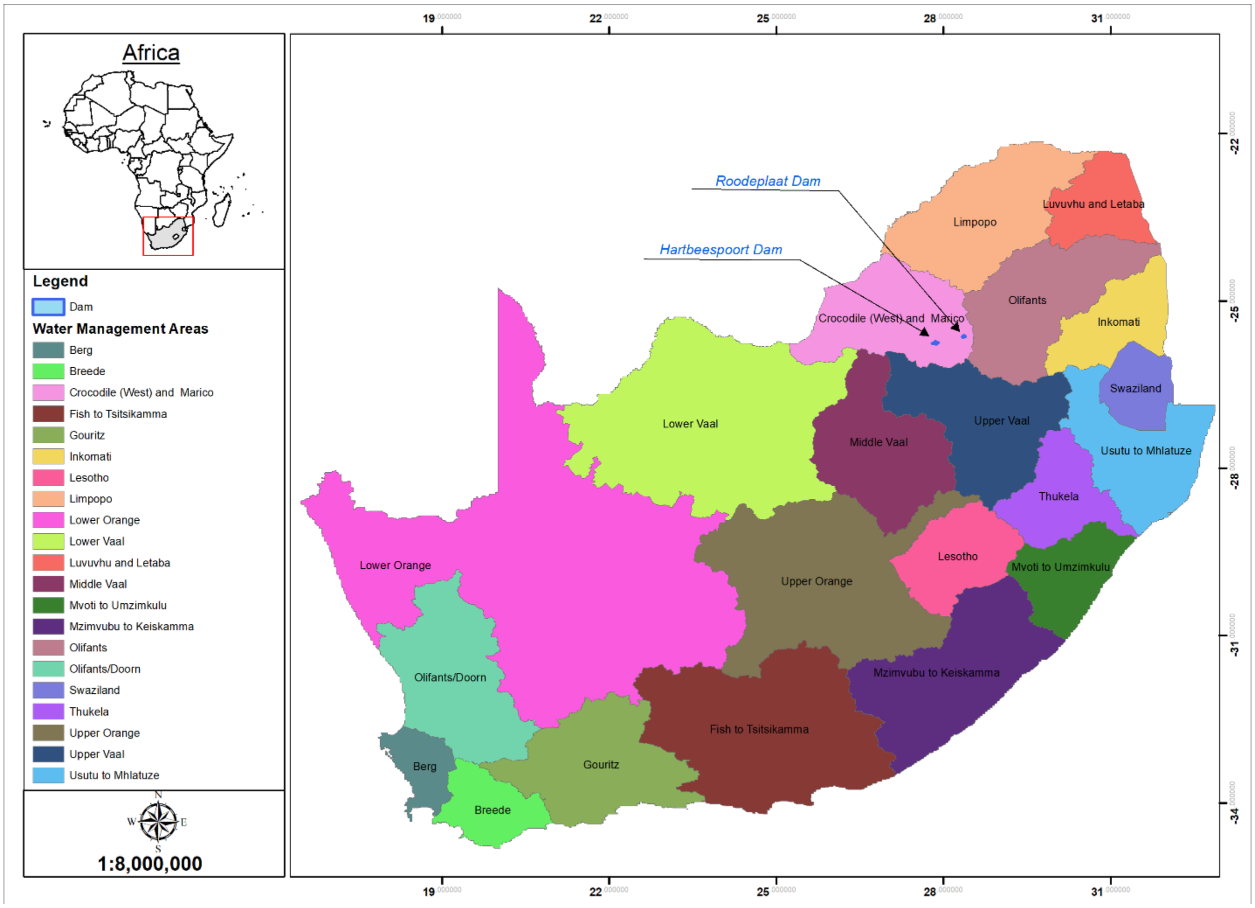

Figure 1. 1. Map of South Africa, showing the location of the Crocodile (West) and Marico WMA

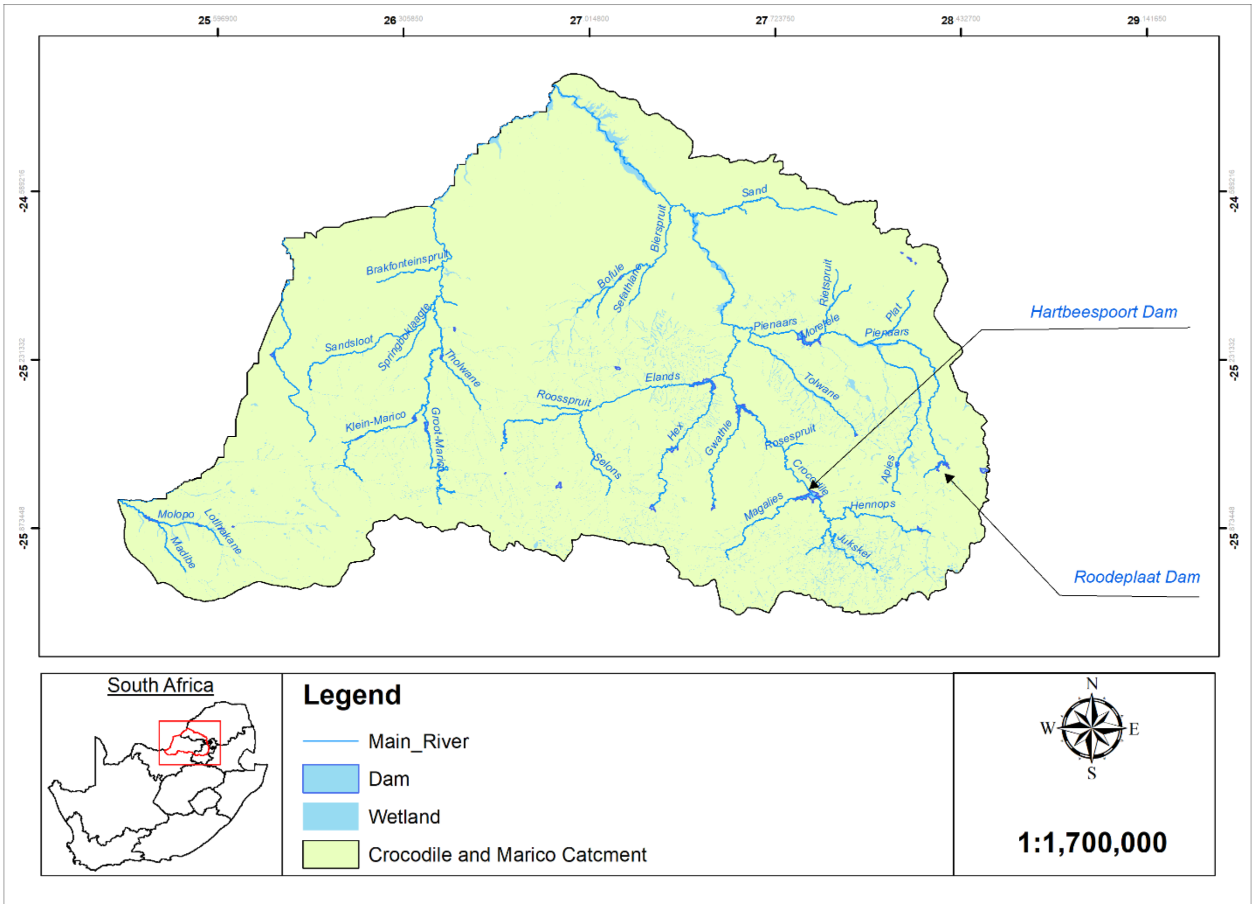

Figure 1. 2. Location of Hartbeespoort dam and Roodeplaat Dam in Crocodile (West) and Marico WMA

Agriculture accounts for about 33.5% of the total water use in the Crocodile (West) and Marico catchment and largely utilizes privately-owned dams and Government water schemes (DWA, 2012). According to DWA (2012), extensive irrigation development occurs along the Crocodile River and in the Brits /Rustenburg area. Farmers in this area produce mainly citrus, table grapes, and deciduous fruit as permanent crops. Cash crops like wheat and vegetables are produced during the winter and soybeans, vegetables and maize during summer months. The local citrus and grain cooperatives as well as the Northwest Department of Agriculture in Brits play a significant role in the irrigation management support services rendered to farmers. The deciduous fruit industry consists mainly of pome fruit (apples and pears), stone fruit (apricots, peaches & nectarines and plums) as well as table grapes (Stevens, 2007).

Hartbeespoort Dam supplies irrigation water through a 544 kilometres (338 mi) long network of canals to 159.76 square kilometres (61.68 sq mi) of farmland, on which tobacco, wheat, lucerne, fruit, and flowers are produced. Deteriorating water quality, most notably MCs contamination, is now causing much of the irrigated agriculture land to fall into disuse (Turton, 2016). The fact that most of the dams in this area are classified as eutrophic and 33.5% of the total use of water in this area is for agricultural purposes, made the Crocodile (West) Marico Water Management Area (WMA) an ideal study area.

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CHAPTER TWO: IMPACTS OF CYANOTOXINS AND OTHER EUTROPHICATION-RELATED STRESSORS ON FOOD PLANTS AND APPLICATION OF SPATT FOR MONITORING IRRIGATION WATER: A LITERATURE SURVEY

2.1 Introduction

Many South African rivers and reservoirs are now known to lack the resilience to take up nutrients and toxicants (Harding, 2015). The deterioration of freshwater resources in South Africa has been due to rapid population growth, urbanization, industrial and agricultural activities (Lu, 2018). Rising usage of detergents, pharmaceuticals and other chemicals in homes and industries led to elevated levels of phosphates than the receiving waters can assimilate.

Even though there is no formal structure in reporting and compliance when it comes to HABs in freshwaters in South Africa, Harding (2015), reported widespread and regular seasonal occurrences of HABs and limited data on the resultant toxins (cyanotoxins). Animal deaths as a result of cyanotoxins poisoning have also been reported for decades in the country (Harding, 2015).

According to Lu (2018), a plethora of research assessing the levels and the impacts of eutrophication and the resulting HABs has been conducted in South Africa, and this was led by the Department of Water Affairs monitoring programme of 1985. However, much of the data is inaccurate due to the exemptions which were being granted to local authorities for their failure to comply with phosphorous permissible thresholds (Lu, 2018).

In this literature survey, a study was conducted to assess the potential long-term threats of using cyanobacteria-infested waters for agricultural purposes in South Africa. In addition to cyanotoxins, the survey looked into other pollutants common to eutrophicated waters such as linear alkylbenzene sulfonate (LAS) which probably increases the uptake of cyanotoxins by plants. Recently published scientific literature which includes books, articles in journals, relevant government and agency documents, and grey literature was used to inform this report. Literature was accessed through google scholar, the google search engine, science direct and, Web of Science. The literature search prioritized scientific articles from studies done in South Africa and other African countries and articles published within the last five years, with older but relevant articles also included. (Lu, 2018)

2.2 Cyanobacterial toxins (cyanotoxins)

Cyanobacteria are photosynthetic prokaryotes that have existed naturally for billions of years, inhabiting a wide variety of aquatic environments, including freshwater, brackish and marine ecosystems, and can form dense blooms (Howard et al., 2017). Many cyanobacteria species are capable of producing toxins (Howard et al., 2017). The toxicity of a bloom depends on several factors with the most being the percentage of toxin producing strains over non-toxin-producing strains (Vasconcelos, 2015).

Cyanotoxins belong to a variety of categories, with each category having its structural analogy congeners. Depending on the human organ affected, cyanotoxins are classified as hepatotoxins (microcystins, nodularin, cylindrospermopsin), neurotoxins (saxitoxins, anatoxin-a, anatoxin-a(s), homoanatoxin-a), cytotoxins (aplysiatoxin, debromoaplysiatoxin, lingbyatoxin, lipopolipopolysaccharide), and skin and gastrointestinal irritants (Drobac et al., 2013). Table 2.1 shows the main cyanobacterial toxins, the genera that produce them, and their mechanism of action. The toxins have different chemical structures, for instance, Anatoxin-a (ATX) and Cylindrospermopsin (CYN) are alkaloids, whereas Microcystins (MCs) and Nodularins (NOD) are Poly cyclopeptides (Mashile & Nomngongo, 2017).

From this list, microcystins are among the most potent and commonly encountered (Tyler et al., 2009). According to Mashile and Nomngongo (2017), many studies have identified microcystins (LR, RR, LA, and YR) as the most frequently encountered cyanotoxins in natural waters with cylindrospermopin (CYN) emerging to be an important cyanotoxin in some regions. Among MCs, MC-LR is the widely reported and the more toxic congener commonly found in freshwaters (Bouaïcha & Corbel, 2016) and by far the most potent cyanotoxin (Mashile & Nomngongo, 2017).

Levels of MCs in surface waters range from 1 to 100 µg L^{-1} and the use of water infested with these toxins has been reported in several countries across the globe in countries like Australia, Canada, China, Holland, and the United States, with toxin levels in the range 0.3 to 80 µg L^{-1} reported (Shi et al., 2012). Ettoumi et al. (2011) reports that throughout the world microcystins

(MCs) are more commonly found in 50–75% of cyanobacterial blooms. Microcystins have also been reported in many African countries. According to Mashile and Nomngongo (2017), MCs have been reported in countries such as Kenya, Nigeria, and Uganda, among others. Species of *Microcystis*, *Anabaena*, *Cylindrospermopsis raciborskii* and *Plantolyngbya* have also been reported in Lake Victoria (Kimambo et al., 2019).

Table 2. 1. Main toxins from cyanobacteria, including genera of main producers and action mechanism (Mbiza, 2014)

Toxin	Cyanobacteria genera	Primary target organ in mammals
Hepatotoxins:		
Microcystin	*Microcystis, Anabaena, Planktothrix, (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis*	Liver
Nodularins	*Nodularia*	Liver
Cylindrospermopsins	*Cylindrospermopsis, Umezakia, Aphanizomenon, Raphidiopsis*	Liver, kidney, spleen, lungs
Neurotoxins:		
Anatoxins-a	*Anabaena, Oscillatoria, Aphanizomenon, Cylindrospermum*	Nerve synapse
Anatoxin-a(S)	*Anabaena*	Nerve synapse
Saxitoxins	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis*	Nerve axons
Dermatotoxins:		
Lyngbyatoxin-a	*Lyngbya, Schizotrix, Oscillatoria*	Skin, gastrointestinal tract
Aplysiatoxin	*Lyngbya, Schizotrix, Oscillatoria*	Skin
Endotoxins:		
Lipopolysaccharides	All cyanobacteria	Potential irritants affect any exposed tissue

In South Africa, incidents of cyanobacterial blooms have been reported across the country ((Matthews and Bernard, 2015; Ndlela et al., 2016). Among species reported across the country, *Anabaena* spp. and *Microcystis aeruginosa* are the dominant species. Other species such as the *Oscillatoria* spp. Have also been widely reported in certain parts of the country and *Cylindrospermopsis raciborskii* in the Northern part of the country (Ndlela et al., 2016). Microcystins have been identified as the fifth most important toxin in areas around Mpumalanga and Gauteng (Rastogi et al., 2014).

Several studies investigating their existence and prevalence have been done in the Hartbeespoort Dam, which is found in the Northwest Province (Mbukwa et al., 2012; Ballot et al., 2014; Turton 2015). Among such studies and reports Turton (2015) reported a shocking median concentration of 580 µg L^{-1}, a maximum concentration of 14 400 µg L^{-1}, and the lowest levels consistently exceeding 10 µg L^{-1} of MCs in the Hartbeespoort Dam in the period August 2003 to May 2004. Mbukwa et al. (2012), found MC-RR as the toxicologically dominant congener in Hartbeespoort Dam. Other congeners of MCs detected in the Dam were MC-LR, MC-YR, MC-WR, MC-(H4) YR, and (D-Asp3, Dha7) MC-RR and are all related to *M. aeruginosa* dominance (Mbukwa et al., 2012). A total of 10 microcystins (MC) variants have been described from Hartbeespoort Dam in different studies: MC-RR, MC-LR, MC-FR, MC-YR, MC-LA, MC-YA, MLABAba, MC-WR, MC-(H4)YR and [Asp3, Dha7]MC-RR (Ballot et al., 2014).

2.3 Impacts of cyanotoxins

Large cyanobacterial blooms are classified as “harmful” when they lead to negative environmental impacts such as mortality, ecosystem instability, and the production of highly active toxic compounds known as cyanotoxins (Drobac et al., 2013). These toxins are quite stable and can affect the environment and human health at different levels and can directly poison aquatic animals and mammals, birds, cattle, livestock, and pets that drink from contaminated reservoirs (Delneri, 2014). Cyanotoxins can also be accumulated by many organisms, leading to a potential transfer of those toxins along food chains and eventually reaching humans (Aboal et al., 2005; Delneri, 2014). Not all organisms have the same ability to accumulate toxins and not all toxins are accumulated at the same rate (Aboal et al., 2005). Among cyanotoxins, BMAA (β-Methylamino-L-alanine) and MC seem to be those with higher rates of accumulation (Aboal et al., 2005). Recently, multiple cyanotoxins are being detected simultaneously in some systems, indicating multiple stressors, the risk of which is uncertain because health thresholds are based on exposures to single toxins (Howard et al., 2017).

2.3.1 Cyanotoxins impacts on human health

Cyanotoxins, particularly microcystins have caused human poisoning worldwide. The reported health problems are most likely related to chronic exposure to low microcystin concentrations through consumption of contaminated water and food (agricultural products, fish, prawns, and mollusks), dermal exposure, and inhalation (Zanchett & Oliveira-Filho, 2013). The health threats caused by cyanotoxins, especially MCs, have led the World Health Organization (WHO) to establish a tolerable daily intake (TDI) (0.04 μg kg^{-1}) and a provisional guideline value for MC-LR in drinking water (1 μg L^{-1}) (Bittencourt-Oliveira et al., 2014)

Several studies have assessed the human health threats posed by the bioaccumulation of cyanotoxins (mainly MC-LR) in food plants (Milligan, 2009; El Khalloufi et al., 2011; Pham & Utsumi, 2018). In one such study, Crush et al. (2007) demonstrated the possibility of human consumption of microcystin-exposed salad to exceed the tolerable daily intake of 0.04 μg kg^{-1} of body weight/day recommended by factor of two. The findings of a study by Crush et al. (2007) indicated the potential for the movement of microcystins into human and animal food chains via irrigation water.

There have not been any studies looking at the potential risk of ingesting cyanotoxins through the diet locally. Most studies done in South Africa have looked at the risk of human exposure via the direct route (drinking water). According to Mashile and Nomngongo (2017), many people in South Africa reside in rural areas and in those areas human beings and animals tend to drink water from the same sources mainly rivers, dams and groundwater. Even though humans and animals share the same water sources, there have been cases of animal poisoning but none of human beings (Mashile & Nomngongo, 2017). Cases of fatalities in wildlife have been reported in South Africa, for example rhino fatalities due to cyanotoxin poisoning at the Kruger National Park (KNP) (Mashile & Nomngongo, 2017). Mass fatalities of Lesser Flamingos have also been reported in East African lakes, for example, lake Bogoria, Kenya (Mashile & Nomngongo, 2017) and Lake

Manyara of Tanzania (Kimambo et al., 2019). A post-mortem study following the mass fatalities of Flamingos in the Arusha Region (Tanzania) in the years 2000-2004 linked the deaths to cyanotoxins (Anatoxin-a and Microcystins) (Kimambo et al., 2019).

2.3.2 Effects of cyanotoxins on aquatic plants

Cyanotoxins have been found in fresh water bodies, in brackish waters of Baltic Sea, in phytoplankton of alkaline volcanic lakes and in lichen-associated strain of cyanobacterium *Nostoc* among other environments (Babica, 2006). When cyanobacterial cells are actively growing and healthy, the cyanotoxins pools are mostly retained within the producer cells (Sivonen, 2009). Extracellular release of cyanobacterial toxins is accelerated during cell lysis, such as during natural bloom decay and in water treatment processes if these disrupts the cyanobacterial cells by physical or chemical action (Metcalf & Codd, 2014; Babica, 2006; Li & Pan, 2015). However, active transport (via putative ATP-binding cassette (ABC) transporter encoded by one gene of mcy-operon) of cyanotoxins from growing cyanobacterial cells has also been suggested (Pearson et al., 2004). Several studies have demonstrated the possible negative effects of cyanotoxins on aquatic plants (Pflugmacher, 2002; Mitrovic et al., 2005; Järvenpää et al., 2007) and biomass reduction in aquatic plants is usually observed after absorption of cyanotoxins, which can bioaccumulate in tissues (Bittencourt-Oliveira et al., 2014).

2.3.3 Effects of cyanotoxins on terrestrial plants

Plants are generally not killed by cyanotoxins, but plant growth may be inhibited and result in a yield reduction (Milligan, 2009; Purkayastha et al., 2010). Cyanotoxins ordinarily are unable to penetrate plant cell membranes but accumulate mainly via adsorption of the dissolved toxins in the roots (Pham & Utsumi, 2018). The mechanism of uptake of cyanotoxins by plants has not been fully explored, but it is generally accepted that these toxins are absorbed in the roots and translocated to other organs (Machado et al., 2017a). There is no information on the specific transporters of cyanotoxins, but there are several plant membrane transporters which are known to have an affinity for amino acids and peptides (Machado et al., 2017a). Since MCs are peptides, it is hypothesized that these peptide transporters are responsible for uptake of the toxins by plants.

Mycorrhizal fungi are also known to play a significant role in the uptake of nutrients and water by plants because the hyphae of these fungi spread out in the soil and give them a larger surface area to draw more nutrients and water from the soil (Siddiqui et al., 2008).

Many studies have demonstrated that the accumulation of cyanotoxins in plants is dose dependent (Bouaïcha & Corbel, 2016; Lee et al., 2017; Pham & Utsumi, 2018), and depends on the type of plants ((Bouaïcha & Corbel, 2016; Lee et al., 2017b), route of exposure, length of exposure and target organs/tissues (Pham & Utsumi, 2018). A summary of the crops studied for their accumulation potential of MCs and the concentrations used can be found in Miller and Russell (2017). According to Miller & Russell (2017), the bioaccumulation of MCs in plants has been shown to increase with increasing concentration of the toxin in irrigation water irrespective of the type of irrigation, irrigation conditions or experimental design.

Crush et al. (2007) reported uptake and storage of MCs in shoots of *Brassica napus* than in *Oryza sativa*. Greater quantities of MCs were recovered from the shoots of rape plant than from rice plant, indicating that different plant species may accumulate MCs at different rates when irrigated with water containing MC at a concentration of 1700 µg L^{-1} for 10 days (Crush et al., 2007). In terms of other cyanotoxins besides MCs, Kittler et al. (2012) treated *Brassica oleracea var. sabellica*, *Brassica juncea*, and *S. alba* to different experimental conditions and demonstrated significant uptake of CYN. Kittler et al. (2012) applied 18–35 µg L^{-1} levels of CYN to the roots of the plants and reported 10–21% of the applied concentration in the leaves of the plants. Such findings indicated that crop plants irrigated with CYN-contaminated water may present a risk of the toxin in the food chain.

Of all the cyanotoxins, many studies have focused on the impacts of MCs on plants. MCs are known to be potent inhibitors of protein phosphatases in plants and animals (Milligan, 2009). Protein phosphatases are important regulatory enzymes that catalyse de-phosphorylation of serine/threonine residues in phosphoproteins. These enzymes play an important role in plants by regulating key processes such as photosynthesis, ion channel activity, tissue development and nitrogen and carbon metabolism (Milligan, 2009).

Several studies have demonstrated that plants can enzymatically biotransform MCs, with a number of studies reporting the stimulation of antioxidant and detoxification enzymes in aquatic plants when exposed to MCs (Pflugmacher et al., 2001; Crush et al., 2007; Pham & Utsumi, 2018). Accumulated MCs in plants are detoxified in a phase II biotransformation through the conjugation of Glutathione (GSH) catalysed by Glutathione S-transferases (GSTs) (Crush et al., 2007; Huang et al., 2008).

The bioaccumulation of cyanotoxins in various tissues of different food plants has been extensively covered in literature, for example in Bouaïcha & Corbel (2016). MCs have been found to impair the growth of a variety of crops (D'Anglada et al., 2015) via oxidative stress and cell death (Lefebvre, 2013). Milligan (2009) documented data of 15 common formed plants that were examined from various studies investigating the effects and accumulation of MC-LR in crop plants. Milligan (2009) reports that all the studied plants were able to grow when exposed to realistic levels of MC-LR and accumulate the toxin. Meneely and Elliott (2013), also reported the uptake and accumulation of MCs in food plants including lettuce, rocket, dill, cabbage, runner beans, radish, rice, runner beans, parsley, soybean, pumpkin, sesame, mung bean and sweet potato upon irrigation with cyanobacteria-infested water.

Cyanotoxins in vegetables are known to inhibit plant growth, lower seed germination and growth of seedlings and induce phytotoxicity (El Khalloufi et al., 2011; Manganelli et al., 2012). Saqrane et al. (2008), tested the effects of MCs on the germination of seeds of *Lens esculenta*, *Zea mays*, *Triticum durum* and *Pisum sativum*. Saqrane et al. (2008) using a range of doses of the cyanobacteria aqueous extracts (equivalent to 0, 1.6, 2.9, 5.8, 8.7 and 11.6 μg L^{-1} MC-LR). Saqrane et al. (2008), found a dose-dependent effect of MC on seed germination, with sensitivity differing according to sensitivity of the tested plants. The *Pisum sativum* seeds were reported to be the most sensitive of the four species tested. Based on such findings, cyanotoxins and more so MCs have a negative impact on the productivity, yield and quality of food crops.

Based on the literature reviewed, it is clear that plants can detoxify cyanotoxins to some extent, but no studies have been performed to determine the length of time necessary to completely break down the toxin (Milligan, 2009; Purkayastha et al., 2010). Crops are also known to have the ability to store cyanotoxins in sufficient concentrations not to induce morphological and physiological changes (Pflugmacher et al., 2006) and exposure to cyanotoxin-infested water does not necessarily always result in negative consequences. Corbel et al. (2015) observed that a long exposition of plants to low amounts of microcystins can accelerate their development.

2.3.4 Possible accumulation of cyanotoxins in agricultural soils

Concentrations of MCs in surface waters intended for irrigation range from 4 to 50 µg L^{-1} and in some instances can be as high as 6500 µg L^{-1} (Machado et al., 2017b). Studies reporting levels of other cyanotoxins are very rare, but Machado et al. (2017b) report that levels of extracellular cylindrospermopsin (CYN) can be as high as 126 µg L^{-1}. MCs and other cyanotoxins can contaminate soils through irrigation with cyanobacteria-infested water or when cyanobacterial blooms are used as organic fertilizers (Corbel et al., 2015; Bouaïcha & Corbel, 2016; Wen et al., 2017; Cao et al., 2017). Most cyanotoxins, more so MCs and CYN are chemically stable because of their cyclic peptide structure and leach into the soil after irrigation with contaminated water, resulting in soil and groundwater contamination with these toxins (Bouaïcha & Corbel, 2016; Machado et al., 2017b; Wen et al., 2017). The adsorption mechanism of MCs in soil is not only because of sorption but also because of their chemical binding with metal ions on the surface of soil particles (Bouaïcha & Corbel, 2016; Machado et al., 2017b). Corbel et al. (2014) and Bouaïcha and Corbel (2016) report relatively long half-lives of MCs in agricultural soils ranging from 6 and 17.8 days; however, these are relatively short compared to other organic pollutants' half lives in soils (Cao et al., 2017).

According to Wen et al. (2017), levels of MCs in agricultural soils can be up to 273.2 µg kg^{-1} and such levels can negatively impact soil organisms, soil ecosystems and structure. Sorption of cyanotoxins in soils is low and this results in prolonged bioavailability of the toxins to plants and soil organisms (Corbel et al., 2014; Machado et al., 2017b). Wen et al. (2017) reported halting of earthworm reproduction at concentrations as low as 0.2 mg kg^{-1} of MC-LR. This demonstrated that environmental levels of MCs in agricultural soils in countries such as China could be posing threats to earthworms and other soil organisms.

The possible impacts of cyanotoxins on soil biota have not been getting much attention. However, inhibitory effects of cyanobacterial extracts and purified MCs on the growth of cultured bacteria such as *E. coli* and *Streptoverticillium* species have been reported (Cao et al., 2017). The inhibitory effects can be attributed to antibacterial substance released by cyanobacteria which alter the permeability of cell membranes damages caused inside the cells when these macromolecules enter the cell (Cao et al., 2017). Such studies are evidence that cyanotoxins may pose a threat to soil ecosystems, but more studies are required to assess the impacts of cyanotoxins on soil microbes such as bacteria, protozoa, and fungi and macro-organisms like earthworms, arthropods, and mollusks as well as soil function.

In that regard, the use of myxogastrids (also known as myxomycetes or plasmodial slime moulds) to assess the impacts of cyanotoxins on soil organisms, particularly protozoa could be useful. This is because myxogastrids are common organisms known to be abundant in soils and are sensitive detectors of environmental impact as shown by (Feest & Stephenson, 2014). Because studies investigating the fate and effect of cyanotoxins on soil microfauna are scarce (Bouaïcha & Corbel, 2016) and mycorrhizae are important components of plant roots, the use of myxogastrids may further play an important role in the study of cyanotoxin–mycorrhizae interactions and the possible effects of cyanotoxins on mycorrhizae, and uptake of nutrients and water by plants.

Lee et al. (2017b) reported MC persistence in agricultural soils long after harvesting, thus prompting the need for routine monitoring of cyanotoxins in agricultural soils. The fact that cyanotoxins are persistent in agricultural soils is of particular importance because this may imply significant accumulations of these toxins in soils following consecutive planting and watering cycles and crops can be exposed to cyanotoxins that were already in the soil prior to planting (Cao et al., 2017; Machado et al., 2017b). The fact that soils cannot protect groundwater from cyanotoxins originating from surface waters (Corbel et al., 2014; Machado et al., 2017b) also warrants the monitoring of cyanotoxins in both irrigation water and soils. Machado et al. (2017b) suggested research looking into the risks of contamination of groundwater as a result of irrigation with cyanotoxin-infested water, taking into consideration the seasons and soil characteristics.

In soils, cyanotoxins are removed by various mechanisms including hydrolysis, photochemical degradation, and biodegradation (Corbel et al., 2014; Bouaïcha & Corbel, 2016). However, microbial degradation is the main removal/breakdown process for cyanotoxins in soils and numerous soil bacteria such as *Brevibacterium* sp., *Rhodococcus* sp. and *Arthrobacter* sp. have the ability to degrade MCs (Corbel et al., 2014; Machado et al., 2017b).

2.3.4 Accumulation of cyanotoxins in plant tissues and potential human health risk

Cyanotoxins enter the human body through various routes including drinking water, food products made from cyanobacteria, and through recreational contact (Bouaïcha & Corbel, 2016). Of recent concern is indirect exposure via consumption of crop plants irrigated with contaminated water. Even though there have not been any cases of poisoning reported via this route (Bouaïcha & Corbel, 2016), there is need to pay particular attention to it because numerous studies have reported a number of crop plants containing MC levels exceeding the tolerable limits (Crush et al., 2007; Pham & Utsumi, 2018). It is thus recognised that agricultural crops irrigated with cyanotoxin-contaminated water may pose health risks to both human beings and livestock.

Cyanotoxins have various effects in mammals, MCs are known to modify cytoskeletons of hepatocytes, induce intrahepatic haemorrhage and cause hepatic insufficiency of liver tissues (Cordeiro-Araújo et al., 2016). Serious health impacts in mammals including colorectal and liver cancers have also been linked to chronic exposure to MCs and this makes a thorough evaluation of potential exposure routes of importance (Cordeiro-Araújo et al., 2016). In that regard, numerous studies have been conducted to assess the potential risks associated with irrigating agricultural food crops with cyanotoxin-contaminated water.

From a human health perspective, most of these studies were conducted using vegetables in which the edible parts are predominantly leaves and in most of these studies the MCs accumulating in the plants would exceed the tolerable daily intake (TDI) of 0.04 μg kg^{-1} of body weight/day, assuming a body weight 60 kg and consumption of each vegetable (Machado et al., 2017b). In studies where roots were the edible portion of the vegetable, roots were proved to accumulate higher levels of cyanotoxins compared to leaves and thus root vegetables will need more attention when considering food safety (Machado et al., 2017b).

The bioaccumulation of different MC congeners in plants has been poorly investigated (Romero-Oliva et al., 2014). Romero-Oliva et al. (2014) reported that the bioaccumulation of different MC congeners and total MCs varies in plants. Under natural conditions, water used for irrigation may contain several MCs congeners, in addition to other cyanotoxins. Furthermore, laboratory results suggest that cyanobacterial strains/species can produce more than one MCs congener (Bittencourt-Oliveira et al., 2014; Puddick et al., 2014). This implies that crops irrigated with cyanobacterial and cyanotoxins-contaminated water may be exposed to more than one MC congener per time.

However, the simultaneous uptake and bioaccumulation of these congeners in vegetables and other crop plants is yet to be extensively studied (Bittencourt-Oliveira et al., 2014). This will need to be fully understood to assess the real risks posed by consuming such plants and the acceptability thereof and future studies are thus required in this regard. There is limited data on the potential accumulation of MCs and other cyanotoxins in livestock even though livestock are commonly exposed to these toxins through consumption of water or contaminated plants.

2.4 Lack of guidelines and policies

It is evident that the consumption of edible plants exposed to cyanotoxins via irrigation may have health risks. Drobac et al. (2013) documented several studies that have shown that cyanotoxins and MCs can be detected in the tissues of exposed terrestrial and aquatic plants. For certain critical routes of exposure, the WHO and certain other institutions provided guideline values for MC-LR based on tolerable daily intake. The TDI is the amount of a potentially harmful substance that can be consumed daily over a lifetime with negligible risk of adverse health effects (Drobac et al., 2013). According to Adamovský, (2010) at least 100 variants of microcystins have been identified but only a few have been found to occur frequently in high concentrations (MC-LR, -RR, -YR). Microcystin-LR is among the most frequent and the most toxic microcystin congeners. Based on numerous studies, tolerable daily intake (TDI) 0.04 µg (MC-LR) kg^{-1} of body weight / day (0.04 µg kg^{-1}) was established (Adamovský, 2010) and a provisional guideline value for MC-LR in drinking water (1 µg L^{-1}) (Crush et al., 2007). The TDI value was based on liver pathology observed in a 13-week study in mice and applying an uncertainty factor of 1000, taking into consideration limitations in the database, in particular lack of data on chronic toxicity and carcinogenicity (Adamovský, 2010).

In South Africa, water quality is regulated by the South African National standard: Drinking Water SANS 241, 2015) (Drinking Water, 2015). According to SANS (2015) a guideline value of ≤1 µg L^{-1} for MC-LR has been set for the maximum permissible concentration for cyanotoxins in domestic water. It is important to note that this guideline is only for microcystins and where the exposure route is drinking water but there is no cyanotoxins guideline in food or dietary intake. According to SANS 241 (2015) the MC only need to be measured where an algal bloom (> 20 000 cyanobacteria cells per millilitre) is present in a raw water source. In the absence of algal monitoring, an algal bloom is deemed to occur where the surface water is visibly green in the vicinity of the abstraction, or samples taken have a strong musty odour (Drinking Water, 2015).

The only regulation that guides and addresses irrigation water quality is the South African Water Quality Guidelines Agricultural Use: Irrigation Volume 4 of 1996 Department of Water Affairs and Forestry (DWAF, 1996). However, these guidelines only cover physicochemical aspects and coliforms bacteria and how these impact on crops and human health. Cyanobacteria and their toxins are not covered in these guidelines.

In a country such as South Africa, water for irrigation of agricultural plants is essential yet it is becoming less available, and the quality is deteriorating. Controlling eutrophication and harmful cyanobacterial blooms seem to best management practice to eliminate risks to agricultural productivity and human health. However, the mismanagement of wastewater treatment from urban zones, a rising human population and climatic conditions favouring an increase in proliferation of toxic cyanobacterial blooms (Machado et al., 2017b) are presenting challenges to regulatory authorities in their bid to control eutrophication and cyanobacterial blooms.

However, Ibelings et al. (2014) postulate the lack of chronic toxicity data as a hurdle in improving cyanotoxin regulations via this route. In the absence of legislation, scientists and authorities can only encourage farmers to employ better practices. This presents a challenge as many freshwater bodies used for irrigation purposes are infested with cyanobacteria and many producers do not have the capacity to monitor nor control cyanobacteria and their toxins. As is the case with drinking water quality regulations, there is lack of monitoring programs for cyanobacteria or their toxins due lack of resources and skilled staff in many countries including South Africa (Newcombe, 2009) and this leaves consumers of irrigated crops at a high risk.

2.5 The risk of multiple stressors in South African waters

The bulk of water allocated to agriculture in South Africa goes to commercial farming, with small-scale farming mostly relying on hand watering and rain-fed agriculture (Turton et al., 2016). For crop production to be profitable and sustainable, irrigation water must be of acceptable quality and irrigating with inferior quality water can lead to reduction in yields and unacceptability of the products. Deteriorating water quality is a huge problem in South Africa as a number of anthropogenic sources including mining and mineral processing (mainly toxic metals); untreated and partially treated sewage; coal-based power plants (causing acid rain) and effluent from industrial processes (including endocrine disrupting chemicals (EDCs)) are negatively impacting on water quality (Oberholster & Botha, 2014).

Among the key challenges faced by developing countries including South Africa is the increase in urban runoff coming from malfunctioning and in some cases overloaded municipal wastewater treatment plants and direct discharge of human waste in waterways (De Villiers, 2007). These are major sources of nutrients and other contaminants in rivers, reservoirs, and groundwater. The prevailing high levels of nutrients in South African waters tend to promote the proliferation of cyanobacteria and increase the likelihood of human exposure to cyanotoxins (DWA (Department of Water Affairs, 2011). In addition to cyanotoxins, other water quality problems facing irrigated agriculture in South Africa include salinity, high pH, high electrical conductivity (EC), high chloride levels and high sodium absorption ratio (DWA, 2012). Some of these are discussed in detail here.

2.5.1 Linear Alkylbenzene Sulphnates (LAS)

Linear alkylbenzene sulfonates (LAS), is a group of anionic surfactants characterized by polar heads and hydrophobic chains, and have widely been in use for over 40 years (Wang et al., 2012). LASs are very important anionic surfactants, because of their use in several important applications, most noticeably in detergents and laundry (Nomura et al., 1998; Anachkov et al., 2015). Surfactants are widely used as emulsifiers, foamers, detergentes, solubilizers and wetting agents in many pharmaceutical, personal care, home care and food products.

After use, LAS can enter into the aquatic environment (Wang et al., 2012). Large quantities of laundry detergent ingredients enter the environment continuously, either through waste water streams or as a direct result of detergent product use in, or near to, surface water bodies (Gordon, 2011) and because of their enormous consumption LASs affects various aquatic environments in which they are ultimately disposed (Nomura et al., 1998).

In most urban and peri-urban areas in developing countries, there is poor provision of wastewater treatment facilities, and in cases where they are available, they are sometimes malfunctional as is the case in many parts of South Africa. This means that LAS and other surfactant concentrations are often high in urban water courses close to emission points (Eniola, 2007), although there is evidence that concentrations decrease rapidly beyond the urban fringe (Gordon, 2011). According to Gordon (2011) no guidelines have been developed for assessing the effects LAS in South Africa.

LAS has a polar narcotic mechanism of toxicity which means that they act by nonspecific disruption of the functioning of cell membranes (Gordon, 2011). Anionic surfactants such as LAS bind to various bioactive macromolecules, such as starch and proteins, by inserting into various phospholipid membranes, thus causing malfunction, or by accumulating in lysosomes and inhibiting lysosomal enzymes (Gordon, 2011). These interferences can alter the folding of polypeptide chains, change the surface charge of molecules, and modify the activity of various enzymes or other cell constituents (Gordon, 2011; Wang et al., 2012). Since LAS can alter the permeability of membranes, the likelihood of accumulation and toxicity of other toxicants in the organisms increases (Wang et al., 2012). Wang et al. (2012) reports several studies where accumulation increased due to the combined exposure of LAS and other pollutants, for example lead bioaccumulation in *Ruditapes philippinarum* was found to be significant in organisms exposed to a mixture of LAS and lead than to lead only: combined toxicity of LAS and other pollutants such as the toxic metal ion Hg^{2+} and pyrene.

Most cyanotoxins, for example MCs are large molecules, with molecular weight (~1000 Da). This makes it difficult for them to easily penetrate biological membranes and bioaccumulate (Wang et al., 2012). The combined presence of LAS and cyanotoxins in the study area may affect the toxicity and accumulation of cyanotoxins in the crop plants. Wang et al. (2012) reports that the combined pollution of cyanotoxins and LAS in aquatic environments such as eutrophic lakes is common due to toxic cyanobacterial blooms and exogenous organic chemicals pollution.

In another study, Wang et al. (2015) further demonstrated that low LAS ($\leq$10 mg L^{-1}) concentrations improved the growth of *M. aeruginosa* after 12 days of exposure. Furthermore, Wang et al. (2015) also found that LAS increased the microcystin production of *M. aeruginosa* and extracellular and intracellular microcystin contents were significantly increased after *M. aeruginosa* was exposed to high LAS concentrations. These findings thus necessitate the need for future research to investigate possible synergetic impacts of cyanobacteria and cyanotoxins in combination with other pollutants such as LAS. This is particularly relevant in areas such as the Crocodile (West) and Marico catchment since most reservoirs in this catchment are hypereutrophic and LAS is likely to be found under such conditions and thus increasing the risk of *Microcystis* bloom and microcystin production and uptake of the toxins by crops.

2.5.2 Toxic metal species

Toxic metals species are introduced in freshwater environments from both natural and anthropogenic sources. Some metals such as Zn are regarded essential biological nutrients but are regarded toxic when they exceed certain thresholds. Others such as Pb are regarded hazardous at any concentration (Shozi, 2015). Anthropogenic activities such as agricultural practices, mining, industrial, municipal and urban waste discharge, have for long been recognised to elevate naturally present metal concentrations and present a risk to freshwater environments and humans (Snaddon, 1998; Ebenebe et al., 2018; Shozi, 2015). Among major contributors of toxic metals in South Africa, is mining. Mining contributes significantly to the country's economy but also pollutes the environment as a huge number of mines' residue deposits (tailing dams) contain harmful metals that pose a health risk in the environment (Ebenebe et al., 2018). Such toxic metals are transmitted through various environmental media and when they reach humans, plants, and animals they cause diseases and to some extent death.

Irrigating of food plants/crops with water contaminated with toxic metals can affect the quality of the crops and pose a health risk to consumers. Crops have the ability to take the contaminants from soil and accumulate them in the edible parts of the crops hence presenting health risks to humans and animals (Musvoto & de Lange, 2019). Metals are not naturally bio-degradable and remain persistent in freshwater systems since they are normally deposited in bottom sediments and are continuously sequestered between the sediment surface and overlying water column (Shozi, 2015). Trace elements and toxic metals cannot be broken down further, and are, therefore, persistent pollutants. Many of the dangerous metals form charged ions in the water column, which then adsorb onto suspended particles. On settling out, these particles take the adsorbed metals into the sediment, where they can remain indefinitely (Snaddon, 1998).

Impacts on irrigated crops include reduction in quality and productivity and changes to the types of crops that can be grown. The consequences depend on a number of factors including what is being mined, chemicals used, methods of extraction used, and life stage of the mine and environmental practices implemented (Musvoto & de Lange, 2019). Elevated levels of toxic metals AS, Zn, Pb and Cd have been recorded in vegetables and soils in areas close to a mine in Portugal (Musvoto & de Lange, 2019). In South Africa, toxic metals are found in water bodies because of human activities, especially mining. Metals such as Hg, Be, Cd, Pb, Ni and Cu are fairly problematic in South Africa, and several cases of severe toxic metal species pollution have been reported in the mining areas, such as in wetlands in Gauteng and Mpumalanga (Snaddon, 1998).

Gzik et al. (2003) reported elevated levels of toxic metals in soils of the Rustenburg area (North-west of Pretoria). The area is renowned for its endowment with mining industries. Ebenebe et al. (2018) also reported deposits are of great health concern; containing enormous amounts of toxic metals, such as U, As, Ra, Ni and Zn in the Witwatersrand Basin of South Africa. Elevated levels of toxic metals were also reported in the soil of heavily industrialized Vanderbijlpark area (Mtunzi, 2015).

The level of contamination was found to differ with locations and the seasons of the year. The concentrations of the toxic metals increased in rainy seasons due to the rapid transportations from their sources to the water bodies. This resulted to rise of the toxic metals in some sources to levels slightly above the set limits. The accumulation of these toxic metals in the water sources in Chief Albert Luthuli Local Municipality, Mpumalanga is believed to be sourced from both natural and anthropogenic activities which include weathering of rich metal containing rocks and unregulated disposals of metal containing waste materials as the landfilling (Nthunya et al., 2017).

Given the history of mining and related industries in South Africa and the harmful impacts on crop production through irrigation with contaminated water it is thus important to assess the risks posed by cyanotoxins together with other pollutants of importance such as toxic metals. This will assist to develop strategies for maintaining agricultural livelihoods and minimizing human health risks in the face of these risks.

2.5.3 Other pollutants

Other water quality problems facing irrigated agriculture in South Africa include salinity, high pH, high electrical conductivity (EC), high chloride levels and high sodium absorption ratio (DWA, 2012). High salinity levels are known to be a threat to biota and can destroy soil structure and the affected soils may negatively impact on crop yields (van der Laan et al., 2012; Rooyen et al., 2016; Musvoto & de Lange, 2019). Soil macro-porosity and hydraulic conductivity are all lowered by the water quality of the irrigation water (Musvoto & de Lange, 2019). According to Musvoto & de Lange (2019) irrigation with water containing elevated levels of sodium increases soil sodicity, change pore geometry, cause clay dispersion and reduce hydraulic conductivity. Soil dispersion causes clay particles to plug soil pores, resulting in reduced soil permeability; and reduced water infiltration; and effects of reduced infiltration include reduced plant available water; increased runoff and soil erosion. Furthermore, irrigation with contaminated water could damage irrigation equipment through encrustation and corrosion (Musvoto & de Lange, 2019).

In addition to inorganic pollutants, the emerging organic pollutants of concern in South Africa are EDCs (Olujimi et al., 2010). South Africa's agricultural productivity is the highest in the continent and there are more than 180 different pesticide ingredients registered in the country of which some of these are known to be EDCs (River, 2015). In South Africa, pesticides in the environment are mainly from agricultural sources and malaria vector control. According to Bornman et al. (2017) pesticides have been detected in water around agricultural areas in South Africa, with in vitro bioassays of the water samples revealing high estradiol equivalent (EEq) values. Water runoff cattle feedlots in the same area also demonstrated estrogenic activity (Bornman et al., 2017).

Another example for EDCs is nonylphenol (NP) which is a product of incomplete biodegradation of alkylphenol ethoxylates which are among the most used surfactants. NP enters the agricultural system via contaminated irrigation water and laboratory experiments with NP impaired lettuce seedling germination, suggesting that it could adversely affect food quality and could reduce yield of crops (Bornman et al., 2017).

Improper management, outdated infrastructure and over-burdened sewage treatment plants all compromise agricultural water quality in South Africa (Bornman et al., 2017). This implies that under natural conditions, plants are simultaneously exposed to a variety of chemical contaminants and the combined effects of the mixture of different chemicals could result in unexpected effects compared to when individual chemical component is applied individually, for example, changes in the uptake and accumulation rates (Machado et al., 2017b).

2.6 SPATT technology as a monitoring tool for cyanotoxins in irrigation water

2.6.1 Traditional sampling technologies

Sampling is an important aspect when it comes to monitoring cyanobacterial toxins in both basic and applied research (Mashile & Nomngongo, 2017). Traditional monitoring programs of cyanotoxins are based on collection of individual samples at specific single spot and time points. However, these traditional sampling techniques have several drawbacks such as large sample volumes are needed to recover sufficient mass of toxin and this consumes both time and labour for the clean-up prior to instrumental analysis (Kohoutek et al., 2010). Further, cyanotoxin concentrations may vary over time, and episodic events may be missed in the traditional monitoring scheme. Although increase of the sampling frequency or installing automatic sampling systems may provide a solution, it may be complicated, especially in remote areas (Kohoutek et al., 2010). Therefore, it may be difficult to formulate the time-weighted average (TWA) concentration of the contaminant, which forms a fundamental part of an ecological risk assessment process.

Accurate assessments of contaminant concentrations based on traditional grab sampling methods are not always possible. Very large sample volumes are often required to accurately sample contaminants at low levels and there is often low recovery of polar compounds in liquid to liquid extraction techniques (Brown, 2010). Volatilization, adsorption to container walls, and chemical degradation are also of concern when using grab sampling techniques (Brown, 2010). Due to the short sample collection period along with transport and storage implications, discrete sampling only provides information on the instantaneous concentration, in contrast to data regarding time weighted average (TWA) concentrations provided by integrative passive samplers (Greenwood et al., 2009).

Automated sampling methods seem to be a solution as they give a better indication of average water constituents than grab sampling. Automated samplers are designed to take samples at specified intervals, which can provide a clearer indication of variation in pollutant concentration over time. However, this process is also subject to high levels of contamination via sampling tubes, valves, and pumps (Brown, 2010) and may be complicated, especially in remote areas (Kohoutek et al., 2010). Due to the shortfalls of grab and automated sampling techniques, new sampling techniques are being developed.

For sampling cyanobacteria, these include remote sensing to determine the horizontal distribution of cyanobacteria in freshwater ecosystems and spectrofluorometric probes to reveal the vertical distribution of these cyanobacteria in the water column (Mashile & Nomngongo, 2017). The disadvantage of these in situ sampling tools is their cost. For this reason, grab, sampling method becomes the most widely used method. However, the disadvantage of this sampling method is that it can provide poor estimation of cyanobacterial abundance due to the spatial and temporal differences in the distribution of cyanobacteria in the sampling site (Mashile & Nomngongo, 2017).

With regards to cyanobacterial toxins, passive samplers are becoming a popular alternative. The information from passive sampling could be more representative of general bioavailability (Greenwood et al., 2009). Passive sampling methods (PSMs) have the potential to eliminate logistical, cost and sediment disturbance pitfalls associated with traditional sediment pore-water assessment methods (Maruya et al., 2015).

2.6.2 Passive sampling technologies

Passive sampling can be defined in its broadest sense as any sampling technique based on free flow of analyte molecules from the sampled medium to a receiving phase in a sampling device, as a result of concentration gradient between the chemical potentials of the analyte in the two media (Kohoutek et al., 2008). Passive samplers have been used in environmental monitoring since the beginning of the 1970s (Greenwood et al., 2009). The early designs were used to measure concentrations of gaseous pollutants in air and this technology is now widely used in monitoring ambient air quality and workplace exposures to potentially harmful compounds such as volatile organic solvents (Brown, 2010).

The first passive sampling of liquid media was used to monitor dissolved inorganic compounds in the surface water in an enclosed dialysis membrane in the 1970s (Křesinová et al., 2016). First use of semi-permeable membrane devices (SPMDs) for sampling organic compounds was reported in 1990s and since then, many passive sampling devices have been developed and many of them are now available commercially (Křesinová et al., 2016).

2.6.2.1 Theory of passive sampling

Passive methods may generally be classified as either adsorptive or absorptive (Kot et al., 2000). Adsorptive methods take advantage of the physical or chemical retention by surfaces and key parameters involve surface binding and/or surface area, whereas absorptive methods involve not only surface phenomena but also analyte permeation in the interceding material (Kot et al., 2000).

A number of parameters can affect passive sampling of analytes (for example water temperature, fluctuation of analyte concentrations) thus it is valuable to determine the parameters responsible for the uptake on the basis of experimental work (Kot et al., 2000). The mass of a contaminant accumulated is determined by its concentration in the water, the length of exposure, and the sampling rate (Rs) of the sampler. The latter is determined by a number of factors including the area of sampler available for diffusion, the properties of the diffusion-limiting layer (for example, thickness and resistivity), and the properties (for example, size and polarity) of the chemical (Greenwood et al., 2009).

Passive sampling is based on the diffusion of analyte molecules from the sampled environmental medium (water or sediment pore water) to a receiving phase in the sampling device. The diffusion occurs because of a difference between chemical potentials of the analyte in the two media (Figure 2.1). Figure 2.1 shows the concentration profile of a compound during diffusion, the accumulation from the bulk of the sampled medium to the sorbent (receiving phase) through a permeable (porous or non-porous) membrane. High affinity to the sorbent inside the sampler drives the diffusion of analyte molecules from the sampled medium into the sampler until the thermodynamic equilibrium is established (Vrana et al., 2006).

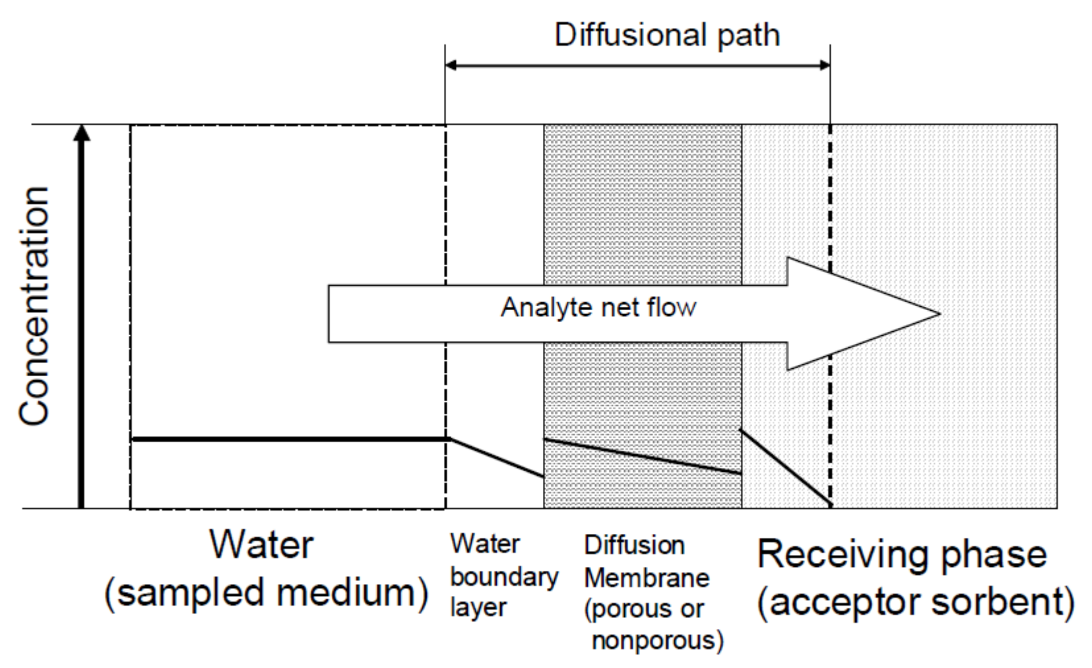

Figure 2. 1. Functional principle of a passive sampling device (Adapted from Vrana et al. (2009)

Diffusion-based passive samplers rely on this method to monitor chemical uptake. These samplers consist of a porous hydrophilic membrane that allow for accumulation of certain organic contaminants, while rejecting others. Fick's first law of diffusion describes the flow of contaminant during passive sampling (Brown, 2010).

$$NA = \frac{DS}{L}C \qquad [2.1]$$

Where:

NA is the mass flow rate, *C* is the analyte concentration, *S* and *L* are surface area and diffusive length, respectively and *D* is the analyte diffusive coefficient in air (Brown, 2010).

Pollutant sorption from water (or other media) to most passive sampling devices follows the general uptake pattern shown in Figure 2.2.

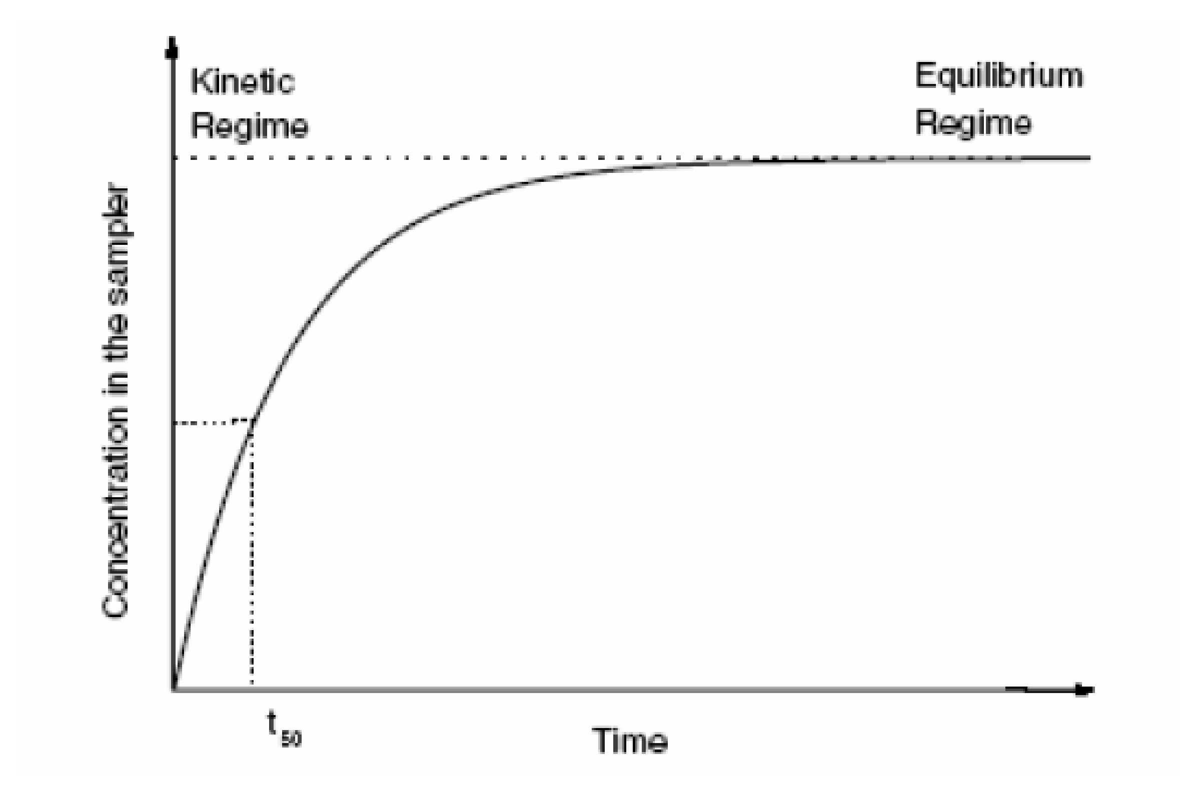

Figure 2. 2. Uptake of analyte by passive sampling device (Kohoutek, 2010)

The process of compound accumulation on the sorbent media is a first order reaction (Alvarez et al., 2004). First-order kinetic models include an integrative phase, curvilinear phase, and equilibrium partitioning phase. During the integrative phase, the sampler acts as an infinite sink for contaminants with log-linear uptake (Alvarez et al., 2004).

In order to use the sampler quantitatively, an uptake rate (Rs) must be determined experimentally for the compounds of interest (Alvarez et al., 2004). The uptake rate can be determined as:

$$Rs \frac{Dw}{Lw} A \qquad [2.2]$$

Where:

The uptake rate Rs is in units of (L/d), Dw is the compound-specific aqueous diffusive coefficient (m^2/s), Lw is the aqueous film layer thickness (m), and A is the available surface area (m^2).

Once an uptake rate has been calculated, the time-weighted average water concentration of the contaminant of interest can be calculated as:

$$\mathrm{Cw} = \frac{\mathrm{CsMs}}{\mathrm{Rst}} \qquad [2.3]$$

Where: *Cw* (ng L^{-1}) and *Cs* (ng/g) are the analyte concentration in water and sorbent, respectively; Ms (g) is the mass of the sorbent, *Rs* (L d^{-1}) is the uptake rate determined from equation 3 above; and t (d) is the exposure time (Brown, 2010).

For most applications sampling rate is independent of concentration in the medium but is characteristic for individual analyte. *Rs* is affected by water flow, turbulences, temperature and biofouling (influence of living creatures, especially microorganisms) (Kohoutek et al., 2010).

In most cases sampling rate for individual analytes must be determined by performing calibration for sampling device. In theory, calibration in the kinetic regime requires the reproduction of conditions in the field. Therefore, water-flow and the temperature of the exposure media should be reproduced. During the calibration it is essential to ensure constant concentration of analytes in the media (Kohoutek et al., 2010).

2.6.2.2 Types of passive samplers

There are two types of passive sampling devices, samplers in which target analytes dissolve (for example, absorption) and those in which analytes are adsorbed (for example, surface bonding); but the sampling process is very similar in both types of sampler (Křesinová et al., 2016). Both inorganic and organic groups of contaminants can be sampled by passive sampling. Inorganic pollutants can be sampled by DGT (Diffusive Gradient in Thin film) or Chemcatchers. For organic pollutants, several passive sampler designs have been developed: POCIS (Polar Organic Chemical Integrative Sampler), SPMD (Semi-Permeable Membrane Device), MESCO (Membrane-Enclosed Sorptive Coating), Chemcatchers and others (Vrana et al., 2006; Charriau et al., 2016). The Chemcatcher® (polar organic version) and polar organic integrative sampler (POCIS) are designed to monitor concentrations of polar (log $K_{ow} < 4$) organic pollutants (Greenwood et al., 2009). In both samplers the diffusion-limiting membrane is a polyether-sulphone sheet with water-filled micropores, and the receiving phases comprise a range of adsorbent materials, either bound in an Empore^{TM} disk, or in a free particulate form (Greenwood et al., 2009). These have been used for measuring the TWA concentrations of a range of polar herbicides, pharmaceuticals, and personal care products (Greenwood et al., 2009).

With most of these passive samplers, contaminants accumulate in a receiving phase, by diffusion followed by sorption, with an integrative step before equilibrium is reached. Passive samplers can be used during the integrative accumulation phase or at equilibrium. POCIS, DGT, Chemcatchers and SPMD are for instance commonly applied in the integrative accumulation phase in order to calculate time-weighted average concentrations (TWACs) of pollutants (Charriau et al., 2016). Passive Diffusion Bag Samplers (PDBSs), polyoxymethylene (POM) and also POCIS or Chemcatchers, with longer deployment periods, can be used at equilibrium (Charriau et al., 2016).

2.6.3 Solid Phase Adsorption Toxin Tracking (SPATT): As a monitoring tool

Owing to the quite high and temporal variability of the occurrence and subsequent development of algal blooms and hence potentially of co-occurring toxin production, passive samplers may prove to be a useful tool for monitoring of natural toxins (Vrana et al., 2009). For most work involved in algal toxins, conventional grab sample collection followed by laboratory clean-up and analysis still remain the common approach by researchers and monitoring crews (Zhang & Zhang, 2014). Nevertheless, considerable progress has been made toward the use of passive sampling for time-integrated concentrations of algal toxins.

The first use of integrative passive sampling for algal toxins is described in the work of MacKenzie et al. (2004). MacKenzie et al. (2004) developed a passive sampler (solid-phase adsorption toxin tracking (SPATT) bag) based on synthetic resin enclosed in porous sachets and used it for monitoring of a group of marine toxins known as paralytic shellfish poisons (Kohoutek et al., 2008). The solid-phase adsorption toxin tracking (SPATT), is conceptually similar to semipermeable membrane device (SPMD) or polar organic chemical integrative samplers (POICS) that have already been used for other trace contaminants in water (Zhang & Zhang, 2014).

The device was designed as early warning of developing algal blooms to protect consumers and harvesting of contaminated seafood products (Kohoutek et al., 2008). This work was continued by other authors. Fux et al. (2008) evaluated various sorbents in the SPATT system. Rundberget et al. (2009) redesigned the device and used it for monitoring of various natural toxins of the southern coast of Norway.

Shea et al (2006 in Vrana et al., 2009) described the development of a monophasic device for monitoring brevetoxins, highly toxic compounds produced during red tide events. Devices constructed of polydimethylsiloxane sheets were successfully used for integrative sampling (Kohoutek et al., 2008).

The SPATT consists of bags sewn from polyester mesh containing activated polystyrene divinylbenzene resin, which can adsorb lipophilic toxins dissolved in water. Like any other passive samplers, SPATT provides time-averaged algal toxin concentration prior to, or during algal blooms. This device was later improved by designing the frame in which the DIAION HP20 resin is retained using disks between two layers of nylon mesh, and clamped tightly in the embroidery frame so as to form a thin layer of resin between the layers of mesh (Zhang & Zhang, 2014).

Many different sorbents have been used for passive sampling all over the world, from DIAION HP20 to SEPABEADS type resins, for the accumulation of microalgal or cyanobacterial toxins of different polarities (Zendong et al., 2014). Cyanotoxins have different chemical properties (such as polarity), and this complicates their simultaneous adsorption and determination. The choice of sorbent therefore plays a significant role in the type of toxins to be sampled and extraction which follows.

The most commonly used sorbents are classified into polar (normal phase), non-polar phases (reserved phase), ion-exchange, and immune-affinity adsorbents, where each one offers unique types of interaction forces. Polar phases are sorbents that are used under normal phase chromatographic condition. They consist of sorbent media such as Florisil, alumina, and polar functionalized silica bonded sorbents (Mashile & Nomngongo, 2017). Another type is the polar-functionalized bonded silica sorbents made up of silica material that has been modified by functional groups, such as cyano (SPE-CN), aminopropyl (SPE-NH3), and diol (SPE-Diol) on the surface of SPE material (Mashile & Nomngongo, 2017). DIAION HP20, a non-polar copolymer styrene-divynilbenzene adsorbent resin has been proved to be an efficient sorbent in accumulating ciguatoxin and maitotoxin in *Gambierdiscus pacificus* cultures (Zendong et al., 2014) and is commonly applied in SPATT for the passive sampling of cyanotoxins, particularly microcystins.

The SPATT collects a relatively clean sample matrix which simplifies subsequent extraction and analysis using ELISA or LC-MS. The results of SPATT in several field studies have been described, implying its potential for use as an early warning for the onset of algal blooms (Zhang & Zhang, 2014).

2.6.3.1 Applications of SPATT in cyanotoxins monitoring

The adsorbent-based solid phase extraction (SPE) and SPATT have become the preferred device for the concentration of analytes at the trace level (Zhang & Zhang, 2014). Several studies have applied passive sampling techniques and have proved it to be a helpful tool for the monitoring of natural toxins such as cyanotoxins because of their high spatial and temporal variability, which is rather difficult to meet with conventional grab sampling (Kohoutek et al., 2008).

Zhang & Zhang (2014) documented an evaluation of the usefulness of the commercially available polymeric Oasis HLB and Strata-X sorbents in both laboratory and field applications for various microalgal toxins. Zhang & Zhang (2014) report that Strata-X and Oasis HLB are fast accumulators and better for daily or on-board evaluation of toxin presence, whereas DIAION HP20 was found to be more appropriate for prolonged exposure period (>5 days).

Since the first application of integrative passive samplers for algal toxins by MacKenzie et al. (2004), they have been successfully used for the monitoring of cyanotoxins in both seawater and freshwater. Howard et al. (2017) applied SPATT to monitor wetlands conditions in San Diego (USA) to provide an insight into the overall toxin prevalence during the summer season in wetland waterbodies. SPATT bags were deployed for approximately 1-month intervals for a period of three months in the year 2012 and grab samples were also collected with each SPATT deployment and retrieval (Howard et al., 2017). In this study, SPATT results indicated a much higher prevalence of MCs throughout the region than the grab sample results, pointing towards the probability of a low-level but chronic exposure via direct as well as indirect pathways (Howard et al., 2017).

Miller et al. (2010) employed Solid Phase Adsorption Toxin Tracking (SPATT) samplers to investigate land-sea flow microcystin intoxication with trophic transfer through marine invertebrates. Because bloom events are often ephemeral and patchy, sensitive methods were required to facilitate source tracking efforts. Miller et al. (2010) used resin-based, Solid Phase Adsorption Toxin Tracking (SPATT) samplers to passively monitor fresh and salt water for microcystin contamination and demonstrated the excellent adsorption characteristics of SPATT resin-based systems for microcystin detection in both fresh and salt water. Miller et al. (2010) found that the SPATT samplers were more sensitive than periodic grab samples for field detection of microcystins and they also found the ability to evaluate samples for the presence of multiple bio-toxins simultaneously as an additional bonus.

New sorbents for optimal sampling of toxins will continue to be developed. One of the aims of the present work was to evaluate the applicability of the SPATT passive sampling approach in monitoring of cyanobacterial toxins microcystins in agricultural water resources. Laboratory and field experiments on the viability of chitosan-based resin in SPATT were also evaluated.

2.7 Conclusions

Literature reviewed here demonstrated that agriculture plays a significant role in the South African economy. However, most farmers rely on untreated surface water for irrigation of their vegetables, crops and for their livestock and this could be contaminated with cyanobacterial toxins and other pollutants. Incidents of cyanobacterial blooms have been on the increase in most of the country’s major reservoirs and rivers because of poorly managed and run municipal wastewater treatment plants, industrial activities, and the changing climate, which seems to promote harmful cyanobacterial blooms.

The literature also showed that most rivers and reservoirs in highly industrialized and populated areas of South Africa such as the Vaal system and Crocodile-West Marico Water Management Area are severely polluted with a range of other pollutants. High salinity, toxic metal species and Persistent Organic Pollutants (POPs) have all been reported to be significant issues of concern. With that in mind, it is thus important to investigate the synergism of multiple stressors in water being used for agricultural purposes and agricultural soils in South Africa and how this could be impacting on productivity and the possible human and animal health impacts.

Despite the predicted increase in incidents of harmful cyanobacterial blooms in South Africa, strategies for monitoring and managing them tend to be reactionary and there is a lack of proactive early warning capabilities. Currently any basic and applied research involving algal toxins, still relies on conventional grab sample collection followed by laboratory clean-up and analysis. This is despite the drawbacks of grab sampling as discussed here. Considerable effort is required towards the use of passive sampling for time-integrated concentrations of algal toxins in agricultural waters and the use of SPATT is suggested. This is because SPATT is cheap, easy to use, sensitive and reliable for the monitoring of these toxins.

With a huge burden of other diseases such as HIV and AIDs, Tuberculosis and diarrheal-related diseases, the South African population could be at substantial risk to the health impacts of cyanotoxins. The major risk could be via long-term exposure to low levels of the toxins through diet. It is thus recommended that the South African water sector, industry, and authorities prioritizes research addressing issues specific to cyanotoxins in irrigation water and development of local guidelines/regulations for cyanotoxins in agricultural water.

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CHAPTER THREE: BIOACCUMULATION OF MICROCYSTINS IN *BRASSICA OLERACEA* AND *SOLANUM TUBEROSUM*

Abstract

This chapter explored the bioaccumulation potential of MCs in distinct parts of the plants *Brassica oleracea* and *Solanum tuberosum*. To investigate MCs uptake, accumulation capacities in edible parts of the plants pot-culture experiments with cabbage (*Brassica oleracea*) and cultivated potato (*Solanum tuberosum*) were conducted. Cabbage (*Brassica oleracea*) and cultivated potato (*Solanum tuberosum*) were selected for the field experiment as they are commonly cultivated in the study area. The results obtained from the pot-culture experiments are expected to contribute in the understanding of the potential transfer of MCs from irrigation water to the plants via the soil, when cyanobacteria infested water is used for irrigation purposes. Data gathered in this chapter is expected to contribute to further understanding of the bioaccumulation of different MCs congeners on *Brassica oleracea* and *Solanum tuberosum*.

Water used to irrigate the plants had MC levels ranging from 0.12 to 2.84 μgL^{-1}. The pH for the water was slightly alkaline (pH 7.29 ±0.71 to 10.03±0.29) and the EC ranged from 296.67±13.87 to 878.67±42.44 $\mu S\ cm^{-1}$. Findings indicated that the levels of MCs and other pollutants in the Roodeplaat dam water have a significant impact on the seed germination of *Brassica oleracea* but did not show significant impact on the general plant growth, nor induce significant oxidative stress as demonstrated by comparable total chlorophyll between the trial plants and the controls.

The findings from this chapter demonstrated that the two plants can bioaccumulate MCs to concerning levels when irrigated with water derived from the Roodeplaat Dam. MCs accumulation levels in the two tested plants ranged from 0.00142 to 0.136 mg kg^{-1} DW for individual MC congeners and this was comparable to the concentrations reported in other studies. In *B. oleracea* leaves, higher levels of the toxins were found in plants exposed to higher levels of MCs, with uptake and accumulation increasing with the concentrations of exposure. The EDI values (derived from the highest mean concentrations from the three treatments) for the plants did not exceed the 0.04 TDI limit set by the WHO. Importantly, the EDI values were calculated for the individual toxins, since the TDI was set for MC-LR only, but if the EDI values for the three toxins were to

be combined, they would have exceeded the total daily intake limit set by the World Health Organization.

In *S. tuberosum* statistically significant higher levels of MC-YR and MC-LR were accumulated in the tubers ($p < 0.05$, Mann-Whitney Test/ Unpaired Student *t* test), whereas no statistically significant differences between the treatments (experimental and controls) were recorded for MC-RR ($p > 0.05$, Mann-Whitney Test/ Unpaired Student *t* test). Much higher levels of MC-LR and MC-YR were accumulated in the experimental plants (exposed to raw dam water) compared to the control plants (exposed to milli-Q water only). Of importance, *S. tuberosum* tubers exposed to the raw dam water accumulated MC-LR to levels exceeding the TDI as recommended by the WHO, thus demonstrating a potential risk to the consumers of such plants if they are irrigated with water from the dam.

The findings, together with findings in other studies discussed here, demonstrates terrestrial food crops can accumulate MCs to levels that can pose human-health risks when exposed to naturally relevant levels of MCs.

3.1 Introduction

There is a possibility that terrestrial plants, particularly those of the human diet, could be contaminated by cyanotoxins contained in irrigation water. Such a possibility has motivated researchers to evaluate the effects of cyanotoxins on these organisms, paying particular attention to microcystins (Bittencourt-Oliveira et al., 2014). Cyanobacterial toxin uptake by crop plants occurs when irrigation is done with cyanobacteria-containing water. Cyanobacterial toxins are phytotoxic. This means that they are toxic to and can induce negative responses in plants. When these are accumulated in crop plants, it poses serious human health risks when they enter the food chain (Purkayastha et al., 2010).

It is evident that the consumption of edible plants exposed to cyanotoxins via irrigation may have health risks. Currently there are no regulatory limits for microcystin-LR loads in plant tissue in the United States (Milligan, 2009). Crush et al. (2007) recommended investigation of the fate of cyanobacterial cells and toxins during and after spray irrigation with water containing cyanobacteria, to contribute to the development of policies on the use of such water and the acceptability of plants for human consumption after irrigation with contaminated water. Purkayastha et al. (2010) state that special attention should be given to the presence of cyanotoxins in crop plants and their degree of accumulation, to take exceptional care in avoiding their contamination in human foodstuff. The potential for bioaccumulation of multiple cyanotoxins in the food web suggests that the influence of toxic cyanobacteria blooms are a much more complex stressor than presently recognized and should be considered a high priority measurement to be included in condition assessments and water quality monitoring programs (Howard et al., 2017).

To investigate MCs uptake and accumulation capacities in the plant tissue, pot-culture experiments with cabbage (*Brassica oleracea*) and cultivated potato (*Solanum tuberosum*) were conducted. Cabbage (*Brassica oleracea*) and cultivated potato (*Solanum tuberosum*) were selected for the field experiment as they are commonly cultivated in the study area. Data obtained from the pot-culture experiments are expected to contribute in the understanding of the potential accumulation of MCs when cyanobacteria infested water such as that derived from Roodeplaat and Hartbeespoort Dams is used for irrigation purposes.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Three congeners of MCs (MC-LR, MC-RR, and MC-YR) (95% purity) were purchased from Cyano Biotech GmbH (Berlin, Germany). Acetonitrile (≥99.9%) and Water HiPerSolv CHROMANORM® (VWR Chemicals, Fontenay-sous-Bois, France)) for LC-MS were purchased from Monitoring & Control Laboratories. HPLC-grade methanol and formic acid (FA) were supplied by Merck (Darmstadt, Germany). For the SPE, Waters Oasis HLB 3cc, 60mg, were purchased from Promolab Pty Ltd T/A Separations (South Africa).

3.2.2 Plant growth conditions

The cabbage seeds were purchased from NTK Agricultural Products & Services, and the potato seeds were purchased from Livingseeds Heirloom Seeds (Pty) Ltd Midvaal, Gauteng. All the potato seeds were first washed with distilled water before being planted into 20 L plant bags filled with non-contaminated soil. The cabbage seedlings were produced and pre-grown in plastic trays with non-contaminated soil. The soils used in the experiments were collected from a field in the

School of Agriculture, University of Venda which was assumed to be free of cyanobacterial contamination. The soils were collected from the top 20 cm, air dried for 48 hours and passed through a 2 mm sieve before use. The soil was mixed with 0.375 g (per kg of soil) of Protek General Fertilizer with N:P:K (%) 2:3:2 (14) before introducing the plants. Upon treating the soil, fourteen-day old cabbage seedlings were transplanted (one plant per pot/bag) into the bags.

The potential for toxin transfer to irrigated crops was evaluated through pot-culture experiments. Replicate cabbage and potato plants were grown with irrigation using raw dam water collected from Roodeplaat Dam. Controls were run concurrently with the treatments (i.e., watered using MCs free water). The plants were watered daily with 500 mL of their respective water. To better monitor the effects of the field water on the plants, a third treatment for *B. oleracea* plants was introduced where the plants were watered with 50% (v/v) field-water/deionised water. Figure 3.1 shows the layout of the plants after 27 days of planting. Four replicates (individual plants/ one seed per pot) and three treatments of the *Brassica oleracea* plant species and the experiment was repeated once i.e., 4X3X2=**24** plants in total. For the *Solanum tuberosum* plant species there were 4 replicates (individual plants/ one seed per pot) and two treatments and the experiment was repeated once i.e., 4X2X2=**16** plants in total. The pots were placed in randomized row design and were rotated after every two days to minimize the micro-effects. The plants received an average daily photosynthetic photon flux density of 1,000 μmol s^{-1} m^{-2} and were exposed to 14 hours of sunlight and average high temperatures of 29°C and minimum of 15°C.

Figure 3. 1. Layout of the experimental set-up showing *B. oleracea* on the left and *S. tuberosum* on the right

Plant leaves for *B. oleracea* and plant tubers for the *S. tuberosum* were harvested after 20 days of exposure to the various treatments and were freeze-dried followed by solid phase extraction (SPE). The subsequent toxin concentrations in the plant tissue was determined using the ELISA method with verification by LC-MS/MS. The entire plants were destructively harvested at maturity (after 84 days for *S. tuberosum* and 120 days for *B. oleracea*) to monitor any visible side effects.

3.2.3 Characterization of the field water

Measured physicochemical parameters of the dam water used for irrigating the plants included levels of chlorophyll a; pH, Electrical Conductivity (EC); Total Dissolved Solids (TDS); and Turbidity.

Physicochemical parameter such as pH, TDS and EC were determined in the field using Jenway pH/Cond meter model (430). Turbidity was determined using TB200 portable turbidity meter model (#TB200-10). All instruments were calibrated following the manufacturers' instructions prior to analysis. Nutrients (nitrates and dissolved phosphates) in the water were determined in the laboratory using a Merck Pharo 100 Spectroquant (using suppliers test kits). (Merck Spectroquant Pharo 100 spectrophotometer, product number 100706, Darmstadt, Germany) and commercially available test kits, using standard methods (Merck Pty Ltd, products: 1.14559.0001, 1.14752.0001, 1.14776.0001, 1.09713.0001, 1.14842.0001, 1.14895.0001). Each sample was filtered through an eight-micron filter paper prior to analysis to remove suspended solids.

Total MCs in the Roodeplaat dam water used were determined using the commercially-available ELISA Microcystin Plate Kits (ENVIROLOGIX INC. (Kit Lot: 071499 Cat No: EP 022)). This assay uses antibodies against microcystin-LR and a microplate reader Spectro-star Nano (BMG LABTECH, 601-1106, Germany) was used to quantify the MCs after the assay. Prior to analysis, 5 mL of each sample was filtered using the 0.20 µm glass fiber syringe filters and 50 µL of the filtered sample was used for the assay. To determine the levels of the different congeners MC (-LR, -YR and –RR) a triple quadrupole LC–MS/MS system (model 8045, Shimadzu Corporation, Japan) was used (section 3.2.5.2).

3.2.4 Monitoring the effects of MCs on plants

Data on general health of the plants including plant height, biomass and oxidative stress known to be induced by MCs in plants were monitored.

3.2.4.1 Estimation of chlorophyll content

To determine oxidative stress induced by MCs in the plants, total chlorophyll estimation was used as an indirect method for the detection of reactive oxygen species (ROS) in the plants. ROS generation in a stressful environment cause the changes in chlorophyll, anthocyanin, compatible solutes, and membrane integrity in plants, therefore, ROS generation can be measured indirectly by measuring the changes in these compounds (Venkidasamy et al., 2019).

Chlorophyll content was measured according to Baskar et al. (2015). In brief, 50 mg of the plant leaves was sliced into small pieces and soaked in 95 % (v/v) ethanol and then incubated for 3 days in the dark. The supernatant was read at 664.2 and 648.6 nm by UV-vis spectrophotometer

(SPECTROstar Nano, BMG LABTECH, Germany). Chlorophyll a and b and total chlorophyll content were calculated using the following equations:

$$Chl\ a = 13.36\ A664.2 - 5.19\ A648.6 \qquad [3.1]$$

$$Chl\ b = 27.43\ A648.6 - 8.12\ A664.2 \qquad [3.2]$$

$$Total\ chlorophyll = Chl\ a + Chl\ b. \qquad [3.3]$$

N.B: Total chlorophyll content was expressed as milligram per gram per fresh matter (FM).

3.2.4.2 Data analysis

To compare the experimental plants and their respective controls, analysis of variance (ANOVA) and/or the Kruskal-Wallis *H* test was used at 95% confidence interval.

3.2.5 Bioaccumulation and plant biochemical responses upon exposure to MCs

3.2.5.1 MCs accumulation capacity

For better understanding of the bioaccumulation of MCs in various plant organs, accumulation experiments with the two plant species using water collected from the Roodeplaat Dam were conducted.

The levels of the toxins in the various plant tissues were determined monthly for the entire cycle of the experiments (using ELISA and LC-MS/MS for the content of MCs). To determine environmental relevant concentrations for the cyanotoxins characterization in raw water and in biomass were conducted with LC-MS/MS.

3.2.5.2 Quantification of MCs in plant material

The bioaccumulation of MCs (MC-LR, MC-RR and MC-YR) in plant tissues was investigated by the ELISA and LC-MS method. Toxin extraction was performed by first freeze-drying 100 g of each plant (leaves for *B. oleracea* and tubers for *S. tuberosum*) for 48 h and -48^{o}C under a constant vacuum of 44 µmHg (Telstar Lyoquest Freeze Dryer, Terrassa, Spain). The freeze-dried material was then ground to powder using a mortar and pestle.

To extract the MCs from the freeze-dried plant material, a modification of a method by Manubolu et al. (2018) was adopted. In brief, 10 mL of 50% Methanol solution was added to 1 g of each freeze-dried sample sonicated for 5 minutes in a water bath (SCITEC Ultrasonic Cleaner, Model 705, South Africa) for 5 minutes. Upon sonication, the plant extracts were then centrifuged for 30 minutes at 1750 rpm. The entire process of sonication and centrifuging and collecting the supernatant was repeated thrice and the supernatant was pooled to give a 30 mL extract of each sample. The 30 mL extract was then cleaned up using solid phase extraction (SPE) with HLB (3cc, 60mg, Waters Oasis).

The HLB cartridges were preconditioned with 6 mL of methanol, followed by 6 mL of ultrapure water. Samples (approximately 30 mL) were applied to the column slowly, and then rinsed afterwards with 20% methanol. Samples were then eluted from column using 10 mL of 80% methanol. The final eluent was evaporated to dryness under a gentle stream of nitrogen (N_2) gas, with the residue suspended in 1 mL of 80% methanol and then subsequently filtered through 0.2 µm Polypropylene (PP) Syringe Filters (Stargate Scientific, South Africa). MC-LR, MC-RR and MC-YR, content in the extracts was then measured using the ELISA method and a triple quadrupole LC–MS/MS system (model 8045, Shimadzu Corporation, Japan).

3.2.5.2.1 Chromatographic Conditions

A triple quadrupole LC–MS/MS system (model LCMS-8045, Shimadzu Corporation, Japan), was used in this work for development of LC-MS/MS method. Data acquisition and data analysis were performed with LabSolutions. Analysis of Microcystins (LR, RR and YR) was conducted on LCMS-8045 with a Shim-pack Velox SP-C18, 2.7 µm, with dimensions 2.1 * 100 mm (Shimadzu, Japan). The injection volume of samples was 10 µL and the mobile phases used were 0.1% FA in water (A) and 0.1%FA in acetonitrile (B). A flow rate of 0.5 mL min^{-1} and a 5-minute binary gradient was used with an elution profile of: 5% B (0.4 min), linear gradient to 95% B (3.1 min), 100% B (0.5 min), and, finally, 5% B (1 min). The interface conditions of LCMS-8045 were set as follows: the ESI interface temperature at 300 ºC, DL temperature at 235 ºC, nebulizing gas flow at 3 L.min^{-1}, drying gas flow at 10 L.min^{-1} and heating gas flow at 10 L min^{-1}. The interface voltage was set at 3.0 kV for positive (ES^{+}) electrospray.

The final concentration of the toxins in the samples was determined by using equation 3.4:

$$\text{Conc in sample } (\mu g/mL) = \left(\frac{\text{Co} * \text{Vol of extract used (L)}}{\text{Volume of sample used (L)}}\right) \quad [3.4]$$

Where: Co = the concentration of sample determined from the calibration curve (µg mL^{-1})

MCs in plant material, were also quantified using a commercially available ELISA Microcystin Plate Kits supplied by Envirologix (Kit Lot: 071499 Cat No: EP 022) and EUROFINS (Kit Lot No: 19I1120: PN 520011) using SPECTROstar Nano (BMG LABTECH, 601-1106, Germany) for quantification. Prior to analysis, 5 mL of each sample was filtered using the 0.20 µm syringe filters.

3.2.5.3 Microcystin accumulation capacity data analysis

One way analysis of variance was used to detect significant differences in bioaccumulation as a function of different MCs treatment. All analyses were conducted at 5% significance level.

3.2.4.4 Estimated Daily Intake (EDI)

To estimate the daily intake of MCs for an average size human we are using equation 3.5:

$$\text{EDI} = \frac{T * C}{W} \quad [3.5]$$

Where:

T = The concentrations of toxins in the edible fractions of the cabbage (T, µg kg^{-1} fresh weight)
C = the daily consumption amounts of cabbage (*C*, kilograms per day)
W = weight of an average-sized human (*W*, 60 kg adult).

N.B: We assumed consumption of cabbage is similar to that of lettuce and used 85 dry weight (DW) grams of cabbage and 148 g dry weight (DW) for potatoes based on the U.S. FDA (2017) suggested serving size (Bartos, 2020). An EDI of > 0.04 exceeds the total daily intake limit set by the World Health Organization.

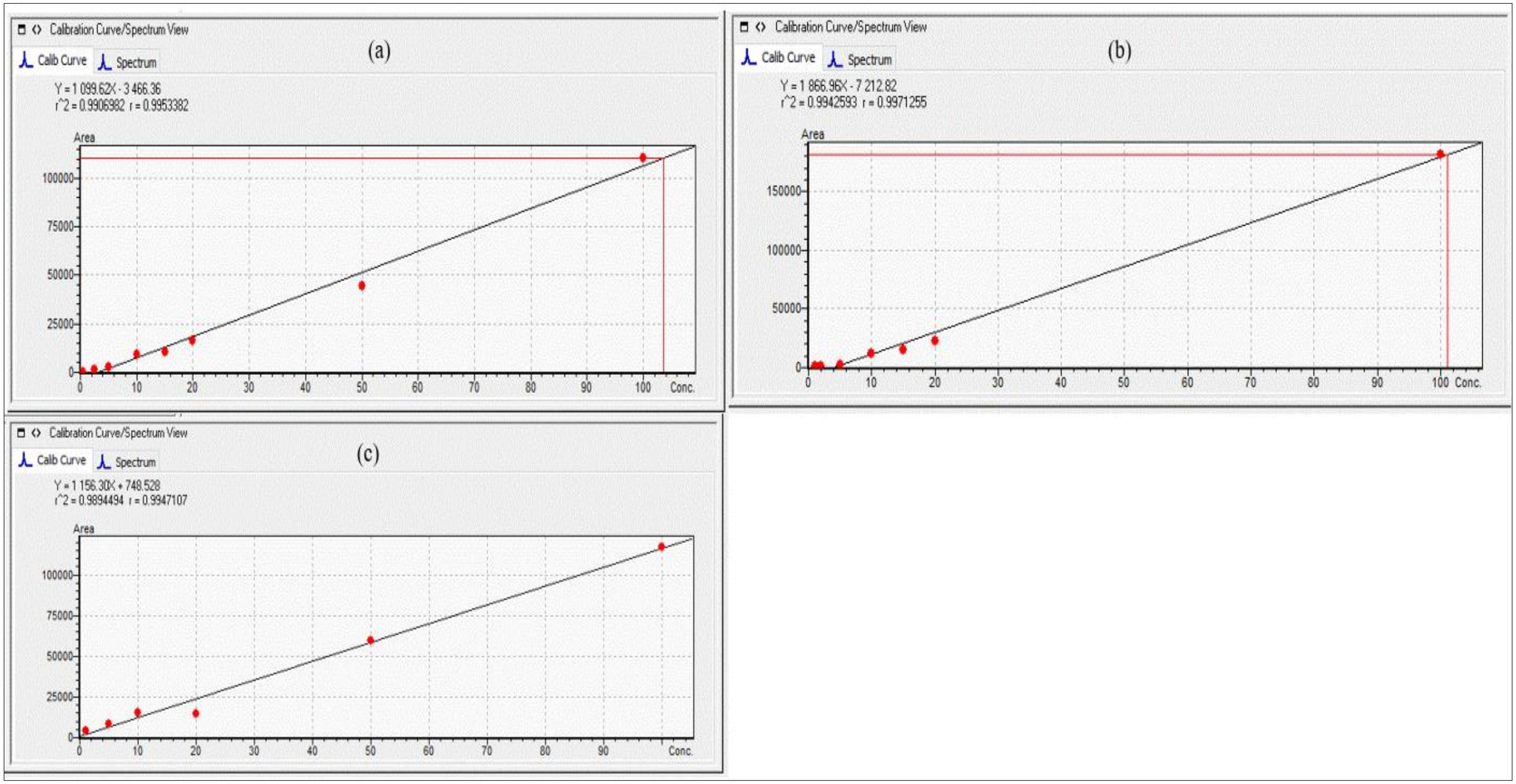
3.3 Results and discussion

3.3.1 Bioaccumulation of MCs in *Brassica oleracea* and *Solanum tuberosum*

To identify and quantify MCs, two multiple reaction monitoring (MRM) transitions for MC-LR and MC-YR were selected and optimized, with the most abundant product ion for quantitation and the other one for confirmation whereas for MC-RR only one transition was used. For MC-LR and MC-YR, the single protonated molecular ions $[M+H]^+$ were formed, and this is as a results of the presence of one arginine moiety, which is the most preferred protonation site for these compounds (Zervou et al., 2017). The MRM transitions employed for MC-LR were 996.0078/996.00 and 498.5078/162.90; for MC-RR: 520.0078/134.90; for MC-YR: 1046.5078/1046 and 523.7578/127.00, with the first one being used for quantification and the second one for confirmation (for MC-LR and MC-YR).

With respect to all the three MCs monitored, the m/z signal 135 was the main product ion. Like other polypeptides, MCs form sodium replacement ions which results in ion envelopes at each charge state apparent in mass spectra (Draper et al., 2013). For MC-RR the transition with a m/z of 520.0078 corresponding to the double charged protonated molecular ion $[M+2H]^{2+}$ precursor ions, as they contain two arginine residues in their molecular structure. (Draper et al., 2013; Zervou et al., 2017).

Standard solutions at 7 different concentrations (1, 2, 5, 10, 20, 50 and 100 µg L^{-1}) were prepared in cabbage leaves extracts and in potato tuber extracts from control plants and these were used to quantify the toxins in the plant samples (Figure 3.2). The MRM chromatograms of the quantification ions for the 3 MCs at a concentration of 100 µg L^{-1} are shown in Figure 3.3.



(a)

Y = 1 099.62X - 3 466.36
r^2 = 0.9906982 r = 0.9953382

(b)

Y = 1 866.96X - 7 212.82
r^2 = 0.9942593 r = 0.9971255

(c)

Y = 1 156.30X + 748.528
r^2 = 0.9894494 r = 0.9947107

Figure 3. 2. Calibration curves obtained for (a) Microcystin-RR (MC-LR); (b) MC-YR; and (c) MC-LR; in plant media

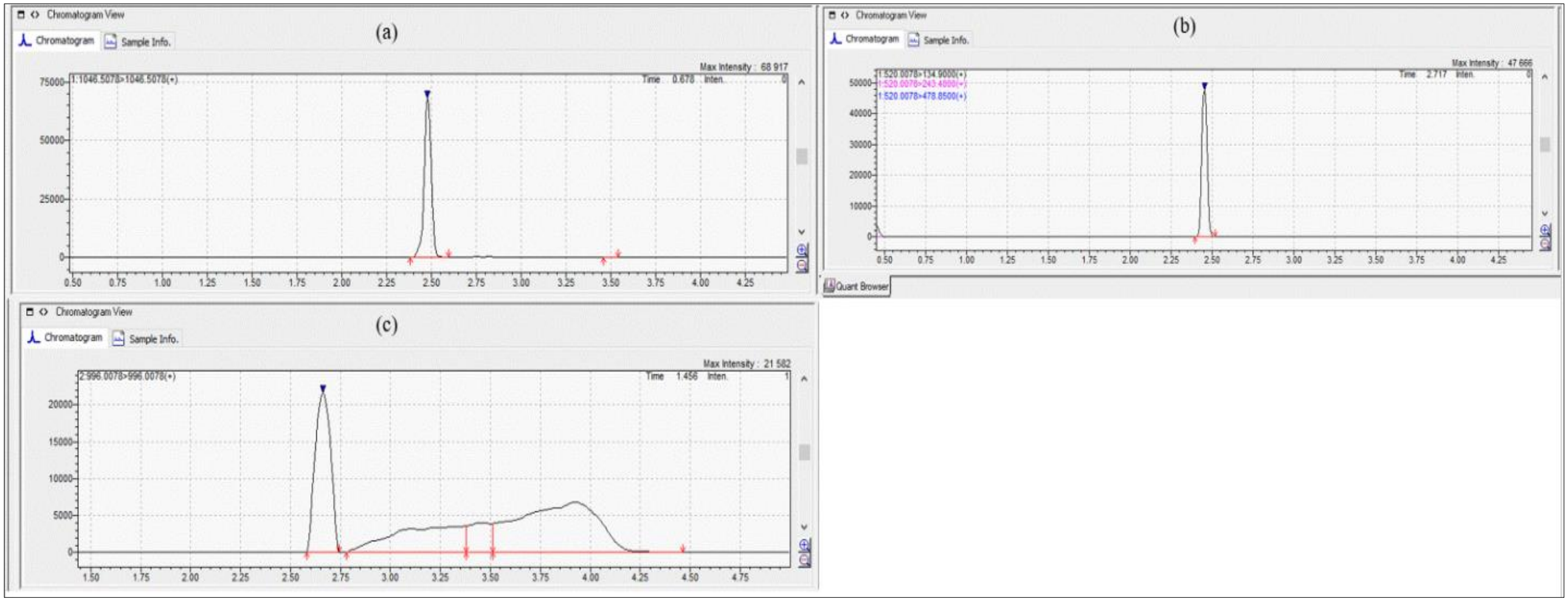


Figure 3. 3. MRM chromatograms of quantification ions for the 3 MCs, (a) MC-RR; (b) MC-YR and (c) MC-LR at a concentration of 100 µg L^{-1}

The raw dam water used had the following mean concentration (± standard deviation) of MC-LR: 60.920 (±10.879); 6.158 (± 4.127) for MC-RR and 8.160 (± 2.544) for MC-YR. With regards to the accumulation of the toxins by the two plant species, results from the ELISA tests where in most cases affected by interferences. Tables 3.1 and 3.2 shows the MCs levels accumulated in in *B. oleracea* leaves and in in *S. tuberosum* tubers upon 20-day exposure to the 3 and 2 treatments, respectively. No statistically significant differences were found in the mean levels of MC-RR and MC-LR among the three treatments even though higher levels of MC-RR were found in the plants exposed to diluted dam water and higher levels of MC-LR were reported in plants exposed to raw dam water (Mann-Whitney test/ unpaired Student *t* test at 0.05 level of significance. Statistically significant differences were found in the mean levels of MC-YR among the 3 treatments, with plants exposed to raw dam water having accumulated statistically higher levels ($p< .05$, ANOVA and/or the Kruskal-Walli's test) of the toxin compared to the other two treatments (diluted dam water and controls (Milli-Q water only).

In as much as higher levels of the toxins were found in plants exposed to higher levels of MCs, with uptake and accumulation increasing with the concentrations of exposure, the EDI values (derived from the highest mean concentrations from the three treatments) for the plants did not exceed the 0.04 total daily intake limit set by the World Health Organization. Importantly, the EDI values were calculated for the individual toxins, since the TDI was set for MC-LR only, but if the EDI values for the three toxins were to be combined, they probably would have exceeded the total daily intake limit set by the World Health Organization.

Table 3. 1. Mean (±SD) of MCs accumulated in *B. oleracea* leaves upon 20-day exposure to the 3 treatments

	Raw Dam Water	**Diluted Dam Water**	**Control**	***P*-value**	**EDI** (mg kg^{-1} of body mass day^{-1})
MCRR (μg g^{-1} DW)	0.00562 (±0.00480)^{a}	0.00672 (±0.00695)^{a}	0.00039 (±0.00027)^{a}	0.1784 (*n.s*)	0.010
MCYR (μg g^{-1} DW)	0.00439 (±0.00074)^{a}	0.00275 (±0.00140)^{b}	0 0.00050 (±0.00018)^{c}	< 0.0001***	0.006
MCLR (μg g^{-1} DW)	0.00207 (±0.00320)^{a}	0.00171 (±0.00169)^{a}	0.00001 (±0.00001)^{a}	0.3371 (*n.s*)	0.003

Note: *P < 0.05, **P <0.01, ***P < 0.001, n.s = not significant.
N.B: Data labelled with different small letters (a- c) differed significantly at $p < 0.05$ in each row.
See appendices 3.1-3.3 for details of the ANOVA/ Kruskal-Wallis tests analysis

The mean levels of MCs accumulated in *S. tuberosum* are shown in Table 3.2. Statistically significant higher levels of MC-YR and MC-LR were accumulated in the tubers ($p < 0.05$, Mann-Whitney Test/ Unpaired Student *t* test), whereas no statistically significant differences between the treatments (experimental and controls) were recorded for MC-RR ($p > .05$, Mann-Whitney Test/ Unpaired Student *t* test). Much higher levels of MC-LR and MC-YR were accumulated in the experimental plants (exposed to raw dam water) compared to the control plants (exposed to Milli-Q water only). Of importance, *S. tuberosum* tubers exposed to the raw dam water accumulated MC-LR to levels exceeding the TDI as recommended by the WHO, thus demonstrating a potential risk to the consumers of such plants if they are irrigated with water from the dam.

Table 3. 2. Mean (±SD) of MCs accumulated in *S. tuberosum* tubers upon 20-day exposure to the 2 treatments

	Experimental	**Control**	***P*-value**	**EDI** (mg kg^{-1} of body mass day^{-1})
MCRR (µg g^{-1} DW)	0.00142 (±0.00262)^{a}	0.00014 (±0.00026)^{a}	0.8361 (*n.s*)	0.002
MCYR (µg g^{-1} DW)	0.00869 (±0.00279)^{a}	0.00044 (±0.00017)^{b}	< 0.0001***	0.012
MCLR (µg g^{-1} DW)	0.13551 (±0.09443)^{a}	0.01413 (±0.01000)^{b}	0.0003***	0.192

Note: *P < 0.05, **P <0.01, ***P < 0.001, n.s = not significant.
N.B: Data labelled with different small letters (a- c) differed significantly at $p < 0.05$ in each row.
See appendices 3.4-3.6 for details of the Unpaired *t* test/ Mann-Whitney test analysis

The findings appeared not to demonstrate an increase in toxin levels in the plant tissue based on the levels of toxins *B. oleracea* and *S. tuberosum* were exposed to, even though we were seeing elevated levels of toxins in plants exposed to raw dam water, but there were no significant differences with diluted dam water for all the three MCs assessed here. This was contradictory to the findings of Bartos (2020) who found increasing levels of MCs in shoots and roots of lettuce as the concentrations of MC-LR in treatments increased. Exposing lettuce plants to concentration of MC-LR which were at least ten times higher (620 to 12500 µg L^{-1}), than what we applied in this study, Hereman & Bittencourt-Oliveira (2012), found that the humans could be exposed to MC-LR levels as high 0.33 to 7.11 mg per day, which easily exceeded the 0.04 mg kg^{-1} of body mass day^{-1} recommended by the WHO.

The findings of this study indicated that *S. tuberosum tubers* were accumulating higher levels of MCs compared to *B. oleracea leaves.* Previous studies for example Crush et al. (2007), have reported high bio-accumulation in roots compared to other parts of the plant. This places food crops such as potatoes and other plants where the root is the edible part as plant crops which are likely to pose a greater risk of transfer of MCs via diet.

This section, reported MCs accumulation levels in the two tested plants to be ranging from 0.001415 to 0.135508 mg kg^{-1} DW for individual MC congeners and this was comparable to the concentrations reported by Crush et al. (2007), who reported mean MCs concentrations of 0.79 mg kg^{-1} DW in commercial lettuce crops irrigated with cyanobacteria infested water. The findings, together with findings in other studies discussed here, demonstrate terrestrial food crops can accumulate MCs to levels that can pose human-health risks when exposed to naturally relevant levels of MCs.

3.3.2 Physicochemical parameters of the soil and field water

Water used to irrigate the plants was collected from Roodeplaat Dam (Pretoria), next to the dam wall in 20L containers and frozen till use. The water was collected in June, September, and November 2020. The raw dam water used had the following mean concentration (± standard deviation) of MC-LR: 60.920 (±10.879); 6.158 (± 4.127) for MC-RR and 8.160 (± 2.544) µg L^{-1} for MC-YR. The winter period (month of June 2020) having higher levels of MCs compared to water collected in spring (September 2020) and summer (November 2020). The levels of MCs in the sampled water were lower than anticipated. Previous studies have reported median concentrations of MCs of 580 µg L^{-1} and a maximum concentration of 14 400 µg L^{-1}, with the lowest levels consistently exceeding 10 µg L^{-1} (Turton, 2015). Mbiza (2014) found total MCs at Roodeplaat Dam to be as high as 2.5 µg L^{-1} during the summer months. Contrary to the findings of this study, Mbiza (2014) reported high toxin levels in the summer months in the same reservoir, whereas in this study, higher levels were reported in the winter months.

The pH for the water was slightly alkaline (pH 7.29 ±0.71 to 10.03±0.29). With regards to pH the water used to irrigate the plants was within the permissible limit according to the South African and FAO guidelines. The EC for the sampled water used ranged from 296.67±13.87 to

878.67±42.44 µS cm^{-1} and was in most cases higher than the South African guideline and FAO limits for irrigation water.

In this study, chlorophyll-*a* was used to give an estimate of phytoplankton biomass (Ramaraj et al., 2013). South Africa like other countries across the globe, has no regulations or policies on cyanobacteria and MCs in water intended for irrigation of food crops (Pindihama & Gitari, 2020). Phytoplankton biomass as chlorophyll-*a* was relatively high in the sampled water, ranging from 49.86±76.26 to 153.70±177.54, with high readings reported in the June 2020 sample.

3.3.3 Possible effects of MCs and other contaminants on *Brassica oleracea* and *Solanum tuberosum*

Previous studies have found that MCs have various effects on plants and these include: the induction of oxidative stress; a reduction in germination rate and the inhibition of growth (Bittencourt-Oliveira et al., 2014; Machado et al., 2017b).

3.3.3.1 Effects on germination

A total of 231 cabbage seeds were grown in cavity germination trays. Of these, 121 seeds were watered with milli-Q water (control) and 110 with cyanobacteria infested dam water (experimental). Of the seeds watered with milli-Q water, a total of 102 (84.3%) had successfully germinated by the 8th day and only 14 (12.7%) successfully germinated (Table 3.3 and Figure 3.4).

Table 3. 3. Effect of dam water on *B. oleracea* seed germination

	No. of seeds	Germinated	Success %
Control	121	102	84.29%
Experimental	110	14	12.73%

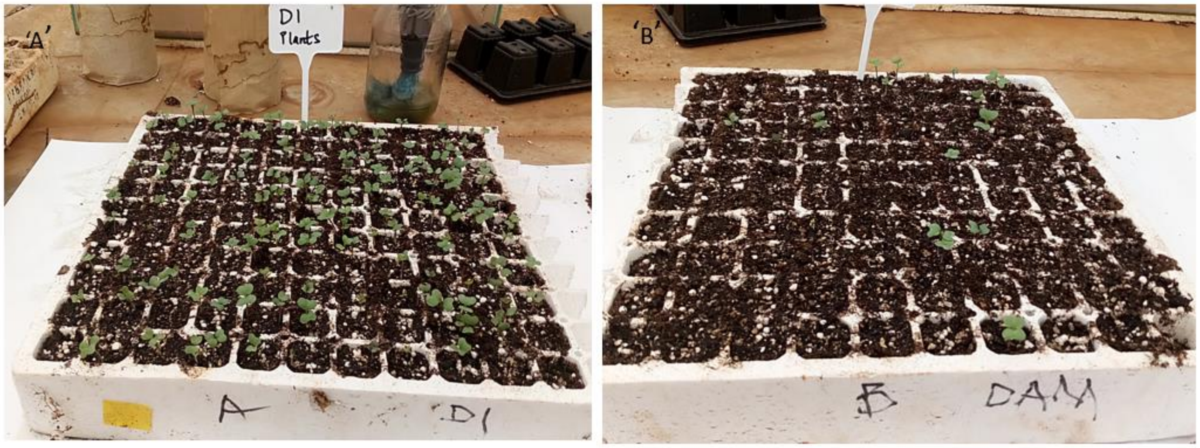


Figure 3. 4. Images of germinated seeds after 8 days of watering (a) with milli-Q water (b) with dam water

In consistent with these findings Nery et al. (2021) reported effects of cyanotoxins in seeds germination. Nery et al. (2021), found that the effect of cyanotoxins on seed germination is dependent on the sensitivity of the species being tested. Nery et al. (2021), used ultrapure water contaminated with extracts of the cyanotoxin, saxitoxin and MC-LR and found that the seeds of lettuce were most affected by saxitoxins and coriander was mostly affected by microcystin-LR (MC-LR). Even though they did not report much effects of MC-LR on germination rates as compared to saxitoxins, Nery et al. (2021) reported more incidence of seedling necrosis in MC-LR than in saxitoxin treatments.

Purkayastha et al. (2010), further reported that germination rates in trial seeds was lower compared to the control groups in numerous studies. According to Purkayastha et al. (2010), resistance to cyanotoxins varies with different plants. For example, rice seeds have been found to be more resistant than rape seeds. *Medicago sativa* showed inhibition of germination when exposed to cyanobacterial toxins (microcystins and anatoxin-a) and cyanobacterial cell-free crude extract. Reduction of germination rate was observed in *lens esculenta*, *zea mays*, *triticum durum* and *pisum sativum* when exposed to MC-LR (Purkayastha et al., 2010).

Saqrane et al. (2008) found a dose-dependent relationship in both germination inhibition and length decrease of the primary root of seeds of *pisum sativum L.*, *L. esculenta*, *Z. mays* and *T. durum* at concentrations between 1,600 -11,600 mg L^{-1} of cell-free extract containing MC-LR. According to Janiele et al. (2021), the main cause of limitation in germination by microcystins is related to inhibition of protein phosphatases. These are regulatory enzymes, and their inhibition causes hyper-phosphorylation of proteins, changing their activity status.

3.3.3.2 Effects on plant growth

MCs are known to affect plants' general well-being. The visual observations for the duration of monitoring did not show any significant differences between the two groups (control and experimental) for both plants *Brassica oleracea* and *Solanum tuberosum*. Figure 3.1 shows an image of the plants after 30 days of the experiment. Among other variables monitored to compare the plant growth of the two groups, was the plant height of *Solanum tuberosum* after 49 days of the running the experiment (Figure 3.5). The results showed that the experimental plants were growing better than the control plants, but the difference was not significant (unpaired Student's *t* test., two-tailed *p* value of 0.4541, considered not significant. The better growth of experimental plants watered with raw dam water compared to the control plants watered with milli-Q water only can be attributed to the nutrients in the dam water and not in the milli-Q water.

Several studies have reported plant growth inhibition due to exposure to cyanotoxins. Growth inhibitory effects have previously been reported by several (McElhiney et al., 2001; Pflugmacher et al., 2006; Crush et al., 2007). In most cases, effects on growth, and leave and root development were recorded. For example, growth of potato (*Solanum tuberosum*) cultures was reduced at 0.005 mg kg^{-1} MC-LR in a solid culture medium and completely inhibited at 0.5–5 mg kg^{-1} MC-LR. Growth of bean (*Phaseolus vulgaris*) plants in culture was inhibited by MC-LR at 1.12 mg kg^{-1} (McElhiney et al., 2001). A significant decrease in leaf and root lengths and in productivity of *Lepidium sativum* seedlings (Gehringer et al., 2003) were caused by cyanobacterial MC-extracts. These investigations suggest that exposure to cyanotoxins through irrigation of cyanotoxin contaminated water can pose a threat to the quality and yield of crop plants.

However, consistent with the findings of the current study, Järvenpää et al. (2007) did not observe any negative effects on the growth and chlorophyll content of the plants when exposed to cyanotoxins. Corbel et al. (2015) also studied the effects of MC-LR in tomatoes following irrigation with water containing 5–100 µg L^{-1} for 90 days and demonstrated that the toxin did not disturb the global growth of the tomatoes. Freitas et al. (2015) also suggested that lettuce plants can cope with low concentrations (1 and 10 µg L^{-1}) of MC-LR, CYN and an MCLR/CYN mixture by ensuring the maintenance of and even increasing their fresh weight. The growth increase promoted by low concentrations of cyanotoxins can be explained by the hormesis concept, which is characterized by an inverted U-shaped dose response (Machado et al., 2017). In explaining similar findings, Machado et al. (2017b), suggested that when a more realistic experimental design is established (i.e., environmentally relevant concentrations, longer exposure period and comparable soil growth conditions), the effects on plant growth are less pronounced. Since the current study used raw dam water and natural soil conditions, it is thus not surprising that less effects on plants were reported.

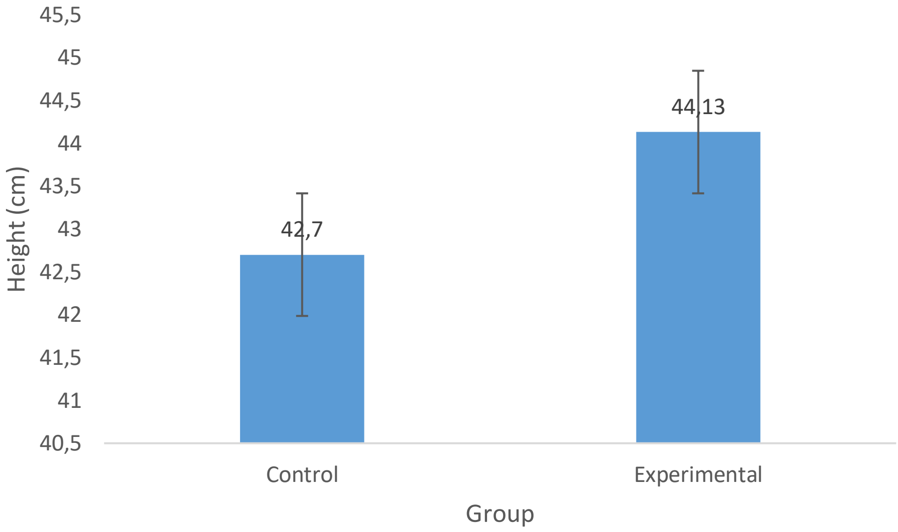


Figure 3. 5. Plant height of *S. tuberosum* after 49 days

3.3.3.3 Oxidative stress

The induction of oxidative stress by the production of reactive oxygen species (ROS) seems also to be an important biochemical mechanism of MCs toxicity in plant cells (Saqrane et al., 2008; Machado et al., 2017b). Since ROS generation in a stress environment is known to cause changes in chlorophyll, anthocyanin, compatible solutes, and membrane integrity in plants. ROS generation can thus be indirectly determined by measuring the changes in these compounds. To monitor oxidative stress possibly induced by cyanotoxins in the dam water, we thus measured and compared total chlorophyll in the two groups for both plants, after 57 days of the experiment.

The total chlorophyll of the plants after 57 days of the experiment is shown in Figures 3.6 and 3.7. In Figure 3.6, the total chlorophyll of *Solanum tuberosum* was found to be higher in the experimental plants compared to the control plants, even though the difference was not significant (unpaired Student's *t* test, two-tailed *p* value is 0.8377, considered not significant at 95% confidence interval (CI)). The higher total chlorophyll content in the plants watered with raw dam water can be attributed to the nutrients in the dam water compared to the control plants which relied solely on nutrients in the soils.

Findings in Figure 3.7 show higher total chlorophyll content for *Brassica oleracea*, in the plants watered with raw dam water, followed by plants watered with diluted raw dam water, with the lowest total chlorophyll content in the control plants. However, the differences in the total chlorophyll of the three treatments was not significant (one way ANOVA, p value is 0.6149, considered not significant at 95% CI).

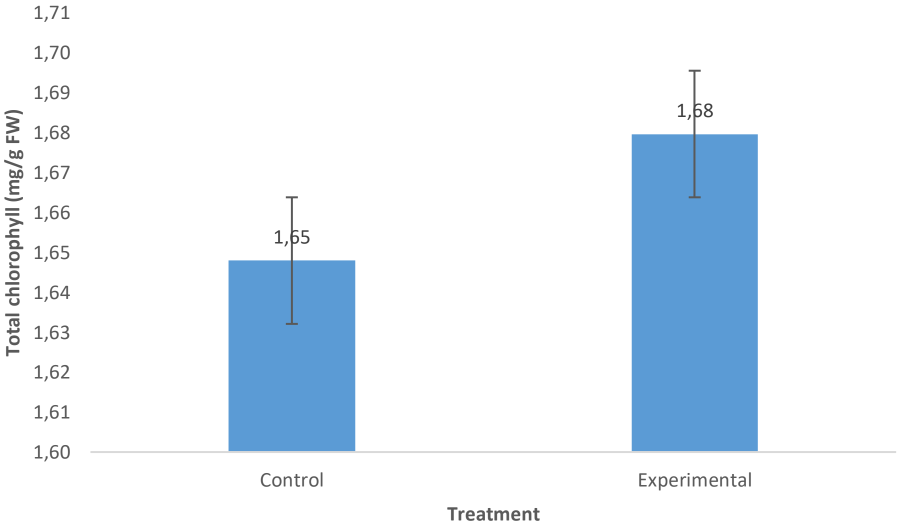


Figure 3. 6. Total chlorophyll of *S. tuberosum* watered with milli-Q water and dam water

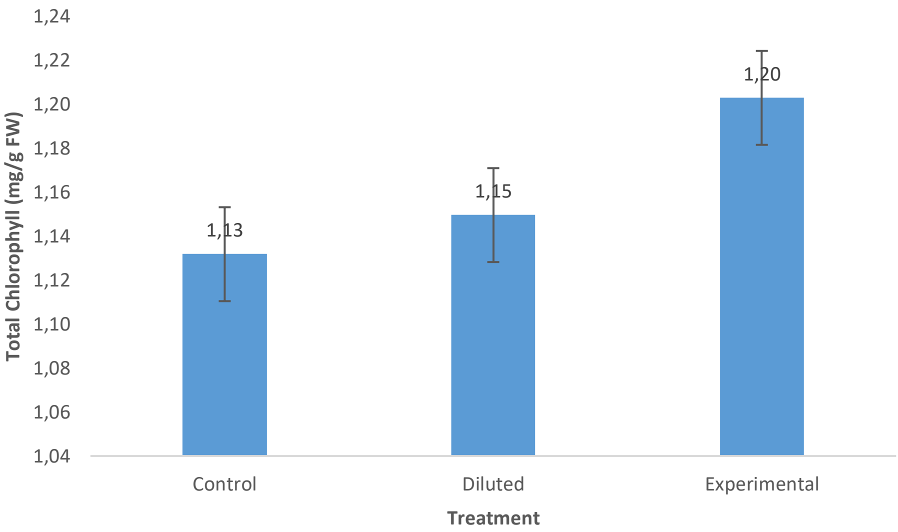


Figure 3. 7. Total chlorophyll of *B. oleracea* watered with milli-Q water, diluted dam water and raw dam water

McElhiney et al. (2001) reported adverse effects on chlorophyll content of potato shoot plants when exposed to 5-50 µg L^{-1} microcystin-LR. McElhiney et al. (2001) found that, while toxin concentrations of 0.001 - 0.01 µg mL^{-1} had no significant effect on the total chlorophyll content of potato shoots, those exposed to microcystin levels of 0.05 -5 µg mL^{-1} had significantly lower total chlorophyll content after 16 days than the control cultures.

The inhibitory effect of MC-LR on photosynthesis has been described in several plant species, although the mechanism behind this process remains unknown (Machado et al., 2017). The inhibition occurs through an indirect action of the toxin by the induction of oxidative stress in plants (El Khalloufi et al., 2011). Along with the specific inhibition of protein phosphatases (PP1) and PP2A, the increase in antioxidant defenses induced by MC-LR suggests that oxidative stress is a major mechanism contributing to the phytotoxicity of this toxin (Machado et al., 2017). However, although the inhibition of photosynthetic processes due to increased concentrations of ROS has been documented, Garda et al. (2016) have shown that under long-term exposure PP inhibition was the primary cause of MC-LR induced mitotic spindle disorders in *Vicia faba* and not ROS induction. A recent study by Corbel et al. (2015) also demonstrated that, with regard to the photosynthetic process, low concentrations of MC-LR did not alter the concentrations of chlorophyll a and b or the chlorophyll fluorescence (Fv/Fm) of *L. esculentum*, highlighting the possibility that environmentally relevant concentrations as used in this study, might not adversely affect exposed plants.

3.4 Conclusions

Based on the findings, the levels of MCs in the dam water collected from Roodeplaat Dam, had a significant negative impact on the seed germination of *Brassica oleracea* but did not show significant impact on the general plant growth, nor induce significant oxidative stress as demonstrated by comparable total chlorophyll between the trial plants and the controls. The findings demonstrated that the two plants can bioaccumulate MCs to concerning levels when irrigated with water derived from the Roodeplaat Dam. MCs accumulation levels in the two tested plants ranged from 0.001415 to 0.135508 mg kg^{-1} DW for individual MC congeners and this was comparable to the concentrations reported in other studies. These findings, together with findings in other studies discussed here, demonstrate terrestrial food crops can accumulate MCs to levels that can pose human-health risks when exposed to naturally relevant levels of MCs.

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CHAPTER FOUR: EFFECT OF LAS ON THE UPTAKE AND ACCUMULATION OF METALS IN *BRASSICA OLERACEA* AND *SOLANUM TUBEROSUM*

Abstract

The hyper-eutrophic conditions in impoundments used for irrigation around South African major cities promote the co-existence of linear alkylbenzene sulfonate (LAS) and other pollutants such as metals. The combined effects of LAS and metals when such water is used to irrigate crops have not been properly investigated in light of human health risks and the prevailing local conditions. To understand the potential risks, pot-culture experiments were conducted to assess the effect of the LAS, sodium-dodecyl-sulphate (SDS) on the accumulation of aluminum (Al), manganese (Mn) and strontium (Sr) in *Brassica oleracea* (cabbage) and *Solanum tuberosum* (potato) plants. The plants were watered with Roodeplaat Dam water containing 3.48 mg L^{-1} of the LAS (SDS) and Mn (0.257 mg L^{-1}), Al (0.6 mg L^{-1}) and Sr (0.16 mg L^{-1}) as determined by field surveys, for 20 days.

The presence of SDS, at the determined prevailing concentrations in the irrigation water, did not enhance uptake of Sr, Mn, Al in the two plants, as demonstrated by statistically insignificant differences in the means of the treatments (with and without SDS). In addition, the presence of the metals, high pH, EC and presence of cyanotoxins in the water did not affect the total chlorophyll and the growth of the plants. These findings imply that, the prevailing levels of anionic surfactants such as SDS, metals and other contaminants in the hypereutrophic lakes pose little risk to the crop yields, quality of the crops and human health due to the possible accumulation of these contaminants in irrigated plants. Inasmuch the study reported no immediate inherent risk to the plants and human health, continuous monitoring of the contaminants in water, soil and irrigated plants is recommended since the conditions, the concentrations and other factors can quickly change if the management of the catchment does not improve in the near future.

Keywords: *Brassica oleracea*, combined effects, sodium-dodecyl-sulphate, metals, *Solanum tuberosum*

4.1 Introduction

South Africa (S.A) is a water scarce country and heavily relies on surface waters for the bulk of its irrigation, urban and industrial needs. Metal contaminants in soil are known to have the ability to migrate and accumulate (Sulaiman & Hamzah, 2018). The accumulation of metal contaminants in soil is of concern, since these can affect the well-being of plants, animals, and humans. In plants, increased levels of metals can induce oxidative stress and also hinder the plants' ability to produce chlorophyll (Sulaiman & Hamzah, 2018).

Linear alkylbenzene sulfonates (LAS) are anionic surfactants that are a common ingredient in a variety of industrial and domestic processes (Wang et al., 2015) and are a commonly used in making detergents because of their ease to synthesize and low costs (Pierattini et al., 2018). Discharge of treated, untreated and improperly treated wastewater are major routes of LAS into the aquatic environment. The elimination of these anionic surfactants is mainly through biodegradation and adsorption, but in oxygen-deficient environments, their degradation is very slow and accumulation of these pollutants is common in such aquatic conditions (Wang et al., 2012). This implies that hypereutrophic water bodies have the right conditions to promote the co-existence of LAS, harmful algal blooms (HABs) and other pollutants. This based on the fact that the accelerated growth of HABs in eutrophic reservoirs takes up oxygen and upon their death, their degradation consumes more oxygen and results in such reservoirs becoming anoxic and anaerobic. Such is the cases in dams like Roodeplaat and Hartbeespoort which are found in the Gauteng and North West Provinces of South Africa respectively. These dams are supplied by the Magalies and Crocodile Rivers which are well renowned as hyper-eutrophic and having poor water quality (Pindihama & Gitari, 2020).

According to Wang et al. (2012), the synergic impacts of LAS with other contaminants such as metal pollutants Hg^{2+}, pyrene and oil has been studied. In S.A, particularly in areas such as the Crocodile (West) and Marico catchment where the Hartbeespoort, Rietvlei, Roodeplaat and Bospoort Dams are all found have been classified as hypertrophic for many decades (van Ginkel, 2004), the co-existence of LAS and other pollutants such as cyanotoxins and metals may require examination since water derived from these dams is mainly used for irrigation. This aim of this study was to assess the effect of the LAS, sodium-dodecyl-sulphate (SDS) on the accumulation of metals aluminum (Al), manganese (Mn) and strontium (Sr) in *Brassica oleracea* (cabbage) and

Solanum tuberosum (potato) plants when exposed to environmentally relevant concentrations of the pollutants.

4.2 Materials and methods

4.2.1 Materials and reagents

Two field surveys were conducted around Roodeplaat and Hartbeespoort Dam sites in June 2019 and September 2019 to determine the levels of metal elements and the water conditions to use in the subsequent experiments. Physicochemical parameters of the water such as pH, Electrical Conductivity (EC) and Total Dissolved Solids (TDS) were monitored in the field using a Thermo-scientific Orion multi-meter model 430, turbidity using a TB200 Portable Turbidimeter, whereas chlorophyll *a*, anionic surfactants, cations and microcystins (MCs) were determined *ex-situ*. The Roodeplaat Dam water used for the experiments was kept frozen at -20^{o}C until needed. The LAS used in this study is Sodium Dodecylbenzene Sulfonate (SDS). The SDS (CAS No. 25155-30-0; molar mass 348.48 g/mol; and chemical formula $C_{18}H_{29}NaO_3S$) was purchased from BYMAZ Pty Ltd, Johannesburg, South Africa.

4.2.2 Pot-culture experiment design

Seeds for *Brassica oleracea* (common cabbage) were acquired from NTK Agricultural Products & Services (S.A) and potato (*Solanum tuberosum*) seeds were sourced from Livingseeds Heirloom Seeds (Pty) Ltd Midvaal, Gauteng. Seeds for *B. oleracea* were thoroughly washed with distilled water prior to their planting in 200 mm plant pots which contained uncontaminated soil. Seedlings for *B. oleracea* were first pre-grown in polystyrene trays with uncontaminated soils before being transplanted into the 200 mm plastic plant pots. Fourteen-day old cabbage seedlings were transplanted (one plant per pot/bag) into the bags. There were 3 replicates and four treatments of both *Brassica oleracea* and *Solanum tuberosum* plant species and the experiment was repeated once i.e., 3X4X2=**24** plants in total of each species and a total of **48** plants for both species.

The soils used were collected from the University of Venda agricultural farm which is located in the Lowveld climate that is typified by deep red soils which are well drained and dominated clay and falls in the Hutton classification, also known as the Rhodic Ferrasol (Mabasa, 2019). The background level of metal elements in the soils used are in Table 4.1. The soils were extracted and determined as detailed in the section on "digestion of plant and soil samples".

With regards to the three main nutrients, P, K, Total N and organic matter, the soils were analyzed at the South African Agricultural Research Council. The organic carbon was analyzed using the Walkley-Black method, Phosphorous was extracted using the ISFEI method as described by Manson and Roberts (2001) and the extract was determined via the molybdenum method and an auto-analyzer. Exchangeable and soluble K was extracted using the USDA (1972) procedure as described by Manson and Roberts (2001) and the K in the leachate was determined by atomic absorption spectrophotometry. Total N in the soils was determined using the wet oxidation procedure, commonly known as the Kjeldahl distillation as described by Manson and Roberts (2001). The P, K, Total N and Organic matter in the soils were 25.86 mg/kg; 184 mg/kg; 0.079% and 2.07% respectively, which are typical of agricultural soils (FAO, 2015). The soil was collected from a depth of 0–50 cm, and 15 kg of the soil was placed into 200 mm plastic pots for the experiments and treated with 0.3 g (per kg of soil) of Protek General Fertilizer with N:P:K (%) 2:3:2 (14) before introducing the plants.

Table 4. 1. Background level of cations in the soil

Element (mg/kg)	Background level	Element (mg/kg)	Background level
B	<280	Sn	0.09±0.01
V	9.14±12.91	Sb	<1
Cr	56.01±33.40	Ba	72.48±2.53
Mn	1709.85±160.28	Hg	0.01±0.01
Co	60.40±18.85	Pb	11.53±1.69
Ni	27.67±9.63	Al	14060.00±1798.88
Cu	126.28±48.07	Fe	33286.00±31726.47
Zn	46.97±1.75	Ca	1877.10±196.43
As	0.79±0.26	K	424.50±129.68
Se	0.09±0.02	Mg	985.30±43.70
Sr	9.83±2.08	Na	290.60±12.16
Mo	<3	P	168.30±0.00
Cd	0.06±0.01	Si	558.00±94.47

To investigate the effect of the LAS, sodium-dodecyl-sulphate (SDS) on metals (Mn, Al and Sr) accumulation in *B. oleracea* and *S. tuberosum*, the plants in pot-cultures were watered daily with Roodeplaat dam water containing 3.4 mg L^{-1} of the LAS, SDS (as determined by the field study, section 4.2.1) a known concentration of MCs (±15 µg L^{-1}, already in the raw dam water collected) and fixed levels of Mn (0.257 mg L^{-1}), Al (0.6 mg L^{-1}) and Sr (0.16 mg L^{-1}) as established from the field study (Table 4.3) for 20 days). During the field surveys irrigation water from the Roodeplaat Dam was monitored twice over a four month period to determine the levels of a range of metallic elements (Table 4.3). Three elements Al, Mn and Sr were consistently detected, and the highest concentrations reported for the three elements were used for the experiments to look at the worst case scenario.

In order to maintain constant concentrations of the LAS, SDS, the media were tested daily using a Hanna HI96769 Anionic Surfactants Portable Photometer, and refreshed accordingly. The accumulation of metals was determined in *B. oleracea* after 5 days and again after 20 days. Metal accumulation in *S. tuberosum* was determined only after 20 days due to the length it takes for the seeds to sprout. Total chlorophyll was determined in leaves of both plants at day 20.

Table 4. 2. Design of the experiment

Treatments
Treatment 1 (T1): Control: Milli-Q water with no contaminants
Treatment 2 (T2): Raw dam water with Mn (0.257 mg L^{-1}), Al (0.6 mg L^{-1}) and Sr (0.16 mg L^{-1})
Treatment 3 (T3): Milli-Q water with 3.4 mg L^{-1} SDS
Treatment 4 (T4): Combined exposure: Raw dam water, SDS (3.4 mg L^{-1}), Mn (0.257 mg L^{-1}), Al (0.6 mg L^{-1}) and Sr (0.16 mg L^{-1})

4.2.3 Determination of total chlorophyll

Reactive oxygen species (ROS) generation in a stress environment in plants causes changes in chlorophyll, anthocyanin and membrane integrity among other effects in plants, therefore, ROS generation can be measured indirectly by measuring the changes in these compounds (Venkidasamy et al., 2019). The total chlorophyll in the plant leaves was determined according to Baskar et al. (2015). Briefly, 50 mg of the plant leaves were chopped into very small pieces (less than 10 mm) and soaked in 95 % (v/v) ethanol followed by incubation in the dark for 3 days. The absorbance of the supernatant was read at 664.2 and 648.6 nm by UVvis spectrophotometer

(SPECTROstar Nano, BMG LABTECH, Germany). Chlorophyll-*a* and *b*, and total chlorophyll content were calculated according to Baskar et al. (2015) using the following formulae:

$$\text{Chl a} = 13.36\ A664.2 - 5.19\ A648.6 \qquad [4.1]$$

$$\text{Chl b} = 27.43\ A648.6 – 8.12\ A664.2 \qquad [4.2]$$

$$\text{Total chlorophyll} = \text{Chl a} + \text{Chl b}. \qquad [4.3]$$

Total chlorophyll content was expressed as milligram per gram per fresh matter (FM).

4.2.4 Digestion of plant and soil samples

Plant and soil samples were acid digested according to Rashid et al. (2016). Soil samples were oven-dried at 60°C in an electric oven for 24 h then grounded by a mortar and pestle into a fine powder. Five grams of the grounded soil was added to a 250 mL conical flask and 10 mL of aqua regia ($HCl:HNO_3$ (3:1)) was added. The mixture was heated at 95°C on a hot plate for 1 h, then left to cool to room temperature. The samples were then diluted with deionized water, followed by filtering through a Macherey-Nagel No.1 filter paper (0.45 µm), Macherey-Nagel, Germany, kept at 4°C in a refrigerator, and then sent to the Stellenbosch University Central Analytical Facility for determination of cations using the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis.

As for plant material, edible portions of both plants (leaves for *B. oleracea* and the tubers for *S. tuberosum*) were first freeze dried, for 48 h at -54ºC under a constant vacuum of 44 µmHg (Telstar Lyoquest Freeze Dryer, Terrassa, Spain). Upon freeze-drying, the plant material was then ground to fine powder using mortar and pestle, then 1 g of the grounded material was suspended in 10 mL of conc HNO_3, left to digest overnight then heated on a hot plate the following day till the mixture became semi-dry. The digested mixture was then left to cool to room temperature followed by filtering through Macherey-Nagel No.1 filter paper (0.45 µm), (Macherey-Nagel, Germany), then dilution to the 50 mL mark using deionized water in a volumetric flask. The samples were then transferred into 50 mL centrifuge tubes, then kept in the refrigerator at 4ºC prior to courier to the

Stellenbosch University Central Analytical Facility for Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis.

4.2.5 Determination of phytoplankton biomass and microcystins

To determine chlorophyll *a* levels, hot ethanol extraction followed by spectrophotometric analysis of absorbency wavelength on a Spectro-star Nano (BMG LABTECH, 601-1106, Germany) according to (Lawton et al., 1999). Briefly, absorbency was monitored at 665 and 750 wavelengths and second readings were further taken upon acidifying the same samples with 10 µL of hydrochloric acid (1 mol l^{-1}) at the same wavelengths to correct for turbidity. The corrected absorbance and turbidity at 750 nm was subtracted from 665 nm absorbance before and after addition of hydrochloric acid. The total chlorophyll *a* was determined according to the following formula provided by Lawton et al. (1999):

Correction for turbidity: $$\text{absorbance } 665a - 750a = \text{corrected } 665a \text{ absorbance} \qquad [4.4]$$

$$665b - 750b = \text{corrected } 665b \text{ absorbance} \qquad [4.5]$$

$$\text{Chlorophyll-a} = \frac{29.62\ (665a - 665b\ ) \text{x Ve}}{\text{Vs x I}} \text{ mg m}^{-3} \qquad [4.6]$$

Where: *Ve* = volume of ethanol extract (mL); *Vs* = volume of water sample (Litre); *I* = path length of cuvette (cm)

Levels of microcystins (MCs) in the Roodeplaat dam water used were determined using the commercially-available ELISA Microcystin Plate Kits (ENVIROLOGIX INC. (Kit Lot: 071499 Cat No: EP 022)). This assay uses antibodies against microcystin-LR and a microplate reader Spectro-star Nano (BMG LABTECH, 601-1106, Germany) was used to quantify the MCs after the assay. Prior to analysis, 5 mL of each sample was filtered using the 0.20 µm glass fibre syringe filters and 50 µL of the filtered sample was used for the assay.

4.2.6 Data analyses

The mean levels of cations bio-accumulated by the plants and the total chlorophyll content of the leaves from the various treatments were compared using the analysis of variance (ANOVA) and/or the Kruskal-Wallis tests at 95% confidence interval (CI) using GraphPad InStat 3 (GraphPad Software, California, United States). To test the data for normality and homogeneity of the variance, the Kolmogorov–Smirnov and Bartlett tests were used, with data passing the normality test subjected to the ANOVA and data failing the test subjected to the Kruskal–Wallis test at 95% CI. The Tukey-Kramer multiple comparisons test and the Dunn's multiple comparisons test were used as post-hoc tests for data which passed the normality tests and data which did not pass the normality test, respectively. The levels of cations accumulated in the plants are presented as means ± the standard deviation (SD) in the results section.

4.3 Results

4.3.1 Physicochemical parameters of the dam water

The Roodeplaat Dam water used to water the plants had high TDS (228 (±7.51) mg L^{-1}) and EC levels (380 (±16.52) µS $cm^{-1)}$, but within the S.A (DWAF, 1996) guidelines and the Food and Agriculture Organization (FAO) (1985) (Ayers and Westcot, 1985) limits for irrigation water set at $\leq$ 400 µS cm^{-1} and 700 µS cm^{-1} respectively.. The water was also slightly alkaline with a mean pH of 9.02 (±0.29) and above the 6.5–8.4 threshold set for irrigation water in S.A (DWAF, 1996). The Roodeplaat Dam water also had high phytoplankton biomass (chlorophyll *a* 440.24±328.147 µg L^{-1}) and high levels of MCs (13.03±3.599 µg L^{-1}).

Table 4.3 shows the levels of cations in the raw dam water. All the cations in the dam water were within the S.A (DWAF, 1996) and FAO (1985) guidelines for irrigation water. Metals such as Sr, Mn and Al were the most prevalent in the dam water, hence their selection for the pot culture experiments.

Table 4. 3. Concentration of metals in the Roodeplaat raw dam water used in the experiments (Mean ±SD)

Metal (mg/L)	June, 2019	September, 2019	S.A. guidelines (1996)	Food and Agriculture Organization (FAO) guidelines (1985)
Al	0.376 (±0.817)	0.624 (±0.726)	5–20	5.0
As	0.002 (±0.001)	0.001 (±0.000)	0.1–2.0	0.1
B	0.059 (±0.008)	0.068 (±0.005)	0.5–6.0	0.7
Ba	0.049 (±0.015)	0.152 (±0.056)	-	-
Cu	0.006 (±0.003)	0.006 (±0.007)	0.2–0.5	0.2
Mn	0.257 (±0.179)	0.158 (±0.109)	0.02–10	0.2
Ni	0.006 (±0.003)	0.008 (±0.003)	0.2–2.0	0.2
Pb	0.004 (±0.004)	0.001 (±0.001)	0.2–2.0	5.0
Sr	0.144 (±0.116)	0.118 (±0.020)	-	-
Zn	0.083 (±0.080)	0.090 (±0.033)	1.0–5.0	2.0

Note: The 1996 South African and the 1985 FAO guidelines do not have a value for barium and strontium.

4.3.2 Accumulation of Al, Sr, Mn and other cations by *B. Oleracea* in the presence of SDS

Findings of the pot-culture experiments in Table 4.4, show that upon 20 days of exposure to the various treatments, the *B. oleracea* leaves accumulated Mn to a maximum of 69.79 (±22.97) µg kg^{-1} (treatment *2*) and a minimum of 57.69 (±12.52) µg kg^{-1} (treatment *1*). Sr was accumulated up to 127.98 (±26.60) µg kg^{-1} (treatment *3*) and lowest accumulation of 126.22 (±28.26) (treatment *2*). The highest levels of Al were accumulated in treatment *1* (0.18 (±0.04) mg kg^{-1}) and the lowest levels were accumulated in treatments *3* and *4* (0.15 (±0.04) mg kg^{-1}).

The findings indicate higher levels of Sr and Al in *B. oleracea* leaves after 5 days of exposure compared to the leaves harvested after 20 days of exposure, even though there were no significant differences in the levels accumulated (Figure 4.1). With regards to Mn accumulation in *B. oleracea* leaves, higher levels were detected in leaves harvested after 20 days of exposure compared to those harvested after 5 days, even though there were no significant differences in the levels accumulated.

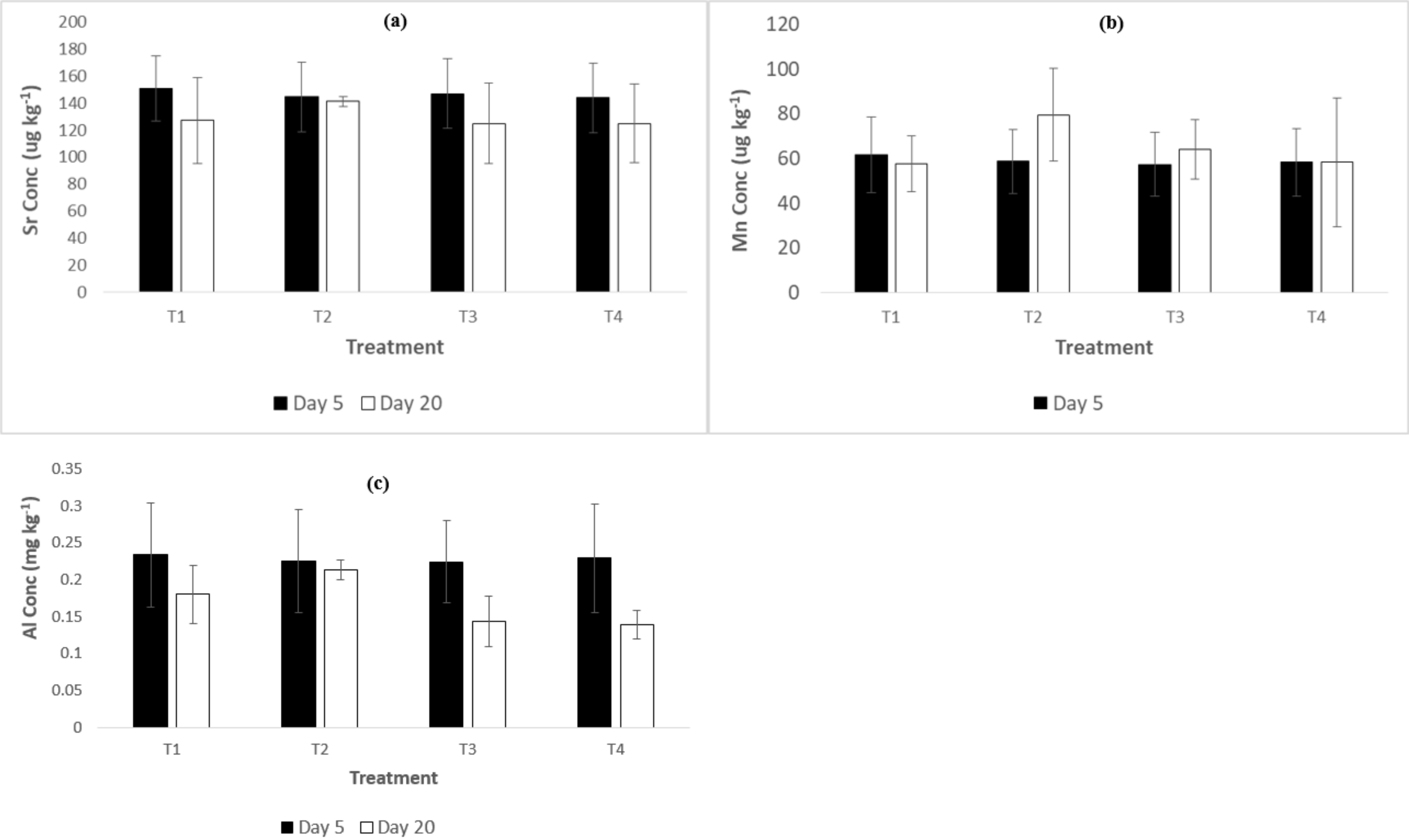

Figure 4. 1. Comparison of accumulation of metals in cabbage (*B. oleracea*) leaves after 5 days vs 20 days of exposure to the various treatments (a) Sr (b) Mn (c) Al

Note: Different lowercase letters indicate no significant difference between groups ($p<0.05$).

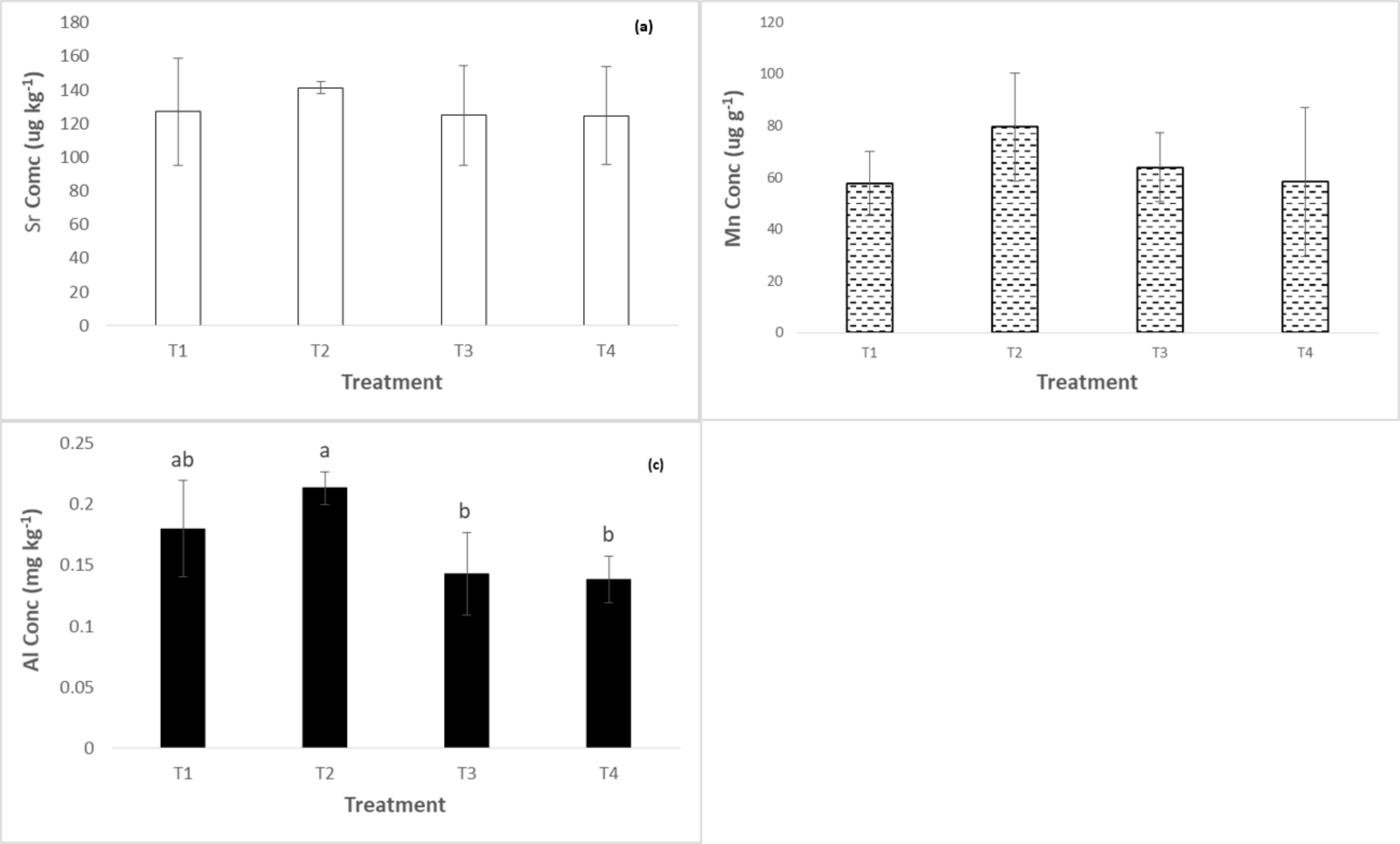

Figure 4. 2. Accumulation of metals in cabbage (*B. oleracea*) leaves after 20 days of exposure to the various treatments (a) Sr (b) Mn (c) Al.
Note: Similar lowercase letters indicate no significant difference among treatments ($p>0.05$).

A comparison of the accumulated metals among the 4 treatments in Figure 4.2 and Table 4.4 shows that no significant differences were observed among the treatments for Sr and Mn ($p > 0.05$). Significantly higher levels of Al were accumulated in plants exposed to treatment *2* (dam water with metals) compared to the other treatments ($p < 0.05$). This implies that accumulation of Al in *B. oleracea* was not affected by the presence of the SDS in treatment *4* which had a combined exposure of metals and SDS.

With regards to the other 19 major and trace cations also assessed in Table 4.4, only Cr, Co, Mg and P showed a significant difference in the levels accumulated among the 4 treatments ($p < 0.05$). For Co, Cr and P, much higher levels were accumulated in plants exposed to treatment *1* compared to the other treatments and for Na, plants exposed to treatment *2* accumulated higher levels of the cation.

4.3.3 Accumulation of Al, Sr, Mn and other cations by *S. tuberosum* in the presence of SDS

Table 4.5 and Figure 4.3 show that the accumulation of the metals in the *S. tuberosum* tubers. Higher levels of Mn were accumulated in treatment *3* (17.34 (±4.93) µg kg^{-1}) and lowest in treatment *1* (11.07 (±2.85) µg kg^{-1}). For Sr, higher accumulation was also in treatment *3* (4.73 (±0.91) µg kg^{-1}) and lowest in treatment *1* (2.93 (±0.38) µg kg^{-1}). Accumulation of Al was highest in treatment *3* (0.17 (±0.06) µg kg^{-1}) and lowest in treatment *4* (0.10 (±0.05) µg kg^{-1}). Accumulation of Mn and Sr was much higher in the edible parts of *B. oleracea* (leaves) compared to the edible parts (tubers) for *S. tuberosum* (highest Mn accumulation of 69.79 (±22.97) in *B. oleracea* and 17.34 (±4.93) in *S. tuberosum* and highest accumulation for Sr, 127.98 (±26.60) in *B. oleracea* and 4.73 (±0.91) in *S. tuberosum*). The accumulation of Al was comparable in the edible parts of the two plant species (maximum accumulation of 0.18 (±0.04) in *B. oleracea* leaves and 0.17 (±0.06) in *S. tuberosum* tubers).

No significant difference was reported among the treatments in the accumulation of Al and Mn in the *S. tuberosum* tubers ($p > 0.05$), but statistically significant different accumulations were found for Sr ($p < 0.05$), with treatment *1* having a significantly lower uptake compared to the other three treatments. Since plants exposed to treatment *1* were not exposed to any SDS nor metals, this probably explains the lower levels of Sr accumulated by the tubers in treatment *1*. With regards to the other major and minor cations also assessed in the tubers (Table 4.5), no significant differences in the accumulation among the treatments were found for all the other cations assessed ($p > 0.05$).

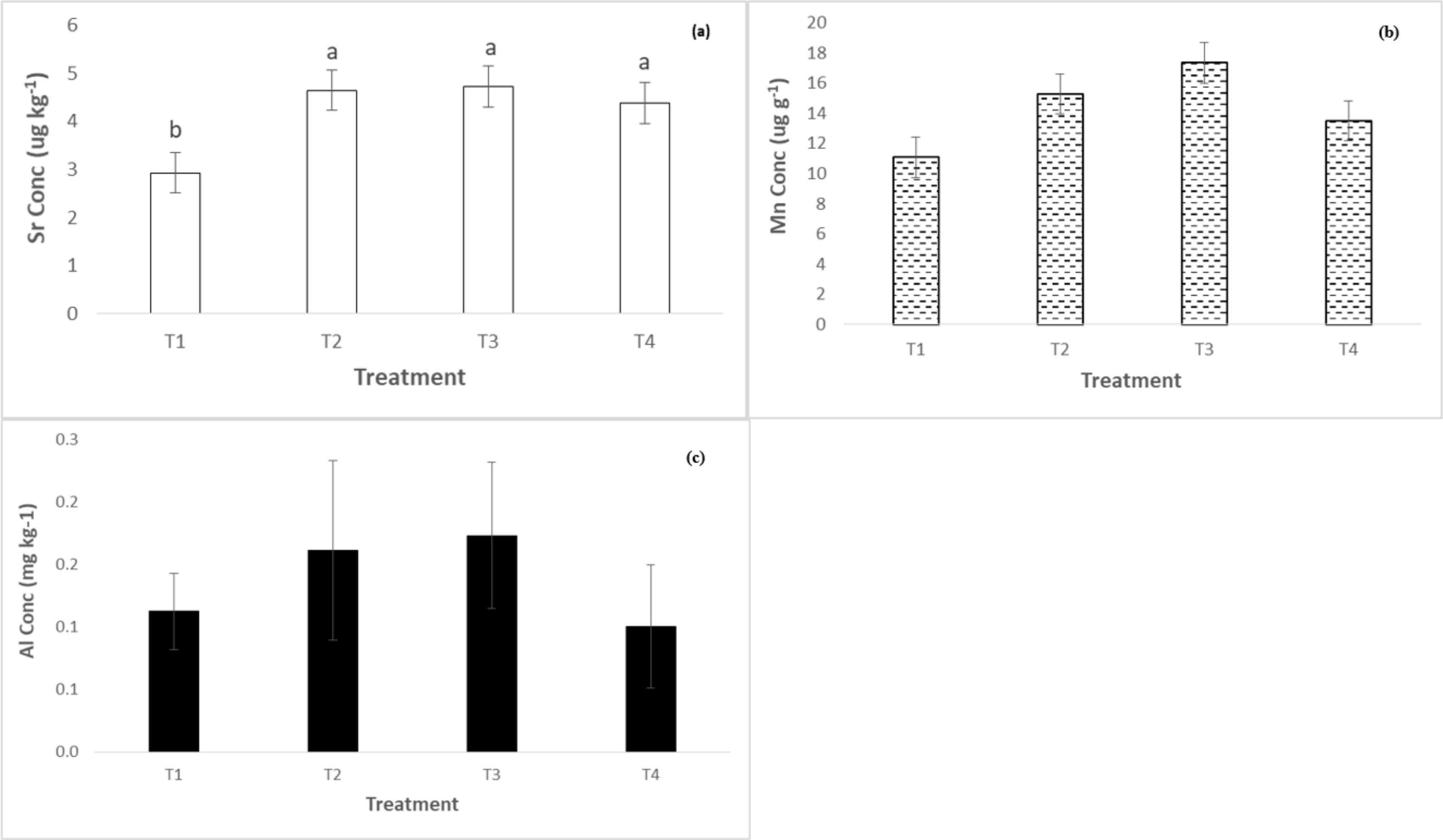

Figure 4. 3. Figure 4. 3. Accumulation of metals in potato (*S. tuberosum*) tubers after 20 days of exposure to the various treatments (a) Sr (b) Mn (c) Al.
Note: Similar lowercase letters indicate no significant difference among treatments ($p>0.05$).

4.3.4 Effects of Al, Sr, Mn and other cations on *B. Oleracea* tolerance and *S. tuberosum*

High levels of metals in plant tissue are known to induce oxidative stress and inhibit chlorophyll production (Shakya et al., 2008; Sulaiman and Hamzah, 2018), total chlorophyll content in the plants was monitored to determine the possible effects of Al, Sr, Mn and other cations on the plants. In addition the dam water used in treatments *2* and *4* also had significant levels of the cyanotoxin, microcystin (±15 µg L^{-1}), high pH (9.02 (±0.29)), high EC levels (380 (±16.52) µs cm^{-1}) and high TDS levels (228 (±7.51) mg L^{-1}). Most of these contaminants for example, high EC and pH (Huang et al., 2017), MCs (Saqrane et al., 2008; Machado et al., 2017) and anionic surfactants (Pandey & Gopal, 2010; Wang et al., 2012), have been reported to inhibit the production of chlorophyll, induce oxidative stress and affect plant growth.

The leaves of *B. oleracea* plants in watered with treatment *1* in Figure 4.4 (a) had higher total chlorophyll content compared to the other three treatments, but there were no statistically significant differences in the mean total chlorophyll content among the four treatments after 20 days of exposure ($p > 0.05$). The total chlorophyll levels of *S. tuberosum* leaves in Figure 4.4 (b) show higher total chlorophyll levels in plants watered with treatments *1* and *4*, but no statistically significant differences were reported among the four treatments ($p > 0.05$). Such findings suggest that at environmentally relevant levels of the three metals and SDS as tested in the current study, and the presence of other cations, MCs and high pH, did not affect chlorophyll production nor induce oxidative stress in the plants. In addition, there were no apparent visual impacts or differences noted on the plants upon exposure to the four different treatments over the duration of the experiments.

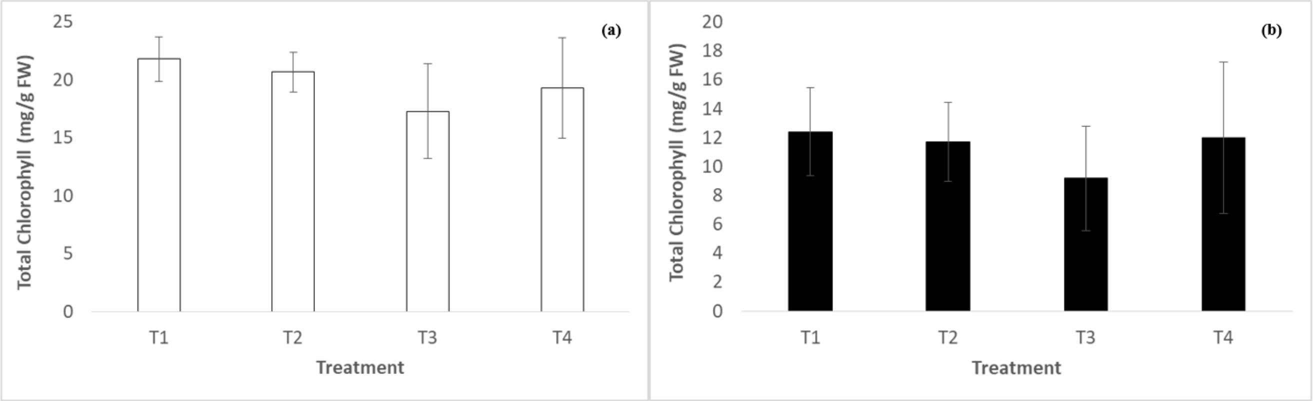

Figure 4. 4. Total chlorophyll in (a) cabbage (*B. oleracea*) leaves (b) potato (*S. tuberosum*) leaves, after 20 days of exposure to the 4 treatments.

Note: Similar lowercase letters indicate no significant difference among treatments (p>0.05).

Table 4. 4. Cations accumulated in *B. oleracea* leaves upon 20 day exposure to the 4 treatments

		T1: Control (Milli-Q water)	**T2**: Roodeplaat Dam water (with Mn (0.257 mg L^{-1}), Al (0.6 mg L^{-1}) and Sr (0.16 mg L^{-1})	**T3**: Milli-Q water with 3.4 mg L^{-1} SDS	**T4:** Roodeplaat Dam water (SDS (3.4 mg L^{-1}) + Mn (0.257 mg L^{-1}), Al (0.6 mg L^{-1}) and Sr (0.16 mg L^{-1}))	Significance
Minor cations (µg kg^{-1})	B	32.42 (±7.01)	26.47 (±4.67)	25.84 ±4.15	26.21 (±5.24)	n.s
	V	1.00 (±0.40)	0.63 (±0.22)	0.59 (±0.29)	0.60 (±0.27)	n.s
	Cr	3.80 (±0.36)^{a}	3.44 (±0.28)^{a,b}	3.43 (±0.19)^{a,b}	3.39 (±0.21)^{b}	*
	Mn	57.69 (±12.52)	69.79 (±22.97)	57.76 (±14.86)	60.16 (±21.10)	n.s
	Co	0.67 (±0.11)^{a}	0.62 (±0.12)^{a,b}	0.51 (±0.11)^{a,b}	0.53 (±0.12)^{b}	*
	Ni	2.17 (±0.29)	1.87 (±0.10)	2.20 (±0.76)	2.13 (±0.71)	n.s
	Cu	3.75 (±0.89)	3.80 (±0.61)	4.08 (±0.51)	3.92 (±0.57)	n.s
	Zn	45.77 (±14.41)	43.23 (±11.49)	38.90 (±10.87)	39.14 (±10.94)	n.s
	As	0.05 (±0.01)	0.14 (±0.17)	0.26 (±0.56)	0.25 (±0.51)	n.s
	Se	0.56 (±0.11)	0.76 (±0.15)	0.73 (±0.10)	0.71 (±0.10)	n.s
	Sr	127.10 (±31.70)	126.22 (±28.26)	127.98 (±26.60)	127.48 (±27.64)	n.s
	Mo	2.83 (±.98)	1.57 (±1.38)	1.70 (±1.62)	1.75 (±1.59)	n.s
	Ba	104.41 (±23.00)	100.90 (±29.52)	105.14 (±30.32)	103.41 (±30.75)	n.s
	Pb	0.20 (±0.04)	0.19 (±0.04)	0.21 (±0.06)	0.19 (±0.06)	n.s
Major cations (mg kg^{-1})	Al	0.18 (±0.04)^{a,b}	0.17 (±0.04)^{a}	0.15 (±0.04)^{b}	0.15 (±0.04)^{b}	***
	Fe	0.30 (±0.11)	0.20 (±0.07)	0.20 (±0.09)	0.19 (±0.09)	n.s
	Ca	35.94 (±10.11)	33.72 (±10.38)	33.63 (±8.31)	32.79 (±9.23)	n.s
	K	19.11 (±3.13)	16.52 (±4.79)	16.86 (±4.76)	17.11 (±4.11)	n.s
	Mg	6.31 (±1.64)	5.83 (±1.38)	5.69 (±1.06)	5.70 (±1.11)	n.s
	Na	0.28 (±0.10)^{b}	0.41 (±0.18)^{a}	0.36 (±0.17)^{b}	0.37 (±0.16)^{a,b}	**
	P	3.81 (±1.45)^{a}	2.74 (±0.91)^{b}	2.66 (±0.93)^{b}	2.65 (±0.94)^{b}	*
	Si	1.39 (±2.96)	0.04 (±0.01)	0.04 (±0.01)	0.04 (±0.01)	n.s

Note: *p < 0.05, **p <0.01, ***p < 0.001, n.s = not significant. *Data labelled with different small letters (a- c) differed significantly at p < 0.05 in each row (Mean ±SD).*

Table 4. 5. Cations accumulated in *S. tuberosum* tubers upon 20 day exposure to the 4 treatments.

		T1: Control (Milli-Q water)	**T2**: Dam water (with Mn (0.257 mg L^{-1}), Al (0.6 mg L^{-1}) and Sr (0.16 mg L^{-1})	**T3**: Milli-Q water with 3.4 mg L^{-1} SDS	**T4:** Dam water (SDS (3.4 mg L^{-1}) + Mn (0.257 mg L^{-1}), Al (0.6 mg L^{-1}) and Sr (0.16 mg L^{-1}))	Significance
Minor cations (µg kg^{-1})	B	5.86 (±0.57)	6.59 (±0.60)	6.51 (±1.65)	10.69 (±11.99)	n.s
	V	0.51 (±0.26)	1.00 (±0.73)	1.18 (±0.66)	2.50 (±4.96)	n.s
	Cr	3.20 (±0.17)	3.28 (±0.19)	3.35 (±0.20)	3.45 (±1.30)	n.s
	Mn	11.07 (±2.85)	15.27 (±6.32)	17.34 (±4.93)	13.51 (±6.51)	n.s
	Co	0.68 (±0.70)	0.51 (±0.22)	0.59 (±0.24)	0.80 (±0.86)	n.s
	Ni	2.47 (±0.44)	2.51 (±0.39)	2.42 (±0.50)	3.61 (±1.52)	n.s
	Cu	5.68 (±1.14)	6.65 (±1.81)	6.19 (±1.09)	8.55 (±2.86)	n.s
	Zn	13.75 (±2.25)	14.07 (±2.60)	12.66 (±1.82)	16.71 (±4.19)	n.s
	Sr	2.93 (±0.38)^{b}	4.64 (±0.99)^{a}	4.73 (±0.91)^{a}	4.38 (±0.77)^{a}	**
	Mo	0.34 (±0.08)	0.33 (±0.05)	0.33 (±0.04)	0.29 (±0.05)	n.s
	Ba	6.29 (±2.15)	9.68 (±4.34)	8.51 (±1.85)	8.99 (±4.23)	n.s
	Pb	0.10 (±0.01)	0.16 (±0.05)	0.16 (±0.04)	0.23 (±0.17)	n.s
Major cations (mg kg^{-1})	Al	0.11 (±0.03)	0.16 (±0.07)	0.17 (±0.06)	0.10 (±0.05)	n.s
	Fe	0.12 (±0.12)	0.30 (±0.27)	0.34 (±0.21)	0.71 (±1.51)	n.s
	Ca	0.70 (±0.19)	1.00 (±0.48)	1.02 (±0.39)	0.75 (±0.30)	n.s
	K	18.08 (±4.06)	18.17 (±3.99)	15.32 (±1.81)	14.64 (±2.43)	n.s
	Mg	1.13 (±0.34)	1.16 (±0.24)	1.16 (±0.25)	1.05 (±0.17)	n.s
	Na	0.14 (±0.03)	0.18 (±0.03)	0.15 (±0.05)	0.18 (±0.06)	n.s
	P	1.94 (±0.76)	1.96 (±0.46)	1.79 (±0.48)	1.90 (±0.41)	n.s
	Si	0.02 (±0.01)	0.03 (±0.02)	0.03 (±0.02)	0.03 (±0.01)	n.s

Note: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s = not significant. Data labelled with different small letters (a- c) differed significantly at $p < 0.05$ in each row (Mean ±SD).

4.4 Discussion

Pollution of aquatic ecosystems and soils by anionic surfactants is common due to their common use in soaps and detergents globally and their presence was confirmed in the sampled dam water from Roodeplaat and Hartbeespoort Dam sites. Levels of anionic surfactants found in the water (0.13 to 3.4 mg L^{-1}) were within the range (0.001 and 20 mg L^{-1}) generally found in surface waters (Wang et al., 2015). Both dams are considered hyper-eutrophic, and warm monomictic impoundments (van Ginkel, 2004). A long history of mining, industrial activities and a rapidly growing urban population in the catchment where these two dams are found promotes the co-existence of pollutants such as the anionic surfactants such as LAS, cyanotoxins and metals in these two dams (Pindihama & Gitari, 2020).

Some of the contaminants observed in the dam water used to irrigate the plants in treatments *2* and *4* have been reported to have adverse effects on plants e.g. MCs are known to induce oxidative stress (Saqrane et al., 2008; Machado et al., 2017), high pH and EC are known to induce oxidative stress and affect chlorophyll production (Huang et al., 2017) and anionic surfactants like LAS are also known to induce oxidative stress, reduce chlorophyll production and affect plant growth (Pandey & Gopal, 2010; Wang et al., 2012).

In this study, exposure to LAS in the form of SDS at environmentally relevant concentrations did not affect the total chlorophyll of the plants. Previous studies reported improved plant growth upon exposure to LAS in the range 0.3 mg L^{-1} - 10 mg L^{-1} and significant stunted growth when the common aquatic duckweed (*Lemna minor*) was exposed to 20 – 30 mg L^{-1} SDS (Wang et al., 2012).

Anionic surfactants like SDS, are amphipathic compounds and can easily interact with the polar and non-polar components of cell membranes, resulting in membrane damage and induce oxidative stress (Forni et al., 2012; Pierattini et al., 2018). Toxic effects such as reduced phenols and chlorophyll content and increased activity of stress related enzymes upon exposure to SDS have been reported in aquatic plants like *L. minor* (Wang et al., 2012; Forni et al., 2012), *Azolla pinnata* (Pandey & Gopal, 2010). In the current study, the plants were exposed to much lower levels (± 3 mg L^{-1}) of SDS compared to those reported in previous studies ($\geq$ 10 mg L^{-1}) (e.g. Pandey & Gopal, 2010; Wang et al., 2012; Forni et al., 2012), hence no significant impacts on total chlorophyll were reported between the treatments and the control plants.

The presence of LAS in the form of SDS, at environmentally relevant concentrations in the irrigation water, did not enhance uptake of Sr, Mn, Al in the two plants tested here, as demonstrated by statistically insignificant differences in the means of the four treatments. In the case of *S. tuberosum*, a statistically significant difference was observed for Sr, with low uptakes reported in the control plants, but plants exposed to metal infested Roodeplaat Dam water without any SDS, to milli-Q water with SDS (without any metals) and to Roodeplaat Dam water with SDS and the three metals (Sr, Mn and Al). This implied that the accumulation of Sr in the *S. tuberosum* tubers was not affected by the presence of SDS and was independent of the presence of the metal in the water used to water the plants.

The presence of SDS also did not affect the uptake and accumulation of Sr, Mn, Al and other 19 major and trace cations in *B. oleracea*. Statistically significant higher accumulation of Al in treatment *2* (dam water with Sr, Mn and Al) compared to other treatments, particularly treatments *3* and *4* which had SDS implies that at the SDS levels tested (3.4 mg L^{-1}), did not enhance the uptake of the metals by plants. With regards to other cations which were not controlled in any of the treatments, but were initially present in the dam water and soils used, statistically significant higher accumulations of Cr, Co, Mg and P in treatments *1* and *2*, which did not have any SDS also supported the finding that uptake and accumulation was independent of the LAS, SDS presence.

Previous studies reported contrasting results on the uptake of metals in presence of anionic surfactants like the LAS, SDS. Hasan et al. (2019) reported increased Cd accumulation in shoots and roots of *Althaea rosea* upon exposure to 348.48 mg L^{-1} of SDS. In another study, (Pierattini et al., 2018) did not report significant total accumulation of Zn by poplar plants *Poplus alba* but observed increased accumulation in leaves when 1mM Zn was applied in combination with 0.5 mM of SDS compared to when 1mM Zn was applied alone. Pierattini et al. (2018) also reported increased translocation of Zn from roots to leaves when the poplar plants were exposed to SDS. Contrary to Pierattini et al. (2018), Almeida et al. (2009) found that the LAS, SDS enhanced Cu accumulation in the salt marsh plant *Halimione portulacoides*, but did not find any Cu translocation to the other parts of the plant.

Consistent to our findings, Zhang et al. (2008) reported a reduction in Cd uptake by soybean plants in the presence of LAS. Zhang et al. (2008) found a reduction in Cd bound to carbonates and exchangeable Cd in the soils when the soils are exposed to LAS hence the low uptakes reported.

Almeida et al. (2009) did not find any influence of LAS on Cu levels in sediments. In addition to data suggesting little influence of anionic surfactants on the solubility of metals, Hasan et al. (2019) and Mao et al. (2015), reported degradation of LAS by strains of *Pseudomonas*, which use the contaminant as a source of carbon. The presence of such bacteria (*Pseudomonas*) to degrade the LAS in the soils in the study area was highly likely given the climate (average temperatures of 29^{o}C) in the study area. In addition the levels of LAS applied here were much lower compared to those applied in studies where significant metal uptakes were reported (e.g. Pierattini et al., 2018; Hasan et al., 2019).

Mao et al. (2015) noted that the accumulation of surfactants occurs at solid to liquid or liquid to liquid interface as monomers and this suggests that when their concentration is increased, they replace the interfacial solvent like water with time and this leads to their decreased polarity in an aqueous environment and a reduced surface tension. According to Mao et al. (2015), high concentrations of surfactants makes pollutants which are dissolved to be more mobile and this promotes their elimination by processes such as microbial degradation and uptake by plants. In addition, the properties of the soils and of the surfactant in question also play a role in uptake or adsorption of the surfactant.

Previous studies have suggested both antagonistic and synergistic interactions of LAS with other pollutants such as metal ions and MCs (Chai et al., 2020). In the current study, no antagonistic nor synergistic effects of a combination of these pollutants were reported. Consistent to our findings, Jensen and Sverdrup (2002) also did not report any synergistic effects of pyrene and LAS on *Folsomia fimetaria* and Zhang et al. (2008) did not report any elevated uptake of Cd by the soybean in the presence of LAS. Combined or synergistic effects are affected by numerous factors, among them the plant species tested, the levels tested, duration of exposure and the types of the pollutants tested (Chai et al., 2020). Considering the warmer climate of the area under study, factors such as faster biodegradation of the SDS, reduced availability of the metals present in the media due to soil interactions and adsorption and the low levels of the anionic surfactant tested, could all have played a significant role in the activity and toxicity of SDS, metals and other contaminants on the plants.

What we also observed was a noticeably higher uptake in the edible parts of *B. oleracea* plants (leaves) compared to the potato (*S. tuberosum*) tubers. According to Hasan et al. (2019), *B.*

oleracea, family *Brassicaceae* belongs to a group of plants known to be hyperaccumulators and suited to grow in soils polluted in with metals. Hyperaccumulators can take up Zn and Mn up to 10, 000 mg kg^{-1}; Cu, Ni and Pb beyond 1, 000 mg kg^{-1} dry weight and up to 100 mg kg^{-1} dry weight of Cd in contaminated media (Hasan et al., 2019).

4.5 Conclusions and recommendations

This study explored the effect of the anionic surfactant LAS (in the form of SDS) on the accumulation of metals aluminum (Al), manganese (Mn) and strontium (Sr) in *Brassica oleracea* (cabbage) and *Solanum tuberosum* (potato) plants when exposed to environmental realistic concentrations of the pollutants. The findings indicated that, when exposed to environmentally relevant concentrations of SDS, metals (Sr, Mn, Al and other cations) common cabbage (*B. oleracea*) and cultivated potato (*S. tuberosum*) plants, the total chlorophyll content of the plants was not affected. Moreover, no visual negative effects could be observed on the plants. The combined exposure of the plants to these contaminants did not result in increased uptake and accumulation of the metals as was anticipated. This implies that, the existing levels of anionic surfactants such as LAS, metals and other contaminants such microcystins being found in hyper-eutrophic reservoirs and lakes such as Roodeplaat and Hartbeespoort Dams in South Africa, poses little risk to the crop yields, quality of the crops and human health due to the possible accumulation of these contaminants in irrigated plants. Inasmuch as there is no immediate inherent risk to the plants and human health, continuous monitoring of the contaminants in water, soil and irrigated plants is recommended since the conditions, the concentrations and other factors can quickly change if the management of the catchment does not improve in the near future.

Data availability

Datasets related to this chapter can be found at: Pindihama, Glynn (2023): The effect of the linear alkylbenzene sulfonates, sodium-dodecyl-sulphate (SDS) on the bioaccumulation of Al, Sr & Mn by *Brassica oleracea* & *Solanum tuberosum*. figshare. Dataset. https://doi.org/10.6084/m9.figshare.22332691.v1

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CHAPTER FIVE: EFFECT OF LAS ON THE UPTAKE AND ACCUMULATION OF MICROCYSTINS IN *BRASSICA OLERACEA* AND *SOLANUM TUBEROSUM*

Abstract

Hypereutrophic conditions in major water reservoirs used for irrigation purposes in South Africa promote the co-existence of cyanotoxins and other pollutants such as linear alkylbenzene sulfonate (LAS). LAS is known to alter the permeability of membranes and promote the uptake of other pollutants by plants. In light of the potential human health risks and prevailing hypereutrophic conditions in some catchments in South Africa, we investigated the combined effects of LAS and microcystins (MCs) when cyanobacteria infested water is used to irrigate terrestrial crops.

To understand the potential risks, pot-culture experiments were conducted to assess the effect of LAS on the accumulation of MCs in *Brassica oleracea* (cabbage) and *Solanum tuberosum* (potato) plants. The plants were watered with dam water containing 3.48 mg L^{-1} of the LAS (sodium dodecyl sulfate) and MCs (MC-LR: 10.47 ± 3.879; 6.158 ± 4.127 for MC-RR and 8.160 ± 2.544 for MC-YR µg L^{-1}) for 20 days.

The presence of LAS, at environmentally relevant concentrations in the irrigation water, did not enhance the uptake of MCs in the two plants, as demonstrated by statistically insignificant differences in the means of the treatments (with and without LAS). In addition, the presence of LAS, high pH, Electrical Conductivity (EC), and cyanotoxins in the water did not affect the total chlorophyll or the well-being of the plants. However, in some cases the levels of MCs bioaccumulated by the two plants exceeded the WHO recommended Tolerable Daily Intake (TDI). These findings imply that the tested levels of LAS and MCs did not have any synergic effects on the two plant species, but irrigating food crops with such water still poses a human health risk.

Keywords: Linear alkylbenzene sulfonate, Microcystins, *Brassica oleracea*, *Solanum tuberosum*, combined effects

5.1 Introduction

Cases of harmful algal blooms (HABs) have been on the rise globally in the last few decades. Such increases have been linked to growing urbanization and the resultant increase in nutrients loads into the aquatic environment, higher atmospheric temperatures and salinity, all of which are worsened by human induced climate change (Zhao *et al*., 2013; Howard *et al*., 2017; Díez-Quijada *et al*., 2018).

Literature reviewed in chapter two has suggested that hypereutrophic lakes and reservoirs ideal environments for the co-existence of toxic cyanobacteria since the excessive growth of cyanobacteria in eutrophic lakes consumes oxygen and their eventual death and degradation makes water bodies anoxic and anaerobic (Wang *et al*., 2012). Previous studies have reported enhanced MCs uptake by plants in the presence of LAS (Wang *et al*., 2012). Furthermore, Wang *et al*. (2015) also found increased production of MCs by *Microcystis aeruginosa* in the presence of LAS.

In South Africa, the Crocodile (West) Marico Water Management Area (WMA) which covers parts of Gauteng and Northwest Provinces, houses dams such as Hartbeespoort, Rietvlei, Roodeplaat, and Bospoort. Dams such as Hartbeespoort, Roodeplaat and Bospoort found in this WMA have been reported to have total phosphorus levels above 130 µg L^{-1} and have been classified as hypereutrophic (Van Dyk et al., 2012). The Roodeplaat and Hartbeespoort Dams have also been reported to have enormous populations of algae and are in the top 10 of impoundments needing eutrophication management in South Africa (Van Dyk et al., 2012). A recent study by Mnyango et al. (2022) also described the Roodeplaat Dam as eutro-hypertrophic.

Thus, the co-existence of pollutants such as LAS, cyanotoxins, metals and many other is likely. It is thus of importance to study the impacts of MCs in combination with other stressors including LAS, on terrestrial food plants since the water derived from these dams is mainly used for irrigation. The aim of this study was to assess the effect of anionic surfactants (LAS) on the accumulation of three MC congeners (MC-LR, MC-RR and MC-YR) in *Brassica oleracea* (cabbage) and *Solanum tuberosum* (potato) plants using realistic concentrations of the pollutants.

5.2 Methods

5.2.1 Materials and reagents

A field survey was conducted from the 23rd to the 25th of June 2019 and again from the 14th to the 16th of September 2019 to collect field water to be used for the experiments. The water was collected from canals and farm dams from the Roodeplaat and Hartbeespoort dam sites. Total Dissolved Solids (TDS), EC, pH, and turbidity of the water were monitored *in-situ* and anionic surfactants, chlorophyll-*a*, microcystins (MCs), and cations were measured *ex-situ*. The water was kept frozen at −20°C until required. The LAS used in this study was sodium dodecylbenzene sulfonate (SDS) (CAS No. 25155-30-0; molecular weight 348.48 g mol^{-1}; and chemical formula $C_{18}H_{29}NaO_3S$ acquired from BYMAZ Pty Ltd, Johannesburg, South Africa).

5.2.2 Pot-culture experiment design

The *B. oleracea* seeds were purchased from NTK Agricultural Products & Services (S.A) and the *S. tuberosum* seeds were purchased from Livingseeds Heirloom Seeds (Pty) Ltd Midvaal, Gauteng (S.A). The *S. tuberosum* seeds were first washed with distilled water before being planted in 200 mm plant pots filled with non-contaminated soil. The *B. oleracea* seedlings were produced and pre-grown in plastic trays with non-contaminated soil. The soil used in this study was collected from the agricultural farm at the University of Venda. The farm lies in the low veld climate and has well-drained deep red soils mostly dominated by clay and falls in the Hutton classification, which is the same as the Rhodic Ferralsol (Mabasa, 2019). Regarding the main nutrients, Phosphorus (P), Potassium (K), total Nitrogen (N) and organic matter, the soil contained 25.86 (mg kg^{-1}); 184 (mg kg^{-1}); 0.079% and 2.07%, respectively. All of which indicated healthy soils for plant growth. The soil was collected from a depth of 0-50 cm, and 15 kg of the soil was placed into 350 mm plastic pots for the experiments and treated with 0.3 g (per kg of soil) of Protek General Fertilizer with N:P:K (%) 2:3:2 (14) before introducing the plants.

To investigate the effect of LAS on MCs uptake and accumulation in *B. oleracea* and *S. tuberosum*, plants were watered daily with dam water with a mean concentration ± standard deviation of MC-LR: 10.47 ± 3.879; 6.158 ± 4.127 for MC-RR, and 8.160 ± 2.544 for MC-YR. In order to maintain approximately constant concentrations of the LAS (3.4 mg L^{-1}), the water treatments were tested daily and refreshed accordingly. After 5 days and 20 days of exposure, the accumulations of MCs in edible parts of *B. oleracea* (leaves) were measured (Table 5.1). For *S. tuberosum*, the accumulated MCs in the tubers were assessed only after 20 days of exposure, since the seeds take longer to sprout. Leaves were also harvested from both plant species on the 20th day of exposure for total chlorophyll determination. There were 3 replicates and four treatments of both *Brassica oleracea* and *Solanum tuberosum* plant species and the experiment was repeated once i.e., 3X4X2=**24** plants in total of each species and a total of **48** plants for both species.

Table 5. 1. Design of the experiment and sampling intervals

Treatments	Parameters monitored
Treatment 1 (T1): Control: Milli-Q water with no contaminants	After 5 days of exposure: MCs in *B. oleracea* After 20 days of exposure: MCs and total chlorophyll in *B. oleracea* & *S. tuberosum*
Treatment 2 (T2): Raw dam water containing MCs (MC-LR: 10.47 ± 3.879 µg L^{-1}; MC-RR: 6.158 ± 4.127 µg L^{-1}; MC-YR: 8.160 ± 2.544 µg L^{-1})	After 5 days of exposure MCs in *B. oleracea* After 20 days of exposure: MCs and total chlorophyll in *B. oleracea* & *S. tuberosum*
Treatment 3 (T3): Milli-Q water with 3.4 mg L^{-1} LAS	After 5 days of exposure: MCs in *B. oleracea* After 20 days of exposure: MCs and total chlorophyll in *B. oleracea* & *S. tuberosum*
Treatment 4 (T4): Combined exposure: Raw dam water containing MC-LR: 10.47 ± 3.879 µg L^{-1}; MC-RR: 6.158 ± 4.127 µg L^{-1}; MC-YR: 8.160 ± 2.544 µg L^{-1} and LAS (3.4 mg L^{-1})	After 5 days of exposure: MCs in *B. oleracea* After 20 days of exposure: MCs and total chlorophyll in *B. oleracea* & *S. tuberosum*

5.2.3 Determination of total chlorophyll

Chlorophyll content was measured according to Baskar *et al*. (2015). 50 mg of the leaves were crushed using a motor and pistil, and then soaked 10 mL of 95 % (v/v) ethanol, followed by incubation in the dark for 72 hours. This was then followed by centrifuging for 30 minutes at 2264 × g, then collection of the supernatant and reading the absorbance at 664.2 and 648.6 nm using a UV-vis spectrophotometer (SPECTROstar Nano, BMG LABTECH, Germany). Equations 1, 2 and 3 (below) were then used to calculate the chlorophyll-*a* and *b*, and the total chlorophyll content.

$$\text{Chl } a = 13.36\ A664.2 - 5.19\ A648.6 \qquad [1]$$

$$\text{Chl } b = 27.43\ A648.6 - 8.12\ A664.2 \qquad [2]$$

$$\text{Total chlorophyll} = \text{Chl } a + \text{Chl } b. \qquad [3]$$

Total chlorophyll content was expressed as milligram per gram per fresh matter (FM).

5.2.4 Quantification of cyanotoxins in plant material

Microcystins (MC-LR, MC-RR, and MC-YR) in plant tissues were determined using a triple quadrupole LC–MS/MS system (model 8045, Shimadzu Corporation, Japan). Toxin extraction was conducted using a modification of the method used by Manubolu *et al*. (2018). Accurately weighed, 100 g of plant material (leaves for *B. oleracea* and tubers for *S. tuberosum*) were freeze-dried for 48 hours at −48°C under a constant vacuum of 44 µmHg (Telstar Lyoquest Freeze Dryer, Terrassa, Spain). The freeze-dried material was then ground to powder using a mortar and pestle.

Ten millilitres of 50% methanol solution was added to 1 g of each freeze-dried sample and sonicated for 5 minutes in a water bath (SCIENTEC Ultrasonic Cleaner, Model 705, South Africa) for 5 minutes. Upon sonication, the plant extracts were then centrifuged for 30 minutes at 2264 × *g*. The whole process of sonication, centrifugation, and collecting the supernatant was repeated thrice and the supernatant was pooled to give approximately a 30 mL extract of each sample. The 30 mL extract was then cleaned up using solid phase extraction (SPE) with HLB (3 cc, 60 mg, Waters Oasis).

For SPE with HLB, the cartridges were first conditioned with methanol (6 mL), followed by ultrapure water (6 mL). The samples (± 30 mL) were the slowly loaded onto the cartridges, followed by rinsing with 20% methanol. The cartridges were then eluted with 10 mL of 80% methanol. Lastly, the eluent was then dried at 50°C under a stream of nitrogen (N_2) gas. The dried samples were reconstituted in 1 mL of 80% methanol prior to LC-MS/MS analysis.

Chromatographic conditions

Levels of microcystins (-LR, -RR and -YR) in the plant extracts were determined on a triple quadrupole LC–MS/MS system (model LCMS-8045, Shimadzu Corporation, Japan) with a Shim-pack Velox SP-C18, 2.7 µm, with dimensions 2.1 × 100 mm (Shimadzu, Japan). The injection volume was set at 10 µL and the mobile phases used were 0.1% formic acid (FA) in water (A) and 0.1% FA in acetonitrile (B). A flow rate of 0.4 mL min^{-1} and a 5-minute binary gradient was used with an elution profile of: 2% B (0.4 min), linear gradient to 70% B (3.1 min), 100% B (0.5 min), and, finally, 2% B (1 min).

The LC-MS/MS interface conditions were: 300^{o}C interface temperature, 3 L min^{-1} for the nebulizing gas flow, 235^{o}C DL temperature, 10 L min^{-1} for both drying gas and heating gas flow and interface voltage of 3.0 kV for electrospray in the positive (ES+) mode.

The final concentration of toxins in each sample was determined using equation 4:

$$\text{Conc in sample } (\mu\text{g L}^{-1}) \; = \left(\frac{C_o \times \text{Vol of extract used (L)}}{\text{Volume of sample used (L)}}\right) \qquad [4]$$

Where C_o = the concentration of the sample determined from the calibration curve (µg L^{-1}).

5.2.5 Estimated daily intake (EDI)

Equation 5 was utilized to estimate the daily intake of cyanotoxins for an average size human (Bartos, 2020):

$$\text{EDI} = \frac{T \times C}{W} \qquad [5]$$

Where:
T = the concentration of toxins in the edible fractions of the cabbage (T, µg kg^{-1} fresh weight).
C = the daily consumption amounts of cabbage (*C*, kilograms per day).
W = weight of an average-sized human (*W*, 60 kg adult).

We assumed that the consumption of cabbage is similar to that of lettuce and used 85 g dry weight (DW) of cabbage and 148 g dry weight (DW) of potatoes based on the U.S. FDA (2017) suggested serving size (Bartos, 2020). An EDI of > 0.04 exceeds the total daily intake limit set by WHO.

5.2.6 Data analysis

To compare the levels of accumulated MCs and the total chlorophyll of the various plant treatments, analysis of variance (ANOVA) and/or the Kruskal-Wallis tests were used at 95% confidence intervals (CI) using GraphPad InStat 3 (GraphPad Software, California, United States; RRID:SCR_000306). Levels of MCs are presented by their means ± the standard deviation (SD). Kolmogorov–Smirnov and Bartlett tests were used to test for normality and variance homogeneity at 95% CI. Data which passed this test was compared using ANOVA while data which did not pass the test was compared using Kruskal–Wallis at 95% CI. The Tukey-Kramer multiple comparisons test and Dunn's multiple comparisons test were used as *post-hoc* assays for data which passed the normality tests and data which did not pass the normality test, respectively.

5.3 Results and discussion

5.3.1 Physicochemical parameters of the dam water

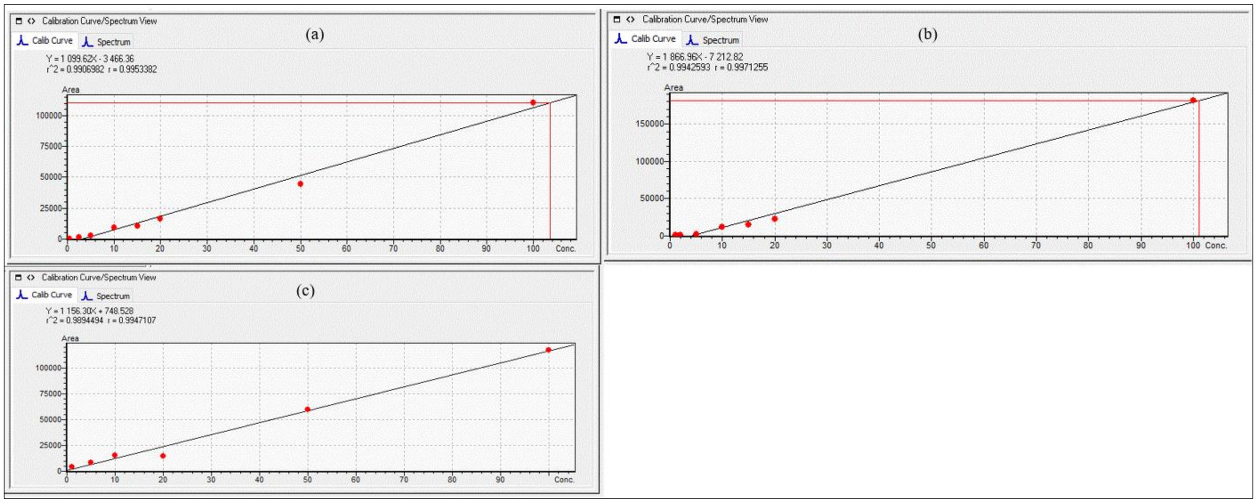
The dam water used to water the plants was alkaline, with a mean pH of 9.02 ± 0.29, had high EC and TDS levels (380 ± 16.52 µS cm^{-1} and 228 ± 7.51 mg L^{-1}, respectively). The water also had a high phytoplankton biomass (Chlorophyll-*a* 440.24 ± 328.147 µg L^{-1}). The raw dam water had the following mean concentration ± standard deviation of MC-LR: 10.47 ± 3.879 µg L^{-1}; 6.158 ± 4.127 µg L^{-1} for MC-RR, and 8.160 ± 2.544 µg L^{-1} for MC-YR. The pH of the dam water used for irrigation was above the 6.5–8.4 threshold for water intended for irrigation in S.A (DWAF, 1996). Even though the EC of the dam water was quite high, it was within the S.A (DWAF, 1996) guideline and the FAO (1985) (Ayers and Westcot, 1985) limits for irrigation water, which are set at $\leq$ 400 µS cm^{-1} and 700 µS cm^{-1}, respectively. The levels of anionic surfactants in the water ranged from 0.13 to 3.4 mg L^{-1}.

5.3.2 Bioaccumulation of cyanotoxins in *S. tuberosum* and *B. oleracea*

To identify and quantify MCs, two multiple reaction monitoring (MRM) transitions for MC-LR and MC-YR were selected and optimized, with the most abundant ionic product utilized for quantitation and the other for confirmation, whereas for MC-RR only one transition was used. For MC-LR and MC-YR, the single protonated molecular ions $[M + H]^+$ were formed, as a result of the presence of one arginine moiety, which is the most preferred protonation site for these compounds (Zervou *et al.*, 2017). The MRM transitions used for MC-LR were 996.0078/996.00 and 498.5078/162.90; for MC-RR: 520.0078/134.90; for MC-YR: 1046.5078/1046 and 523.7578/127.00, with the first one being used for quantification and the second one for confirmation (for MC-LR and MC-YR).

With respect to all the three MCs monitored, the mass-to-charge ratio (m/z) signal 135 was the main ionic product. Like other polypeptides, MCs form sodium replacement ions which results in ion envelopes at each charge state apparent in mass spectra (Draper *et al.*, 2013). For MC-RR the transition with a m/z of 520.0078 corresponding to the double charged protonated molecular ion $[M + 2H]^{2+}$ precursor ions, as they contain two arginine residues in their molecular structure (Draper *et al.*, 2013; Zervou *et al.*, 2017).

Standard solutions at seven different concentrations (1, 2, 5, 10, 20, 50, and 100 µg L^{-1}) were prepared using cabbage leaves extracts and potato tuber extracts and these were used to quantify the toxins in the plant samples (Figure 5.1). The MRM chromatograms of the quantification ions for the three MCs at a concentration of 100 µg L^{-1} are shown in Figure 5.2 (retention times were 2.5 mins for MC-RR; 2.48 mins for MC-YR and 2.7 mins for MC-LR).



(a)

```
Y = 1 099.62X - 3 466.36
r^2 = 0.9906982  r = 0.9953382
```

(b)

```
Y = 1 866.96X - 7 212.82
r^2 = 0.9942593  r = 0.9971255
```

(c)

```
Y = 1 156.30X + 748.528
r^2 = 0.9894494  r = 0.9947107
```

Figure 5. 1. Calibration curves obtained for (a) Microcystin-RR (MC-RR); (b) MC-YR; and (c) MC-LR; in *B. oleracea* leaf extract.

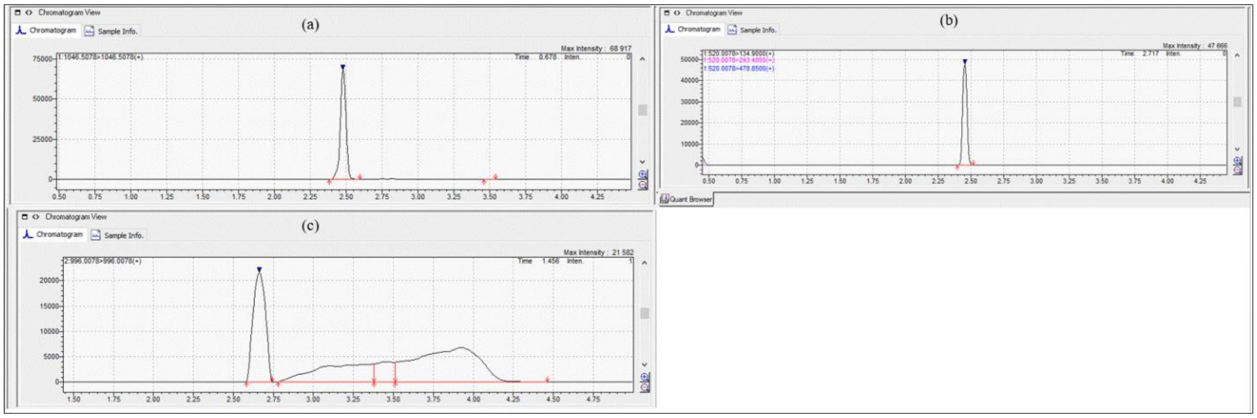


Figure 5. 2. MRM chromatograms of quantification ions for the three MCs, (a) MC-RR; (b) MC-YR and (c) MC-LR at a concentration of 100 µg L^{-1}.

5.3.3 Bioaccumulation of cyanotoxins in *S. tuberosum*

When exposed to the four different treatments for a period of 20 days, Table 5.2 shows the mean ± SE concentrations of MCs accumulated in the tubers of *S. tuberosum*. The accumulation patterns resembled the levels of MCs in the raw water samples, with plants exposed to raw dam water (*T2* and *T4*) showing higher levels of MC-LR, followed by MC-RR then MC-YR. Statistically significant differences among the mean levels of accumulated toxins were reported for MC-LR and MC-YR, whereas MC-RR did show any statistically significant differences (ANOVA/Kruskal-Wallis Test, at 95% CI) among the four treatments. Higher levels of the toxins accumulated in plants exposed to raw dam water (*T2*) compared to the other three treatments. The presence of LAS in the raw dam water in *T4* did not result in higher uptake and accumulation as anticipated.

Except for MC-LR, the levels of MCs accumulated by the tubers did not reach levels high enough to exceed the TDI of 0.04 mg kg^{-1} of body weight recommended by WHO. Since MC-LR is normally the dominant congener in many waters dominated by the *Microcystis* and *Aeruginosa* genera, the raw dam water used was dominated by MC-LR, hence it was the only congener which exceeded the recommended TDI.

5.3.4 Bioaccumulation of cyanotoxins in *B. oleracea*

Regarding the accumulation of the toxins in *B. oleracea* leaves, Table 5.3 and Table 5.4 show the mean levels of MCs accumulated in the leaves of the plants after 5 days and 20 days, respectively.

Based on the findings, a clear increase in the accumulation of the three MCs in the plants from the 5th day to the 20th day is observed. Statistically significant differences in treatments were observed for MC-YR and MC-LR after 5 days of exposure, with significantly higher accumulations observed in *T2* followed by *T4*, compared to the other treatments (ANOVA/Kruskal-Wallis Test at $p = 0.05$).

Findings in Table 5.4 show that statistically significant differences were found among the treatments for all of the three congeners of MCs (ANOVA/Kruskal-Wallis Test at $p = 0.05$). Similar to the patterns observed for *S. tuberosum* tubers and for *B. oleracea* leaves after 5-day exposures, higher levels of MCs accumulated in *T2*, followed by *T4*, compared to the other treatments. The findings imply that the presence of LAS in *T4* did not have any impact on the uptake of MCs from the soil (compared to *T2* raw dam water containing MCs but without LAS) and that the presence of LAS in raw dam water in *T4* did not enhance the uptake and accumulation of MCs by the plants.

Table 5. 2. Mean (±SD) MCs accumulated in *S. tuberosum* tubers upon 20-day exposure to the four treatments.

	Treatment 1	**Treatment 2**	**Treatment 3**	**Treatment 4**	**p-value**	**EDI** (mg kg^{-1} of body mass day^{-1})
MCRR (μg g^{-1} DW)	0.00025 (±0.00035)^{a}	0.00485 (±0.00239)^{a}	0.00054 (±0.00027)^{a}	0.00382 (±0.00296)^{a}	0.0793 (n.s)	0.007
MCYR (μg g^{-1} DW)	0.00055 (±0.00018)^{a}	0.00640 (±0.00262)^{a,b}	0.00117 (±0.00084)^{b}	0.00531 (±0.00181)^{a,c}	0.0007***	0.009
MCLR (μg g^{-1} DW)	0.01604 (±0.02380)^{a}	0.20039 (±0.13240)^{b}	0.02271 (±0.02386)^{a,c}	0.15087 (±0.12120)^{a,b,d}	0.0013 **	0.284

*p < 0.05, **p < 0.01, ***p < 0.001, n.s = not significant. Data labelled with a–d differed significantly (ANOVA/ Kruskal-Wallis Test) at p < 0.05 in each row. See appendices 5.1-5.3 for details of the ANOVA/ Kruskal-Wallis tests analysis

Table 5. 3. Mean (±SD) MCs accumulated in *B. oleracea* leaves upon 5-day exposure to the four treatments.

	Treatment 1	**Treatment 2**	**Treatment 3**	**Treatment 4**	**p-value**	**EDI** (mg kg^{-1} of body mass day^{-1})
MCRR (μg g^{-1} DW)	0.00058 (±0.00117)^{a}	0.01165 (±0.01663)^{a}	0.00088 (±0.00217)^{a}	0.01296 (±0.02318)^{a}	0.5427 (n.s)	0.018
MCYR (μg g^{-1} DW)	0.00059 (±0.00012)^{a}	0.00579 (±0.00071)^{b}	0.00060 (±0.00008)^{a}	0.00547 (±0.00070)^{b}	< 0.0001 ***	0.008
MCLR (μg g^{-1} DW)	0.0000 (±0.0000)^{a}	0.0033 (±0.0015)^{b}	0.0000 (±0.0000)^{a}	0.0011 (±0.0011)^{a}	< 0.0001***	0.005

*p < 0.05, **p < 0.01, ***p < 0.001, n.s = not significant. Data labelled with a–d differed significantly (ANOVA/ Kruskal-Wallis Test) at p < 0.05 in each row. See appendices 5.4-5.6 for details of the ANOVA/ Kruskal-Wallis tests analysis

Table 5. 4. Mean (±SD) MCs accumulated in *B. oleracea* leaves upon 20-day exposure to the four treatments.

	Treatment 1	**Treatment 2**	**Treatment 3**	**Treatment 4**	**p-value**	**EDI** (mg kg^{-1} of body mass day^{-1})
MCRR (µg g^{-1} DW)	0.00016 (±0.00035)^{a}	0.08392 (±0.05950)^{b}	0.00068 (±0.00181)^{a}	0.03497 (±0.04290)^{a,b}	0.0143*	0.119
MCYR (µg g^{-1} DW)	0.0006 (±0.0001)^{a}	0.0069 (±0.0005)^{b}	0.0005 (±0.0000)^{a}	0.0051 (±0.0008)^{c}	< 0.0001***	0.010
MCLR (µg g^{-1} DW)	0.0000 (±0.0000)^{a}	0.0057 (±0.0013)^{b}	0.0000 (±0.0000)^{a}	0.0025 (±0.0019)^{c}	< 0.0001***	0.008

*p < 0.05, **p < 0.01, ***p < 0.001, n.s = not significant. Data labelled with a–d differed significantly (ANOVA/ Kruskal-Wallis Test) at p < 0.05 in each row. See appendices 5.7-5.9 for details of the ANOVA/ Kruskal-Wallis tests analysis

Much of the work on the combined risks of LAS and MCs has been done by Wang *et al* (Wang *et al*., 2011; Wang *et al*., 2012). According to Wang *et al*. (2011), LAS affects organisms by altering their membrane permeability, the activity of enzymes, and the structure of tissues in organisms (Wang *et al*., 2011). Unlike our findings, where the presence of LAS did not impact the accumulation of MC-LR in plants, Wang *et al*. (2011) reported higher accumulation rates when lettuce seedlings were exposed to a combination of MC-LR and LAS compared to MC-LR alone.

Similar to our findings, where we found higher levels of MCs in potato tubers compared to cabbage leaves, Wang *et al*. (2011) reported higher levels in roots compared to other parts of the plants. Similar to our findings, where we found higher levels of MCs in potato tubers compared to cabbage leaves, Wang *et al*. (2011) reported higher levels in roots compared to other parts of the plants. This is because many plants accumulate pollutants in the roots and restrict their transportation to the other plants of the plants (Stefanowicz et al., 2016). This is a very a useful mechanism of phytostabilization, which makes pollutant immobile in plants (Stefanowicz et al., 2016).

In contrast to our findings, Wang *et al*. (2012) found enhanced uptake of MC-LR in duckweed even at the lowest concentration of 3 µg mL^{-1}, which was comparable to the 3.4 µg mL^{-1} used in this study. The major difference between these experiments was the media in which the experiments were conducted, with Wang *et al*. (2012) having used aquatic plants compared to the terrestrial plants tested in this study.

In our study, the presence of microbes with the potential to degrade LAS in the soil could have been a major factor. According to Mao *et al*. (2015) at low concentrations, surfactants build up at the liquid to liquid or at the solid to liquid interface as monomers. Increasing their concentrations, eventually replaces the interfacial solvent, such as water, leading to decreased polarity of the aqueous-phase and a surface tension reduction. In high concentrations of surfactants, dissolved pollutants in the aqueous phase gain more mobility which is conducive for removal and uptake by plants and even degradation by microbes. Also, the properties of the soil and the surfactant itself influence the adsorption of a surfactant (Mao *et al*., 2015). In addition, previous studies have demonstrated that soils have the potential to temporarily make cyanotoxins unavailable for uptake by plants through chemical and physical modification, though this is dependent on the type of soil (Bartos, 2020).

The interaction and combination of LAS and other contaminants including microcystins and metal ions has been found to be both synergistic and, in some cases, antagonistic (Chai *et al*., 2020). Our findings did not suggest any synergistic nor antagonistic effects of LAS in combination with MCs in the water used. Consistent to our findings, Zhang *et al.* (2008) did not find increased uptake of Cadmium (Cd) by soybeans in the presence of LAS. Jensen and Sverdrup (2002) also did not find any combined effect of LAS and pyrene on the *Folsomia fimetaria*. According to Chai *et al*. (2020) synergistic or combined effects are influenced by a number of factors including the types of contaminants tested, the plant species, the concentrations tested, and the duration of exposure. In this study factors such as faster biodegradation of LAS by microbes and the low concentrations of LAS tested could all have affected the activity and toxicity to the plants of LAS, MCs, and other contaminants.

Even though MC-RR was of a lower concentration in the raw dam water compared to MC-LR, the findings of the current study have shown that it can accumulate in cabbage leaves to levels which can exceed the 0.04 mg day^{-1} kg^{-1} of body weight when plants are watered with contaminated dam water. This is of concern since this limit was reached after only 5 days of exposure to the dam water. However the fact that the EDI was not exceeded in the cabbage leaves after 20 days of exposure to the same dam water could be a reflection that the plants were finding ways of copying and bio-transforming the toxins as the exposure was prolonged. Xiang *et al.* (2020) established that the biotransformation and depuration rates of MC-LR in plants can sometimes exceed its uptake and there can also be higher degradation in soils, thus lower bioaccumulation from soil.

It is also important to mention that WHO has provisional TDI values for MC-LR only and not for other MC congeners and here we calculated EDIs for each of the three MC congeners monitored. Based on that, assuming that all the MCs have similar impacts on human beings, the combined EDIs for the MCs monitored here and those not monitored in this study can easily exceed the set TDI values.

5.3.5 Effects of LAS and MCs on *B. Oleracea* and *S. tuberosum*

Since exposure to increased levels of MCs in plants is known to induce oxidative stress, hinder the plants' ability to produce chlorophyll, and inhibit photosynthesis (Campos *et al*., 2021), total chlorophyll levels in the two plant species were monitored to assess the potential effects of LAS and MCs on the plants. In addition to the two stressors (MCs and LAS), the dam water used in treatments *2* and *4* also had a high EC (380 ±16.52 µs cm^{-1}) and TDS levels (228 ±7.51 mg L^{-1}), indicating contamination with other salts. All these contaminants, for example MCs (Saqrane *et al.*, 2008; Machado *et al.*, 2017), high pH, high EC (Huang *et al.*, 2017), and anionic surfactants (Pandey & Gopal, 2010; Wang *et al.*, 2012) are also known to induce oxidative stress, reduce chlorophyll production, and affect plant growth.

Figure 5.3 (i) shows higher total chlorophyll content in the leaves of *B. oleracea* plants exposed to treatment *1* compared to other treatments. There were no statistically significant differences in the mean total chlorophyll content among the plants exposed to the four treatments after 20 days of exposure ($p > 0.05$). This implied that the levels of MCs, LAS, and other pollutants in the raw dam water used were not high enough to impact the synthesis of chlorophyll and other photosynthetic processes in the plants.

Concerning the total chlorophyll content of the *S. tuberosum* leaves, higher total chlorophyll levels were observed in plants exposed to treatments *1* and *4*, but there were no statistically significant differences in the mean total chlorophyll levels among the four treatments ($p > 0.05$). The findings imply that exposure to environmentally relevant levels of MCs and LAS, as applied in this study, and the high EC in the raw dam water did not induce oxidative stress nor inhibit chlorophyll production in the plants. In addition, no significant visual impacts were observed on the plants exposed to the four treatments.

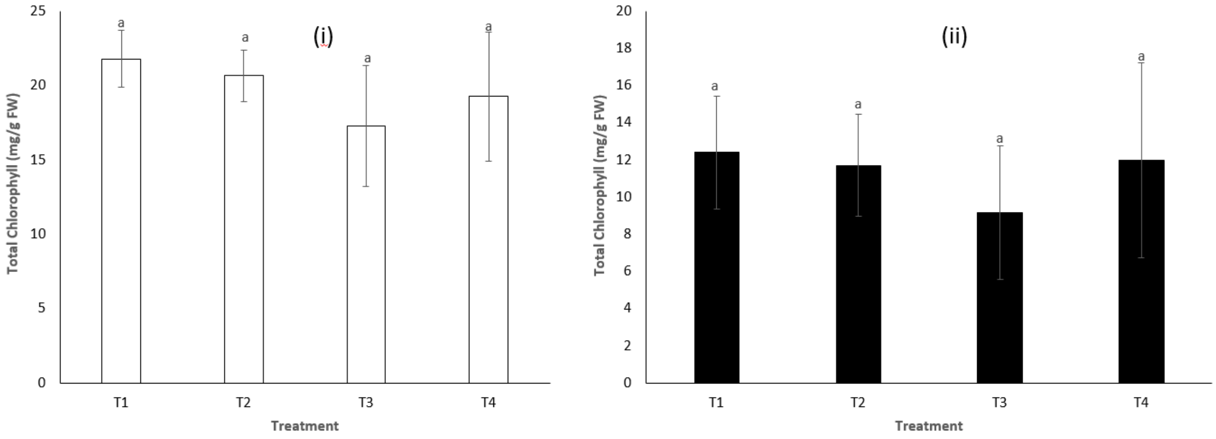


Figure 5. 3. Total chlorophyll in (i) cabbage (*B. oleracea*) leaves (ii) potato (*S. tuberosum*) leaves, after 20 days of exposure to the four treatments.
N.B: Data labelled with a–d differed significantly (ANOVA/ Kruskal-Wallis Test) at $p < 0.05$ in each row.

5.4 Conclusions

Based on the findings, the presence of the anionic surfactant (LAS) did not induce or promote the uptake of MCs by the two plant species. In addition, the presence of LAS and MCs in the irrigation water did not affect the total chlorophyll content and well-being of the tested plants. The study demonstrated that irrigation of terrestrial food plants with cyanobacteria-infested water from dams, such as Roodeplaat, can lead to MCs accumulating in the edible parts of the plants to levels that can exceed the set TDI of 0.04 mg kg^{-1} of body weight. Long-term studies investigating the levels of cyanotoxins in irrigation water in areas such as the Crocodile (West) Marico WMA and their potential impacts on crop productivity and the dietetic acceptability of such plants for human consumption are recommended. Such studies will need to factor in the local climate, soil types, degradation rates of cyanotoxins in the soil, and also consider a variety of cyanotoxin classes for a proper risk assessment.

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CHAPTER SIX: DEVELOPMENT & APPLICATION OF A CROSSLINKED CHITOSAN-BASED SOLID PHASE ADSORPTION TOXIN TRACKING TECHNOLOGY (SPATT) ADSORBENT

Abstract

Since contamination of water, food crops and fish with cyanotoxin have been recognized as a human health risk, there is a need to look into more reliable and sensitive early warning tools for these toxins in water bodies to protect human health. In this study, a composite of glutaraldehyde-crosslinked chitosan and multiwalled carbon nanotubes (ChMWCNT) was synthesized using mechanical stirring and freeze-drying and tested for its potential use as a solid phase adsorption toxin tracking (SPATT) adsorbent for monitoring of microcystins (MCs) in freshwater. The composite was characterized for its functional groups, surface area and pore properties and surface morphology by FTIR, BET and SEM. Batch adsorption experiments to assess the effect of contact time, adsorbent dosage and initial MC-LR concentration were conducted. For desorption efficiency, various methanol solutions were tested. The composite was found to be efficient in adsorbing MC-LR showing 97% removal and maximum adsorption capacity of 4.639 µg g^{-1} under optimized conditions of 5 µg L^{-1} of MC-LR, adsorbent dose of 0.03g 5 mL^{-1}, and contact time of 30 mins. The adsorption kinetics were better explained by the second-order model, meaning that the adsorption was occurring through chemisorption. The isotherm data was better explained by the Langmuir isotherm model, thus inferring a monolayer surface adsorption of MC-LR onto ChMWCNT. In terms of desorption, 100 % methanol was found to be most effective, with the highest mean desorption efficiency of 84.71 ± 6.47 %. When applied for the adsorption of microcystins in raw dam water, the composite was saturated within two days of exposure and effectively adsorbed and desorbed three congeners of MCs (-LR, -RR and –YR) tested. The developed composite showed potential for application in SPATT for monitoring of MCs in freshwater regardless of its lower maximum adsorption capacity compared to other adsorbents used for similar purposes.

6.1 Introduction

The global proliferation of toxin producing cyanobacterial blooms has gained international attention in recent years. These increases have been attributed to a wide variety of environmental factors including nutrient pollution, increased temperature and salinity, many of which will likely be exacerbated by climate change (Zhao et al., 2013; Howard et al., 2017; Díez-Quijada et al., 2018). Toxins produced by algae (cyanotoxins) pose a significant risk for humans, livestock, pets, and wildlife since they can cause illness and mortality (Howard et al., 2017). Among the many types of cyanotoxins that have been documented, microcystins (MCs) are the most frequently occurring in freshwater environment, and hence, have been widely investigated.

While MCs contamination of drinking water and recreational waters can pose an immediate threat to humans, food consumption can also be an exposure route, as MCs can accumulate in the edible tissues of animals and plants (Manubolu et al., 2018). Thus, humans may be orally exposed to cyanotoxins by drinking contaminated water, through the consumption of cyanotoxin-containing freshwater fish, crops, and food supplements, or by ingesting water during recreational activities (Díez-Quijada et al., 2018). Microcystins are commonly found in freshwater ecosystems worldwide and because of their toxicity, in 1999, the World Health Organisation (WHO) set a provisional guideline of 1 μg L^{-1} for microcystin-LR (MCLR) in drinking water (Meneely and Elliott, 2013) and a tolerable daily intake (TDI) of 0.04 μg MC-LR Kg^{-1} body weight (BW) in food (Zhao et al., 2013; Miller et al., 2017; Miller & Russell, 2017). There is thus a need to look into more reliable and sensitive early warning tools for these toxins in water bodies to protect human health and avoid economic losses.

Several countries have routine programmes to monitor these toxins and to predict harmful algal blooms (HABs) proliferation and test possible contamination of food crops and fish. However, the drawback of monitoring HABs lies in the difficulties in integrating the water samples temporally and spatially, the variability in the toxicity of HABs, tentative identification of these HABs, and the amount of time, labor, and expertise required (Li et al., 2011). The sampling and monitoring of these toxins in rivers and large water bodies can also be problematic since their levels can vary rapidly (Wood et al., 2011).

To overcome the drawbacks of grab sampling, MacKenzie et al. (2004) came up with the solid-phase adsorption toxin tracking (SPATT) technology, for possible use in the detection and early warning of the presence of cyanotoxins. SPATT involves suspending small bags containing adsorbent which accumulate toxins in the water body. The toxins can then be extracted and measured, providing information on extracellular toxins over an extended period. Applicability has been demonstrated for a range of lipophilic toxins in seawater, but a range of technical problems remain to be solved for use of SPATT with high water-soluble toxins such as cylindrospermopsin and saxitoxins (Wood et al., 2011; Li et al., 2011). The technique offers numerous advantages including integrated sampling in terms of time and spatial distribution of the toxins, relatively clean sample matrices, provides data on toxin dynamics and its early warning capabilities for biotoxins (Li et al., 2011).

Many different sorbents have been used for passive sampling all over the world, from the DIAION HP20 to SEPABEADS type resins for the accumulation of microalgal or cyanobacterial toxins of different polarities (Zendong et al., 2014). Although passive sampling has been successfully used several times to monitor cyanotoxins using different bulk polymeric sorbents (Zendong et al., 2014), most of these sorbents are synthetic and relatively costly to buy. Studies on the characterization and mechanism of SPATT resins for the adsorption of lipophilic and hydrophilic cyanotoxins are also limited (Zhao et al., 2013).

This study aimed to synthesize and assess the physicochemical properties of a crosslinked chitosan and multi-walled carbon nanotubes composite and investigate its potential applicability to adsorb and desorb MCs for potential use in the solid phase adsorption tracking of MCs in freshwater. Chitin and its de-acetylated product, chitosan, are the world’s second most abundant natural polymers after cellulose (Gang et al., 2010) and the most abundant amino polysaccharide (Crini, 2005). As a sorbent, it poses advantages due to its availability and low cost. Chitosan is nontoxic, biocompatible and hydrophilic hence a suitable option as an adsorbent since it is obtained from renewable natural resources and has a high affinity for a variety of pollutants ranging from metal ions to organic compounds (Gonçalves et al., 2017).

Recent studies for example Gomez-Maldonado et al. (2022) and Tran et al. (2022) have demonstrated that chitosan can be modified and be applied for the adsorption of MC-LR in water purification, but no studies have evaluated its use as a sorbent in the passive sampling of MCs in SPATT format. Insertion of multi-walled carbon nanotubes into the chitosan hydrogel to form a chitosan-multi-walled carbon nanotube (ChMWCNT) composite was hypothesized to have the synergistic effect of improving the physical properties of chitosan and improving its adsorptive characteristics. Thus, the objective of this work was to insert multi-walled carbon nanotubes into the hydrogel structure and evaluate the respective effects on the composite material characteristics, its capabilities for MC adsorption and desorption for possible use in SPATT samplers and for the solid phase extraction of the toxins.

6.2 Materials and methods

6.2.1 Chemicals and reagents

Course ground flakes and powdered high molecular weight chitosan was purchased from Rochelle Chemicals (Johannesburg, South Africa) with a deacetylation percentage of approximately 57.72 %. Glutaraldehyde (GLA) and acetic acid were also purchased from Rochelle Chemicals (Johannesburg, South Africa) and were all analytical-reagent grade. Hydroxyl functionalized Multiwalled Carbon Nanotubes (MWCNTs, Purity: >98 %, Average diameter: 10-20 nm) were purchased from SabiNano (Pty) Ltd (Johannesburg, South Africa). Synthetic aromatic adsorbent DIAION HP20 resin was purchased from Rochelle Chemicals (Johannesburg, South Africa). The ionized water obtained from a milli-Q water purification system (Merck-Millipore Ltd., Germany) of 18.2 MΩ cm^{-1} quality was used to prepare all the solutions. Microcystin-LR (MC-LR) standards, 0.5 mg films (Eurofins Scientific, U.S.A) were purchased from ToxSolutions, Kits and Services (South Africa).

6.2.2 Preparation of the ChMWCNT composite

Chitosan was crosslinked with glutaraldehyde using ratios optimized by Gonçalves et al. (2017) and chitosan to MWCNTs ratios applied by Alves et al. (2019). In brief, chitosan (1 g) was dissolved in 50 mL of 1 % v/v acetic acid. After complete dissolution of chitosan, carbon nanotubes (CNT) (10 % wt) were added to the solution. Then, glutaraldehyde (2 % (v/v)) was used as a crosslinking agent and slowly added to the carbon nanotubes chitosan solution under mechanical stirring (50 rpm) until it formed a gel. The formed hydrogel was freeze dried for 48 hours at -48 °C under a constant vacuum of 44 µmHg (Telstar Lyoquest Freeze Dryer, Terrassa, Spain). The freeze-dried material was then ground to powder using a mortar and pestle, followed by sieving using a 250 µm sieve to get particles above 250 µm only (suitable to be used in SPATT bags).

6.2.3 Characterization

The FTIR spectra of the chitosan (CH), gluteraldehyde crosslinked chitosan (ChGLA) and the chitosan/MWCNT composite (ChMWCNT) were recorded using Bruker Alpha-P FTIR spectrometer equipped with a diamond ATR window (Bruker Optik GmbH, Ettlingen, Germany). All spectra were recorded in the spectral range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}. Surface area measurements were conducted on a Micromeritics TriStar II 3020 Surface Area and Porosity Analyzer (Norcross, Georgia, U.S.A). The surface morphology of the samples was characterized by FEI Nova Nano SEM 230 (U.S.A).

6.2.4 Adsorption and desorption studies

6.2.4.1 Optimization of equilibration time

A 5 µg L^{-1} MC-LR solution was used to investigate the effect of contact time. The toxin solutions (5 mL) were placed in 15 mL glass centrifuge tubes with 0.01 g of the ChMWCNT composite and the mixture was shaken at 145 rpm (Stuart Reciprocating Shaker, SSL2, U.K) for 5, 10, 15, 30 and 60 mins at room temperature and immediately after, filtered through 0.22 µm pore membrane filters and the filtrate analyzed for MCs using Microcystins/Nodularins (ADDA) ELISA kits, EUROFINS (Kit Lot No: 19I1120: PN 520011, Eurofins Scientific, U.S.A) using SPECTROstar Nano (BMG LABTECH, 601-1106, Germany) for quantification.

6.2.4.2 Optimization of dosage

A 5 µg L^{-1} of MC-LR (5 mL) and composite dosages 0.005 g; 0.01 g; 0.02 g; 0.03 g; 0.05g and 0 g (control) were investigated by placing the composite in 15 mL glass centrifuge tubes and shaken at 145 rpm at room temp for 30 mins (as determined from the previous experiment section 2.4.1). Samples were then filtered through 0.22 µm pore membrane filters immediately after agitation and the residual toxin levels in the supernatant determined as described in section 2.4.1.

6.2.4.3 Adsorption and equilibrium studies

Adsorption experiments were conducted to investigate the adsorption isotherms at equilibrium and the kinetics. In all the experiments (except in SPATT bag format), ChMWCNT was the adsorbent and the optimized unit dose (0.03 g) and optimized time of 30 mins as determined in the previous experiments were used. Unless otherwise stated, in all the experiments, 0.03 g of ChMWCNT and

5 mL of 5 µg L^{-1} MC-LR solution were reacted in 15 mL glass centrifuge tubes, and the tubes were shaken for 30 mins at 145 rpm at ambient temperature.

The effect of contact time and the kinetics studies were investigated by placing 0.03 g of ChMWCNT (the optimized unit dose) in 15 mL glass centrifuge tubes with 5 mL of 5 µg L^{-1} MC-LR solution. This was then followed by agitation for 5, 10, 15, 30 and 60 mins, then filtering through 0.22 µm pore membrane filters and the residual toxin levels in the supernatant determined as described in section 2.4.1.

For the equilibrium study, 0.03 g of adsorbent was introduced into seven 15 mL centrifuge tubes with 5 mL of different MC-LR concentrations (i.e., 2.5; 5; 7.5; 10; 15; 20 and 25 ug L^{-1}). The tubes were agitated using a reciprocal shaker for 30 mins at room temperature. Afterwards, samples were then filtered through 0.22 µm pore membrane filters immediately after agitation and the residual toxin levels in the supernatant determined. Two isotherm models of Freundlich and Langmuir were employed to analyze the equilibrium data.

6.2.4.4 Adsorption data modelling

The adsorption capacity (q_t) and equilibrium adsorption capacity (q_e, $\mu g . g^{-1}$) were calculated using equation (6.1) and equation (6.2), respectively (Lim et al., 2021):

$$q_t = V \frac{C_o - C_t}{m} \quad [6.1]$$

$$q_e = V \frac{C_o - C_e}{m} \quad [6.2]$$

Where *V* is the solution volume (L); *m* is the weight of the adsorbent (g); C_o, C_t, and C_e ($\mu g . L^{-1}$) are the concentrations of the adsorbate at the initial, certain, and equilibrium times.

To better understand the factors and mechanisms controlling adsorption of MC-LR onto ChMWCNT, the experimental data obtained at various contact times was fitted into kinetics models namely, pseudo first order and pseudo second order and the Weber Morris intra-particle diffusion models. The mathematical expressions (nonlinear fits) of the pseudo-first- order (PFO) and pseudo second order (PSO) kinetic models are shown in Equations. (6.3) and (6.4), respectively (Choi & Lee, 2022).

$$q_t = q_e(1 - e^{-k_1 t}) \qquad [6.3]$$

$$q_t = \frac{k_2\, q_e^2\, t}{1+k_2\, q_e\, t} \qquad [6.4]$$

Where q_t (mg/g) and q_e (mg/g) are the amounts of MC-LR adsorbed at time *t* (h) and equilibrium, respectively, and k_1 (h^{-1}) and k_2 (g/mg.h) are the PFO and PSO adsorption rate constants, respectively.

Equation 6.5 shows the Weber-Morris intra-particle diffusion model:

$$q_t = k_{id} t^{1/2} + C \qquad [6.5]$$

Where, k_{id} is the intra-particle diffusion coefficient (mg.g^{-1}. min$^{-1/2}$), and C (g.g^{-1}) is a constant associated with diffusion resistance.

Adsorption isotherms models, namely, Langmuir and Freundlich adsorption isotherm models were used to give more insight into the relationship between the adsorbate concentration and the solid phases in the aqueous solution at equilibrium (Braik et al., 2022). The nonlinear mathematical expressions of the Langmuir and Freundlich isotherm models are shown in Equation (6.6) and equation (6.7), respectively (Choi & Lee, 2022).

$$q_e = \frac{Q_{max}\; K_L\, C_e}{1+K_L\, C_e} \qquad [6.6]$$

$$q_e = K_F C_e^{\frac{1}{n}} \qquad [6.7]$$

Where, Q_{max} (mg/g) and K_L (L/mg) are the maximum adsorption capacity of the adsorbent and the Langmuir constant related to the adsorption energy of the adsorbent, respectively. K_F (mg/g $(mg/L)^{-1/n}$ and *n* (-) are the Freundlich constant and adsorption strength, respectively.

For the Langmuir isotherm, the adsorption process efficiency was determined using a dimensionless constant separation factor R_L, calculated using the calculated K_L from equation 6 as follows (equation 6.8):

$$R_L = \frac{1}{1+K_L * C_e} \qquad [6.8]$$

If the R_L values are found to be between 0 and 1, then the process of adsorption is deemed favorable at the temperatures studied (Braik et al., 2022).

6.2.4.5 Desorption optimization

A MC-LR solution (5 µg L^{-1}) was used to optimize the desorption solution. Supernatants were discarded after adsorption equilibration was achieved (based on optimum equilibration time and dosage in sections 6.2.4.1, 6.2.4.2 and 6.2.4.3). The residual adsorbent was extracted with varying percentages of methanol (0, 20, 50, 80 and 100 %) through sonication (SCIENTEC Ultrasonic Cleaner, Model 705, South Africa) for 5 mins to release toxins before centrifugation at 1750 rpm for 10 min. The whole cycle of sonication and centrifuging was repeated three times and the supernatant pooled to give approximately a 15 mL extract of each sample. The pooled samples were then evaporated to dryness under a gentle stream of nitrogen (N_2) gas, with the residue suspended in 1 mL of milli-Q water followed by analysis using the ELISA method.

6.2.4.6 Adsorption and desorption in SPATT bag format

6.2.4.6.1 SPATT construction and activation

SPATT bags were constructed from nylon mesh with approximately 95–100-micron pore size. The nylon mesh was sewn on three sides using a sewing machine and the bag was filled with 0.2 g of the ChMWCNT composite, then the fourth side was sewn to form a finished SPATT bag of approximately 60 × 60 mm dimension (Figure 1a). The SPATT bags were activated by soaking in 100% MeOH for 48 hours, then rinsed thoroughly in milli-Q water for removal of any MeOH residues and kept in milli-Q water at 4-6 ˚C prior to use.

6.2.4.6.2 Adsorption experiment in SPATT bags with spiked milli-Q water

A 20 mL MC-LR solution (5 µg L^{-1}) was used for the SPATT adsorption experiment. The toxin solution (20 mL) was placed in 100 mL glass amber bottles and the SPATT bags packed with 0.2 g (to fill a normal size SPATT bag of 60 mm * 60 mm) of the ChMWCNT composite was introduced to each bottle and the solutions were shaken at 145 rpm (to ensure homogeneity of the solution) for 30 min at room temperature. The SPATT bags were removed after 30 mins, rinsed in milli-Q water, and kept at 4-6 ˚C prior to extraction.

6.2.4.6.3 Adsorption experiment with field water

Raw dam water collected from Roodeplaat Dam was used for the SPATT adsorption experiment with field water. Prior to the experiment, the raw dam water was analyzed for pH, TDS, EC, and turbidity. TDS, pH, and EC were determined using a Jenway pH/Cond meter model (430) and turbidity was determined using a TB200 portable turbidity meter model (#TB200-10). The raw dam water (1 L) was placed in 1 L amber bottles and the SPATT bags packed with 0.2 g of the ChMWCNT composite were introduced to each bottle and the solutions were shaken at 145 rpm for 30 min at room temperature (Figure 6.1b). The SPATT bags were removed after the 30 mins, rinsed in milli-Q water, and kept at 4-6 ˚C prior to extraction.

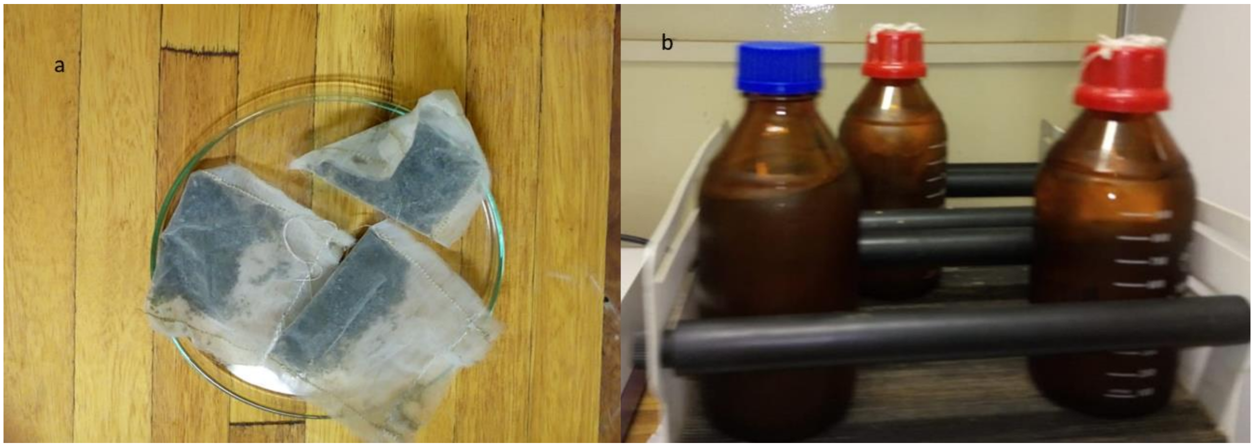

Figure 6. 1. (a) SPATT samplers packed with 0.2 g of the synthesized ChMWCNT composite (b) Laboratory adsorption experiment set-up with field water.

6.2.4.6.4 SPATT Extraction

The SPATT bags were oven dried at 40 ˚C, then cut open and the material was placed in 15 mL centrifuge tubes. The toxins were extracted using 100 % MeOH through sonication (5 mins) to release toxins and centrifugation at 1750 rpm as described in 2.4.4. The pooled supernatant was collected and dried at 50 ˚C under a stream of nitrogen. The dried material was then reconstituted with 1 mL of milli-Q water and analyzed using the ELISA method (ELISA test kits, EUROFINS (Kit Lot No: 19I1120: PN 520011) and LCMS.

6.2.4.6.5 Determination of MC levels in the residues and extracted material

EUROFINS Microcystin/Nodularin Enzyme-Linked Immunosorbent Assay (ELISA) Plate Kit were used for the analysis of total MCs in the samples following the manufacturer's' instructions. The method is based on a direct competitive ELISA for quantitative detection of MCs and Nodularins on the polyclonal antibodies. To determine the levels of some of the congeners of MCs (LR, RR and YR) adsorbed by the composites, a chromatographic method using a Shimadzu triple quadrupole LC–MS/MS system (model LCMS-8045, Shimadzu Corporation, Japan) was used.

Chromatographic Conditions

Levels of Microcystins (LR, RR and YR) in the samples were determined on a triple quadrupole LC–MS/MS system (model LCMS-8045, Shimadzu Corporation, Japan) with a Shim-pack Velox SP-C18, 2.7 µm, with dimensions 2.1 * 100 mm (Shimadzu, Japan). The injection volume was set at 10 µL and the mobile phases used were 0.1 % formic acid (FA) in water (A) and 0.1 % FA in acetonitrile (B). A flow rate of 0.5 mL min^{-1} and a 5 min binary gradient was used with an elution profile of: 2 % B (0.4 min), linear gradient to 70% B (3.1 min), 100 % B (0.5 min), and, finally, 2% B (1 min).

The interface conditions of LCMS-8045 were set as follows: the ESI interface temperature at 300 ˚C, DL temperature at 235 ˚C, nebulizing gas flow at 3 L min^{-1}, drying gas flow at 10 L min^{-1} and heating gas flow at 10 L min^{-1}. The interface voltage was set at 3.0 kV for positive (ES+) electrospray.

The final concentration of the toxins in the samples was determined by using equation 6.9:

$$\text{Conc in sample } (\mu\text{g/L}) = \left(\frac{\text{Co} * \text{Vol of extract used (L)}}{\text{Volume of sample used (L)}}\right) \qquad [6.9]$$

Where: C_o = the conc of sample determined from the calibration curve (µg L^{-1})

To determine the microcystin concentrations adsorbed to the SPATT composite/resin, Equation 6.10 was used:

$$\text{ug/g-resin} = \frac{(\mu\text{g L}^{-1}\text{ in extract x 0.001 L extract vol })}{0.2\text{ g}-\text{composite}} \qquad [10]$$

Where: the extract volume is 0.001 L and are 0.2 g of composite per sampler for ChMWCNT; and 3 g for the DIAION HP20 resin.

6.2.5 Statistical data analysis

To compare the levels of MCs adsorbed and desorbed by different adsorbents and solutions, analysis of variance (ANOVA) and/or the Kruskal-Wallis tests were used at 95% confidence interval (CI) using GraphPad in Stat 3 (GraphPad Software, California, United States). Levels of MCs are presented by their means ± the standard deviation (SD). Kolmogorov–Smirnov and Bartlett tests were used to test for normality and variance homogeneity at 95% CI. Data which passed this test was compared using ANOVA and data which did not pass that test was compared using the Kruskal–Wallis at 95% CI. The Tukey-Kramer multiple comparisons test and the Dunn's multiple comparisons test were used as post-hoc assays for data which passed the normality tests and data which did not pass the normality test, respectively.

6.3 Results and discussion

6.3.1 Physicochemical characterization

6.3.1.1 Fourier Transform Infrared (FTIR) analysis

The FTIR spectra for raw chitosan, glutaraldehyde-crosslinked chitosan (ChGLA) and the crosslinked chitosan-multiwalled carbon nanotubes (ChMWCNT) composite are shown in Figure 6.2. The notable peaks of chitosan can be attributed to: the broad and strong band ranging from 3200 to 3600 cm^{-1} corresponds to the presence of –OH and –NH$_2$ groups, 2927 cm^{-1} (CH$_3$ symmetric stretch), 1634 cm^{-1} (C=O stretching vibration), 1154 cm^{-1} (C–O–C bending vibration), and 1066 cm^{-1} (C–OH stretching vibration) as also observed by Li et al. (2013). Major differences in the spectra of chitosan compared to ChGLA and CHMWCNT were presence of C-O-C band at 1154 cm^{-1} in chitosan but not in the two crosslinked products and the presence of the amide II at

1554 cm^{-1} in the crosslinked material (ChGLA and ChMWCNT) but not in the spectra of chitosan (Figure 6.2). Of importance to note, were the broader peaks of the ChMWCNT compared to the other two material, which probably indicated coating of the chitosan by the multi-walled carbon nanotubes as was also observed by Liu et al. (2007).

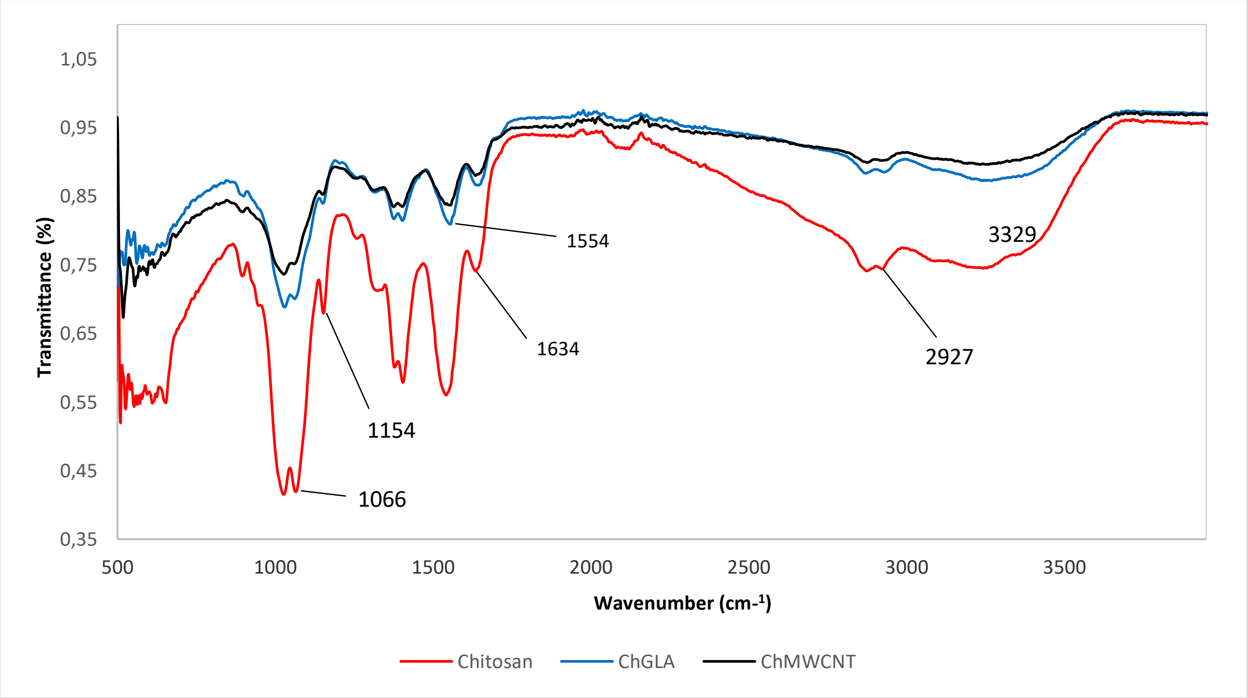

Figure 6. 2. FTIR spectra for chitosan, glutaraldehyde-crosslinked chitosan (ChGLA) and the crosslinked chitosan-multiwalled carbon nanotubes (ChMWCNT) composite.

6.3.1.2 Morphological analysis

Figure 6.3 shows the surface morphology of raw chitosan at different magnifications. The SEM images show a slightly rough and dense surface with large clusters of particles for raw chitosan. After the crosslinking and introduction of multiwalled CNTs (Fig. 4A) the surface structure appears more porous and flaky. The introduction of multiwalled CNTs, seems to have enhanced pore formation. A clear distinction of the ChMWCNT morphology before and after adsorbing MCs can be seen in Figure 6.4 (A vs B) with the structure becoming deformed and irregular upon adsorption. Figure 6.4(B) shows formation of granules in the used material, which could be attributed to the swelling of the material during adsorption with the pores appearing saturated after use.

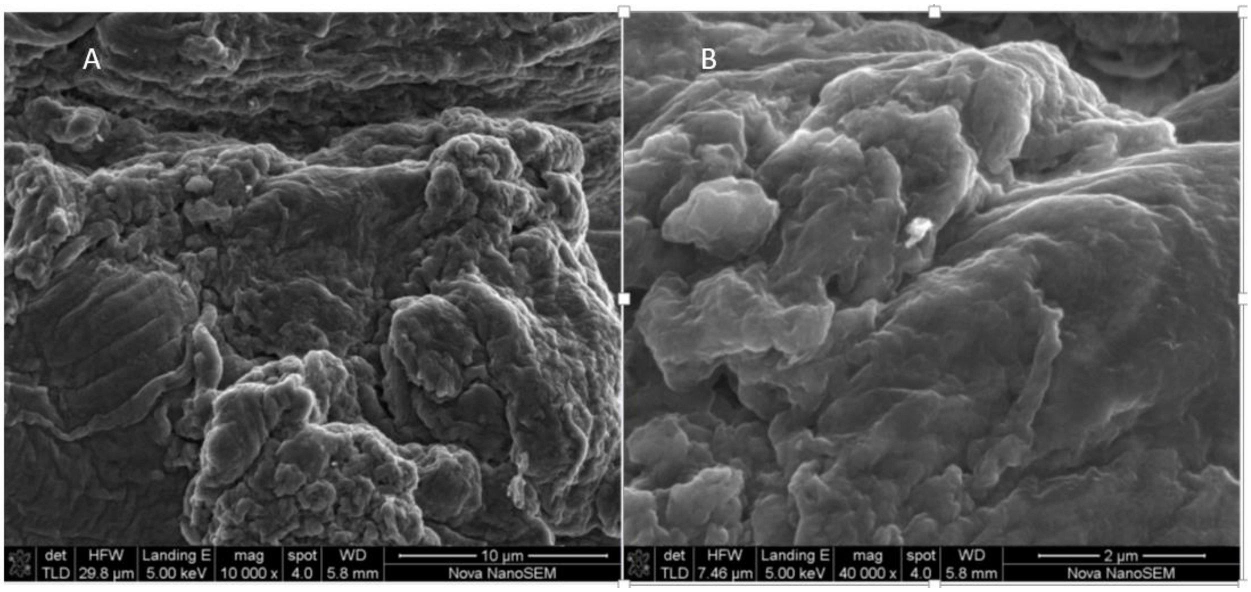

Figure 6. 3. SEM images for Chitosan A) x10 000 magnification B) x40 000 magnification

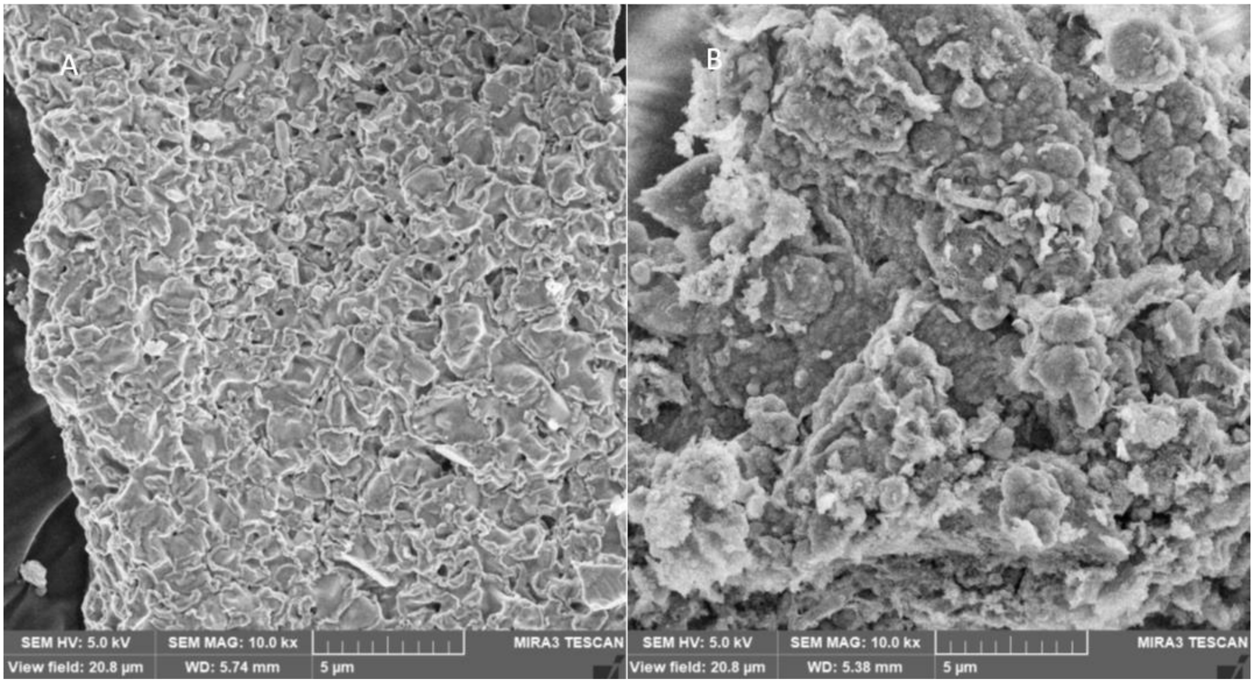

Figure 6. 4. SEM images for ChMWCNT A) before adsorption B) after adsorption x10 000 magnification

6.3.1.3 Surface area, pore volume, and pore size

Specific surface area and pore volume, are very important aspects for any material to be used for the adsorption of MCs since MCs are large molecules and cannot easily enter into the micropores of materials with low micropore sizes (Zhao et al., 2013). Table 6.1 shows the surface area and pore size of the raw chitosan, synthesized glutaraldehyde-crosslinked chitosan (ChGLA), ChMWCNT and the commercial resin DIAION HP20 resin, which were evaluated using the Brunauer-Emmett-Teller (BET) method.

The results indicate that the DIAION HP20 resin had surface area, pore volume, and pore size comparable to what was reported by Li et al. (2011). In terms of the surface area, pore volume and pore size of the synthesised materials, raw chitosan had the lowest, followed by ChGLA and ChMWCNT. The DIAION HP20 resin displays more superior surface area and pore volume compared to any of the synthesized materials, but the ChMWCNT had much greater pore sizes compared to DIAION HP20.

Introduction of multi-walled CNTs onto the ChGLA improved the surface area, pore volume, and pore size of the chitosan, hence ChMWCNT seemed more suitable for the adsorption of MCs compared to ChGLA. Regardless of the lower surface area of the ChMWCNT compared to DIAION HP20, its higher pore sizes make it ideal for the adsorption of MCs. Li et al. (2011), reiterate the importance of materials' pore size instead of surface area in determining the equilibration rates and abilities to adsorb the toxins.

Table 6. 1. Surface area, pore volume, and pore size of DIAION HP20, ChGLA*, ChMWCNT and Chitosan

	DIAION HP20	ChGLA*	ChMWCNT	Chitosan
Surface area (m²/g)				
Single point surface area	618.0379	1.7616	8.0174	0.264
BET Surface Area	653.6041	1.8978	8.377	0.3241
BJH Adsorption cumulative surface area of pores	481.58	0.506	8.424	0.158
BJH Desorption cumulative surface area of pores	475.1997	0.49	10.5357	0.9885
Pore Volume (cm³/g)				
Single point adsorption total pore volume of pores	0.74539	-	0.02248	-
BJH Adsorption cumulative volume of pores	0.999586	0.00022	0.029747	0.001056
BJH Desorption cumulative volume of pores	0.983416	-	0.029914	0.001138
Pore Size (Å)				
Adsorption average pore width (4V/A by BET)	45.6172	-	107.3432	-
BJH Adsorption average pore diameter (4V/A)	83.026	17.397	141.254	267.817
BJH Desorption average pore diameter (4V/A)	82.779	-	113.57	46.043

*ChGLA-Gluteraldehyde Crosslinked Chitosan

6.3.2 Adsorption and desorption characteristics of the composite for microcystins

Results of batch adsorption experiments showing the effects of the effect of contact time, initial adsorbate (MC-LR) concentration, and adsorbent (ChMWCNT) dosage are presented in Figure 6.5 a, ,b and c, respectively.

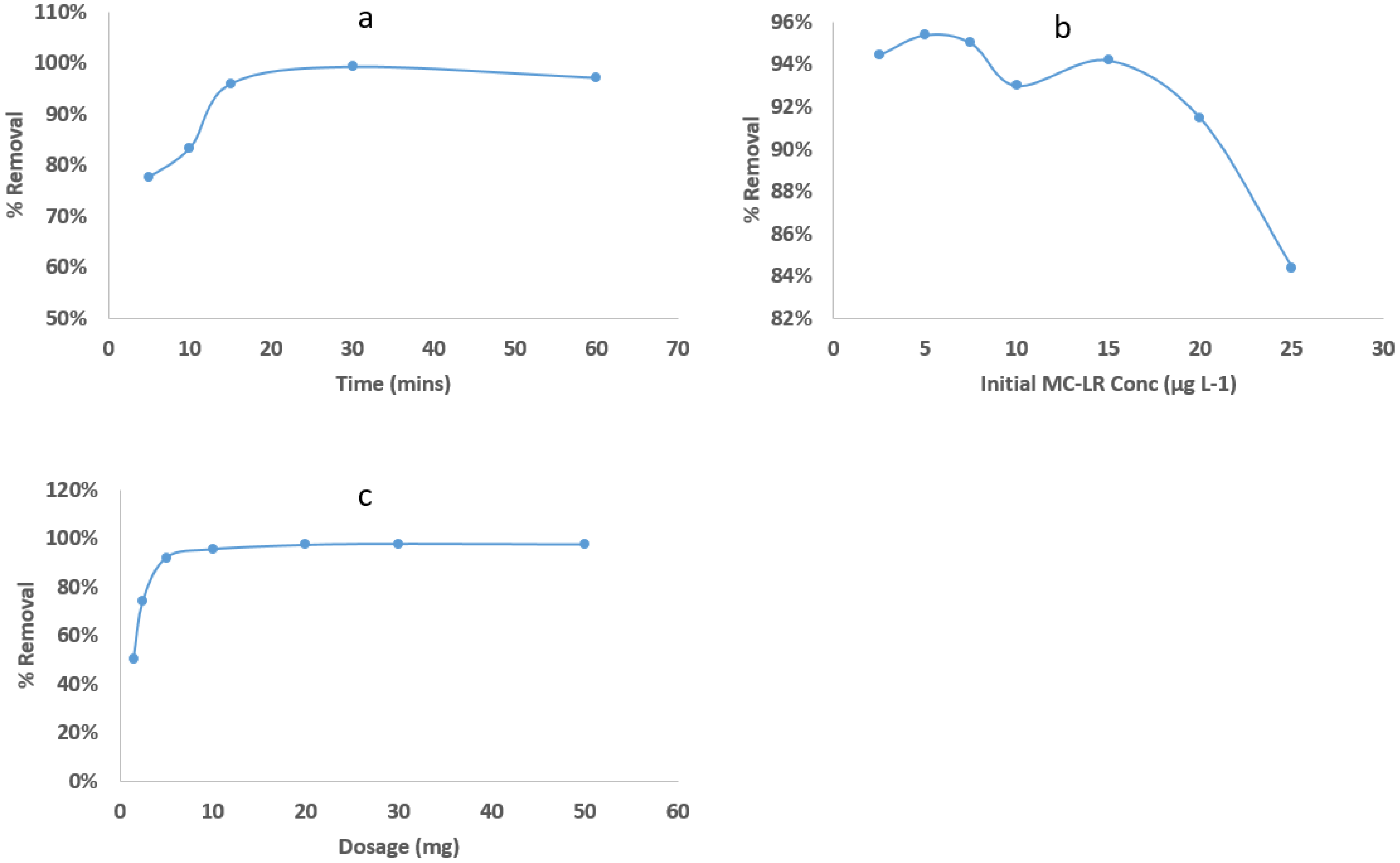

Figure 6. 5. (a) Effect of contact time on the adsorption of MC-LR onto ChMWCNT (5 μg L^{-1} initial concentration, 10 mg 5 mL^{-1} adsorbent dosage, pH 6.5 ± 0.5 and shaking speed of 145 rpm) (b) Adsorption of MC-LR onto ChMWCNT as a function of adsorbent dosage (5 μg L^{-1} initial MC-LR conc, 30 min contact time, pH 6.5 ± 0.5) (c) Adsorption of MC-LR onto ChMWCNT as a function of initial concentration (30 mg 5 mL-1 adsorbent dosage, 30 min contact time, pH 6.5 ± 0.5).

6.3.2.1 Effect of contact time

The effect of contact time was evaluated to establish the equilibrium time for optimum adsorption of MC-LR onto ChMWCNT and establish the adsorption kinetics. The results in Fig 5a show that MC-LR was rapidly adsorbed in the first 15 mins of the reaction and then slowed down as the reaction time increased to 30 mins where optimum uptake of MC-LR was recorded. Thereafter, removal efficiency remained almost constant as the time increased to 60 mins indicating that equilibrium had been reached.

The rapid adsorption of MC-LR in the initial stages (5-15 mins) of contact could be attributed to the binding of adsorbate onto the readily available active sites on the outer surface of the ChMWCNT. In the early stages, there are many sorption sites available for occupation by the adsorbate, thus higher initial rates, then as the reaction continues, the sorption sites and concentration of the adsorbate decreases and the rate of adsorption also lowers (Choi & Lee, 2022). According to Zaidi et al. (2021), equilibrium has been reached when the adsorbate removal does not significantly change regardless of the extended duration of contact.

Based on that, 30 mins was adopted as the reaction time for subsequent experiments. Our findings were similar to those of Zhao et al. (2013) who found the equilibration times of MCs onto the DIAION HP20 resin to be 30 mins, but that of SP700 to be 15 mins. This implies that the synthesized composite adsorbs MCs at a comparable rate with other adsorbents being used for the same purpose.

To further explain the adsorption mechanisms and the adsorption rate-controlling factors, the experimental data was fit into reaction kinetics models namely Pseudo-first order (PFO), Pseudo-second order (PSO) and intra-particular diffusion (Mudzielwana et al., 2019). The nonlinear plot for PFO and PSO are presented in Fig 6a while the constant parameters are presented in Table 6.2. Since the PSO models showed a higher correlation coefficient (R^2 = 0.875), this means that the experimental data is better explained by the PSO model, thus showing that the adsorption was occurring through chemisorption. The adsorption mechanism of MC-LR onto the composite can be explained in terms of the functional groups in chitosan. Chitosan has numerous amino ligands and hydroxyl groups on its surface (Sanford et al., 2012), which are all considered to be active sites for the sorption of MCs.

The experimental data were also fitted into the intra-particle diffusion plot to understand the controlling factors limiting the adsorption of MC-LR onto the synthesized composite (Figure 6.6b). Based on this model, if a graph/plot displays multi-linearity, this is an indication that there are more than one adsorption processes taking place and the rate-limiting step is not the intra-particle diffusion (Zaidi et al., 2021). In this study, a multi-linear plot was obtained (Figure 6.6b) thus showing a two-step diffusion process. Figure 6.6(b) shows a sharp first step (in the first 15 mins) showing external mass transfer through instantaneous sorption. The second and last step (the equilibrium stage) shows intra-particle diffusion and a plateau demonstrating slow diffusion as the levels of the adsorbate decrease. These findings thus show that the adsorption of MC-LR onto ChMWCNT is not only controlled by intra-particle diffusion.

Table 6. 2. Parameters of pseudo first order and second order of reaction kinetics

	Conc. ($\mu g\ L^{-1}$)	**Pseudo-first order model**			**Pseudo-second order model**		
		q_e ($\mu g.g^{-1}$)	K ($L.mg^{-1}$)	R^2	q_e ($\mu g.g^{-1}$)	K ($L.mg^{-1}$)	R^2
ChMWCNT	5	0.801	0.295	0.773	0.852	0.710	0.875

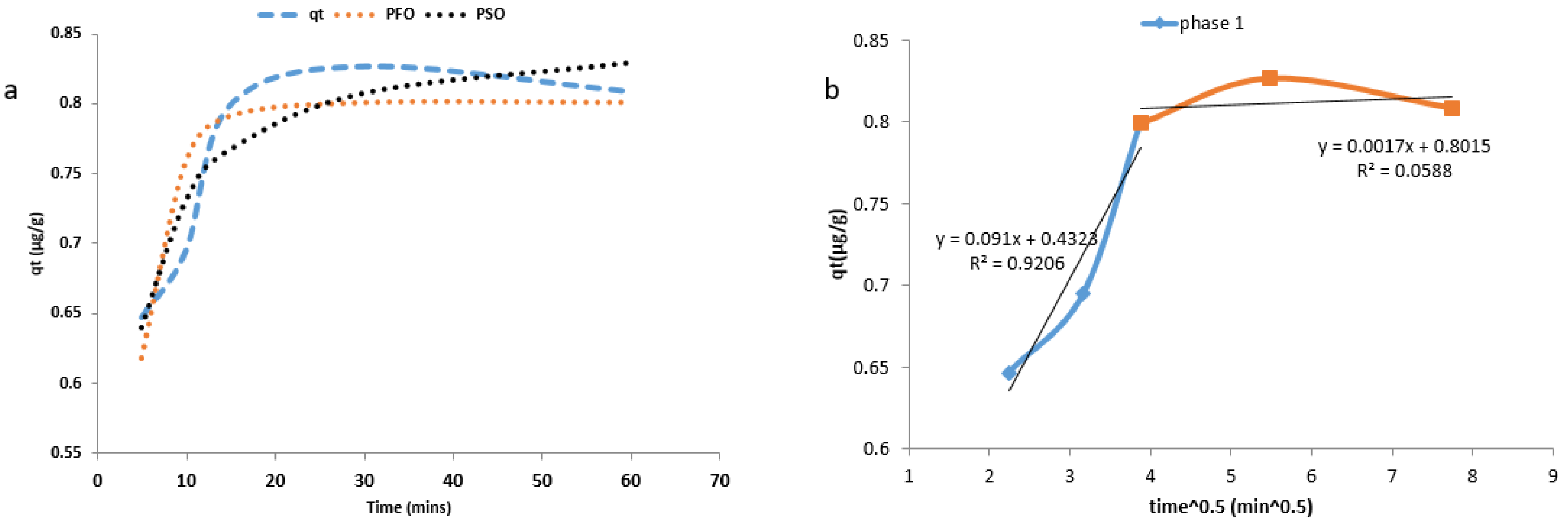

Figure 6. 6. (a) Results of kinetics experiment on ChMWCNT and nonlinear regression analysis of PFO and PSO models (b) intra-particle Diffusion of MC-LR onto ChMWCNT (initial MC-LR concentration: 5 µg L^{-1}, adsorbent dose: 30 mg 5 mL^{-1}, time: 5, 10, 15, 30 and 60 min).

6.3.2.2 Effect of adsorbent dosage

The dosage of adsorbent is known to affect remote oval efficiency of adsorbates since increasing the adsorbent dosage increases the available active sites for adsorption. Findings in Figure 6.5(c) show that the adsorption of MC-LR onto ChMWCNT was increasing with increasing dose of the adsorbent. This is because increasing the dosage of the adsorbent increased the active sites for the MC-LR to be adsorbed thus an increase in removal efficiency. Maximum adsorption was reported to be 97% at a dose of 30 mg/ 5 mL of the solution and this was adopted as the optimum dose and used in the subsequent experiments.

6.3.2.3 Effect of adsorbate concentration

The adsorbate’s initial concentration has a direct effect on the rate of adsorption, and this makes it a parameter of paramount importance to investigate to understand the adsorption process. The effect of initial concentration on the adsorption of MC-LR by ChMWCNT was evaluated using seven initial solution concentrations of MC-LR (2.5, 5, 7.5, 10, 15, 2,0 and 25 µ,g L^{-1}) and a dosage of 30 mg per 5 mL solution. Results in Figure 6.5(b) show that the removal efficiency (as % removal) of MC-LR was declining as the concentration of the adsorbate was increased. The trend observed is due to adsorbent’s active site being exhausted by the adsorbate with an increase in the initial MC-LR concentrations. Similar patterns of MCs adsorption have been reported by numerous other researchers for example, Pavagadhi et al. (2013) and Hena et al. (2014). High The highest removal was reported when 5 µg L^{-1} solution was used, and this was selected as the optimum concentration in subsequent experiments.

The adsorption data obtained when investigating the effect of the adsorbate initial concentration on the process was fitted into nonlinear equations of Langmuir and Freundlich isotherm models. Analysis of the equilibrium isotherms gives data on the adsorption capacity which is crucial when investigating adsorption systems (Choi & Lee, 2022). The Langmuir model assumes that the adsorption process happens on a homogenous surface with single-player adsorption taking place at all the available adsorption sites. The model also assumes no interaction between the adsorbent and the adsorbate (Zaidi et al., 2021). On the other hand, the Freundlich model assumes that adsorption occurs in a multilayer on a heterogeneous adsorbent. The isotherm model assumes adsorption sites to be having unequal energy which differs exponentially and this results in numerous adsorbate layers forming ozone surface of the adsorbent (Mudzielwana et al., 2019).

The nonlinear plots for Langmuir and Freundlich isotherms are shown in Figure 6.7 and their respective constant parameters are shown in Table 6.3. Based on the correlation coefficient values (R^2) (Table 6.3) the data better fitted the Langmuir isotherm model and thus inferring a monolayer uniform adsorption onto the surface of the ChMWCNT composite and no interaction of the adsorbate molecules.

The calculated R_L (dimensionless equilibrium parameter) values for the Langmuir isotherm (in Table 4) and the Freundlich adsorption intensity ($1/n$) in Table 6.3, were all between 0 and 1, thus showing that the adsorption of MC-LR onto the ChMWCNT composite is favorable. However, when compared to the adsorption capacities of other adsorbents applied in SPATT for microcystins as reported by Zhao et al. (2013) (Table 6.3), the adsorption capacities of DIAION HP20 and SP700 were found to be much higher than the developed composite. Even though superior adsorption capacities were observed by Zhao et al. (2013) for the resins DIAION HP20 and SP700, the huge differences with the developed composite could also be due to the huge differences in the experimental conditions, with Zhao et al. (2013) having used microcystin concentrations (MC-LR 1.98–148.86 µg mL^{-1}) which were at least a thousand times more and adsorbent dosages (0.1 g) which were at least 3 times more than applied in this study.

Table 6. 3. Parameters of Langmuir's and Freundlich's isotherm models at 25°C

	Langmuir's isotherm model			**Freundlich's isotherm model**		
	Q_{max} $(\mu g.g^{-1})$	K_L $(L.mg^{-1})$	R^2	K_F $(L.mg^{-1})$	*1/n*	R^2
ChMWCNT	4.639	0.938	0.972	2.012	0.473	0.908

Table 6. 4. The calculated Langmuir isotherm dimensionless constant separation factors

C_o **(ChMWCNT) (μg L^{-1})**	**Calculated** R_L
2.5	0.885
5	0.823
7.5	0.740
10	0.604
15	0.550
20	0.384
25	0.215

Table 6. 5. List of adsorbents used in SPATT for microcystins

Adsorbent	Type of microcystin	Adsorption capacity (μg g^{-1})	Reference
DIAION HP20	MC-LR	335	Zhao et al., 2013
SP700	MC-LR	828	Zhao et al., 2013
ChMWCNT	MC-LR	4.639	This study

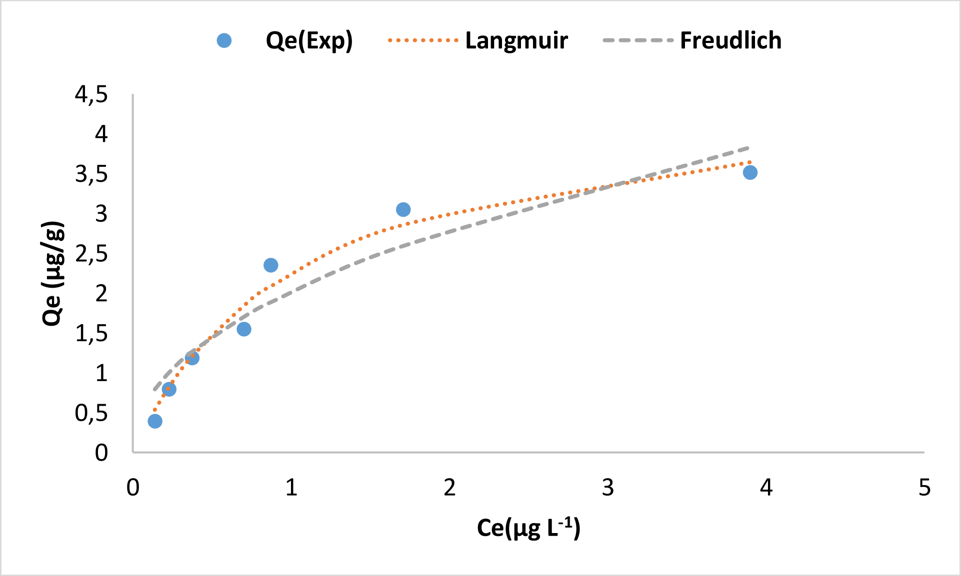

Figure 6. 7. Adsorption equilibrium isotherms for MC-LR on ChMWCNT

6.3.2.4 Desorption study on ChMWCNT composite

The desorption of MC-LR from the ChMWCNT composite was tested in five different aqueous methanol solutions (0, 20, 50, 80 and 100 %). Recoveries of MC-LR from the composite using these solutions are shown in Figure 6.8. Pure water (0 %), 20 % and 50 % methanol solutions proved to be very ineffective in desorbing MC-LR. Higher mean recoveries of 71.99 ± 4.47 % and 84.71 ± 6.47 % were observed for 80 % and 100 % methanol, respectively. Similar findings were reported by Zhao et al. (2013) who reported the highest recovery of 78.1 ± 4.1 % for MC-LR from the resin SP700 using 100 % methanol. However, Zhao et al. (2013) found better recoveries of 91.5 ± 4.6 % for MC-LR with 75 % methanol compared to 100 % methanol (approx. 78 % recovery).

Such findings imply that, compared to DIAION HP20, the synthesized composite has a stronger affinity for MC-LR. However, the fact that some MCs were desorbed when 100% water was used suggests a possibility of MCs leaching from the composite in solution when this material is used for SPATT.

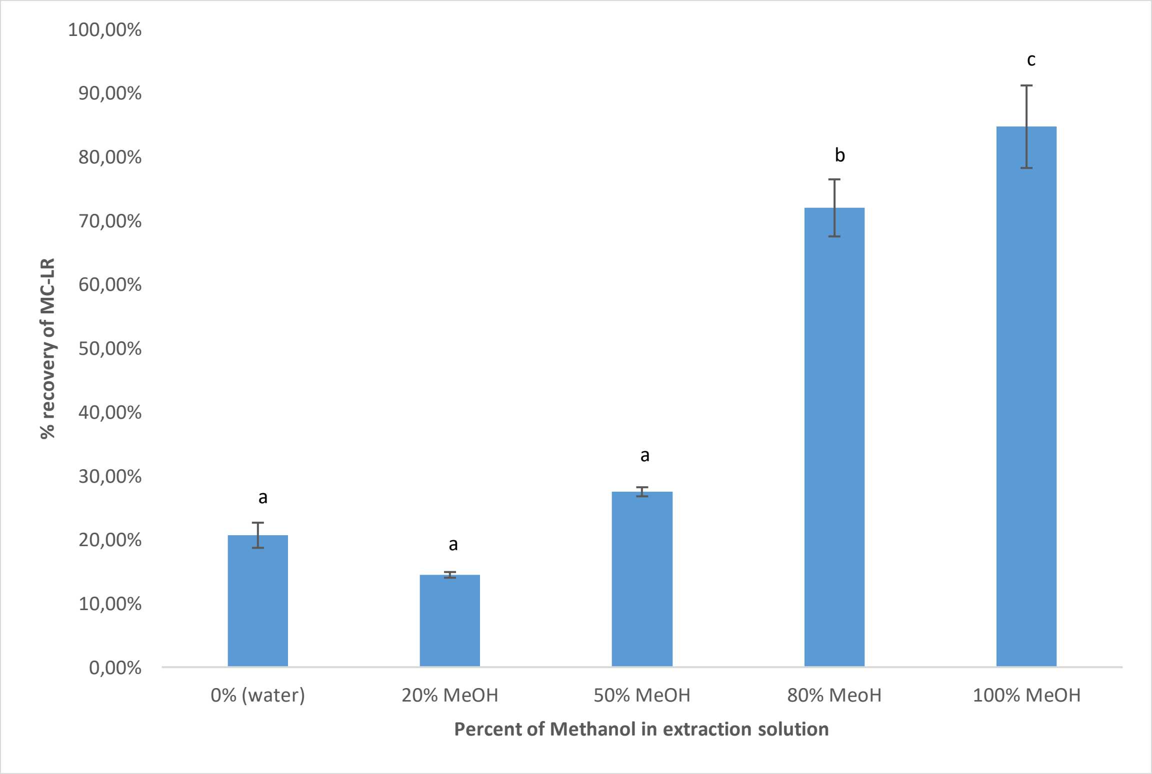

Figure 6. 8. Differences in the desorption efficiency of MC-LR from ChMWCNT composites using various percentages of methanol as the extraction solution. *N.B*: Data labelled with different small letters (a - c) differed significantly at $p < 0.05$ in each bar (n=3).

6.3.3 Application in raw dam water & SPATT bag format

The raw dam water had a mean pH of 8.84 ± 0.71, EC of 346 ± 13.87 µs cm^{-1}, TDS of 234 ± 23.52 mg L^{-1} and turbidity of 34.05 ± 16.04 NTU. The SPATT samplers were loaded with 0.2 g of ChMWCNT and 3 g DIAION HP20 and exposed to raw dam water in 1 L bottles, with continuous agitation to try and estimate the time they took to be saturated and their adsorption behavior when exposed to raw dam water with a mean total MCs concentration of 7.79 ± 1.69 µg L^{-1}. Findings of the laboratory trial in Figure 6.9 (a) show that both adsorbents were saturated within 24 hours when exposed to field water. Of importance to note was the fact that the resin DIAION HP20 retained the MCs through72-hourseriod of exposure, whereas the ChMWCNT composite seemed to leach the adsorbed MCs as indicated by the decline in the recovered MCs as the time of exposure was increased (from 24 hours to 72 hours). This finding was supported by the findings in section 3.2.4 (Figure 6.8) where some MCs were recovered when pure water was used as a desorption solution, thus implying that, the composite might not be suited for longer deployments.

With regards to the residual MCs levels in the water upon exposure to the samplers, Figure 6.9 (b) shows that from an initial MC concentration of ±9 µg L^{-1}, all the MCs were adsorbed by the samplers within 24 hours of exposure by the DIAION HP20 resin. In bottles in which the field water was exposed to the ChMWCNT loaded samplers, the MCs levels in the bottles were gradually going down from an initial concentration of ± 6.59 µg L^{-1} to ± 2 µg L^{-1} after 72 hours of exposure. The differences in the two treatments could be attributed to the huge differences in the masses of the adsorbent used, with 3 g having been used for DIAION HP20 and only 0.2 g for ChMWCNT, since the composite is less dense and bulky compared to DIAION HP20, hence the SPATT bags could not be loaded with more adsorbent material.

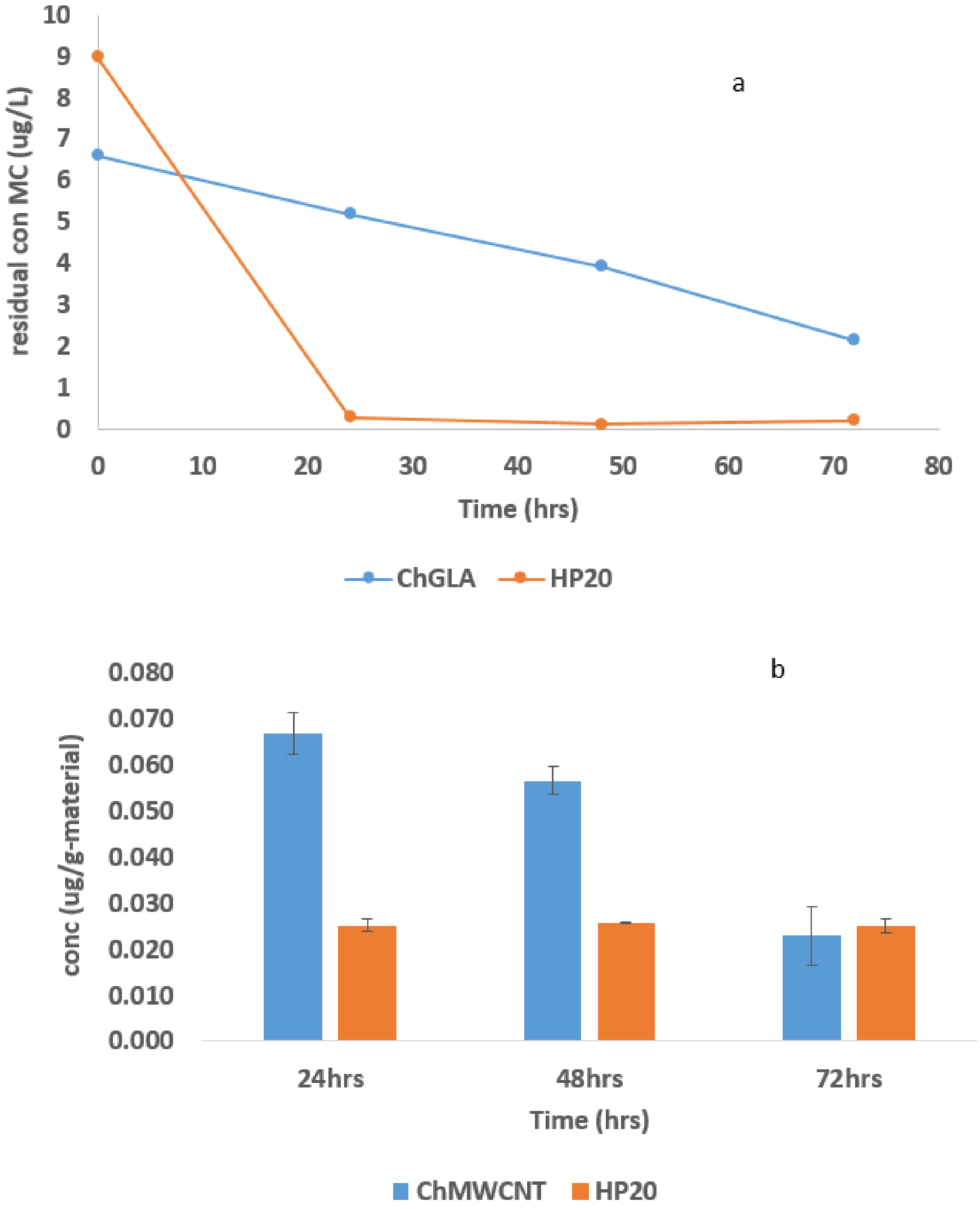

Figure 6. 9. (a) Residual MCs levels in the bottles with SPATT samplers with DIAION HP20 and ChMWCNT over time (b) MCs adsorbed by the sorbents in SPATT samplers (µg g^{-1} of material) over time when exposed to field water during laboratory trails

To determine the potential of the composite to adsorb and desorb different congeners of MCs (MC-LR, RR, and YR) we exposed to SPATT samplers loaded with 0.2 g of the composite to 1 L of raw dam water with a slightly alkaline pH of 7.29 ±0.71, and EC of 878.67±42.44 µS cm^{-1} and MCs with the following mean concentrations: MC-LR 25.14 ± 2.34; MC-YR 10.21 ± 0.41 and MC-RR 7.92 ± 0.10 µg L^{-1}. The findings in Figure 6.10 (a & b) show that except for MC-YR, where the recovery seemed disproportional to the levels in solution, the levels of MC-LR and MC-RR recovered were representative/proportional to the levels in the dam water. This seems to imply less affinity or recoveries of MC-YR compared to the other two congeners monitored. It is also highly possible that the water had other congeners of MCs not monitored here as reported by Mbukwa et al. (2012) for the same catchment, which could have outcompeted MC-YR for adsorption sites onto the composite.

The differences in adsorption efficiencies of the different MCs can also be explained in terms of their varied sizes and structures. Of the 3 MC congeners tested here, MC-YR has the largest molecular weight, and this probably makes it less favorable to be adsorbed on the pores on the surfaces of the adsorbent. The findings also seem to suggest that the composite had a higher affinity for MC-RR compared to the other two variants. Although these MCs may be similar in structure, they have different amino acids MC-RR has arginine and arginine whereas MC-LR has leucine and arginine (Pavagadhi et al., 2013). Arginine being a more polar amino acid compared to leucine, would make MC-RR more polar and a preferred adsorbate onto the ChMWCNT active sites.

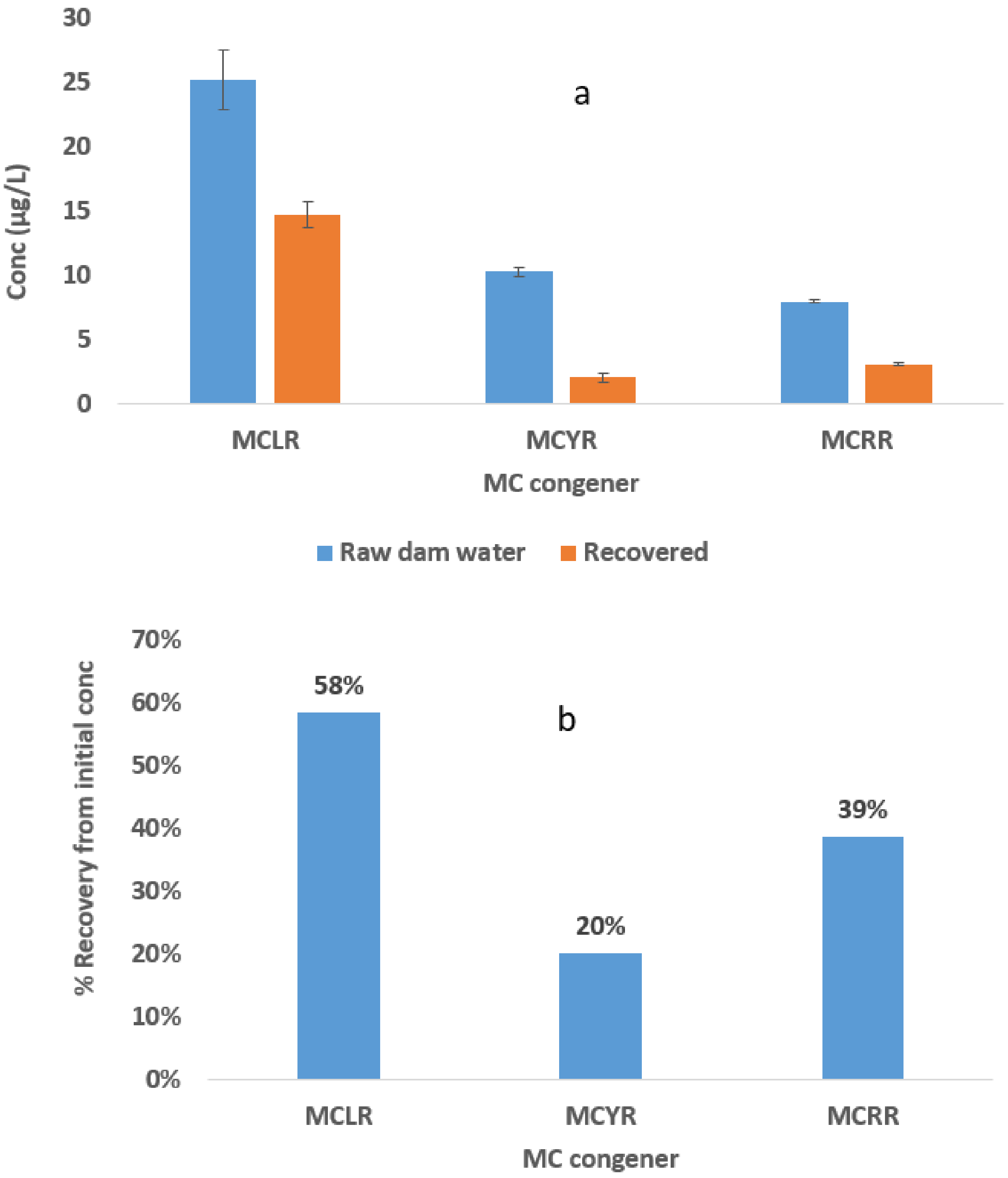

Figure 6. 10. a) The various levels of MCs (LR, YR, RR) adsorbed onto ChMWCNT (b) Percentage Levels of MC (LR, YR, RR) adsorbed from the raw dam water.

6.4 Conclusions

In this study glutaraldehyde crosslinked chitosan- multiwalled carbon nanotube composite was synthesized and evaluated for its adsorption and desorption of MC-LR in batch experiments and then later applied in raw dam water to assess its applicability in the passive sampling of MCs in SPATT samplers. The optimum conditions for MC-LR adsorption were 30 min contact time, 5 µg L^{-1} dosage, and 30 mg per 5 mL dosage at ambient room temperatures and natural pH water. The composite was found to be efficient in adsorbing MC-LR with up to 97% removal of the toxin under optimized conditions. The kinetics data for the adsorption process was better explained by a pseudo-seconded-order model, meaning that the adsorption was occurring through chemisorption. The experimental data was fitting better into the Langmuir isotherm compared to the Freundlich isotherm, thus inferring a monolayer surface adsorption of MC-LR onto ChMWCNT. Adsorption capacity of 4.639 µg g^{-1} was reported and R_L (dimensionless equilibrium parameter) values for the Langmuir isotherm, were between 0 and 1, inferring that adsorption of MC-LR onto the ChMWCNT composite is favorable. In terms of desorption, 100% methanol was found to be most effective with the highest mean desorption efficiency of 84.71 ± 6.47% reported. When applied for the adsorption in raw dam water, the composite was saturated within two days of exposure and adsorbed and desorbed the three congeners of MCs (-LR, -RR and –YR) relatively well. Based on the findings, we recommend further studies looking into the effect of other parameters such as pH effect of co-existing pollutants and ions and field SPATT monitoring of MCs using the developed composite to assess its performance on different MCs.

Data Availability

Datasets related to this chapter can be found at https://doi.org/10.6084/m9.figshare.20992291.v1, an open-source online data repository hosted at Figshare (Pindihama, 2022).

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CHAPTER SEVEN: FIELD APPLICATION OF CROSSLINKED CHITOSAN-BASED ADSORBENTS IN SOLID PHASE ADSORPTION TOXIN TRACKING (SPATT) OF MICROCYSTINS

Abstract

This Chapter describes the successful field deployment of SPATT samplers using the two newly synthesized chitosan sorbents namely, glutaraldehyde crosslinked (ChGLA) and gluteraldehyde crosslinked chitosan modified with multi-walled carbon nanotubes (ChMWCNT). Due to their less density, 0.2 g of the sorbents were loaded into 60 * 60 mm SPATT bags, whereas 3 g of the commercially available DIAION HP20 resin was used for the field deployments.

In the field, the 3 types of SPATT samplers were deployed for 48 hours since low levels of MCs were anticipated due to the excessive rainfall in the study sites during the first field sampling visit. The findings indicated that MCs were detectable in both sites (Roodeplaat and Hartbeespoort), in all six sampling points and, by all three types of samplers and grab samples.

The sites sampled in this study are of critical importance as the water is directly used for irrigation and other agricultural purposes, hence the likelihood of the toxins being taken up by crops consumed by humans. The three samplers were easily detecting MCs even in areas where grab samples detected traces of the toxin.

The findings showed a good correlation of the toxins detected by the two newly synthesized and in-grab samples, and a strong positive correlation was also reported between the two newly synthesized materials (ChGLA and ChMWCNT). Among physicochemical parameters monitored, there was a strong positive correlation between DO and pH and MCs in grab samples, ChGLA and ChMWCNT, making DO and pH better predictors of toxin loads in the study area and also factors determining the suitability for the application of the newly synthesized samplers. The levels of MCs detected by the DIAION HP20 resin did not correlate to any of the physicochemical parameters monitored. There strong positive correlation between gab sampling and the ChGLA and ChMWCNT samplers, suggests a better prediction of toxin loads by the newly synthesized material compared to the DIAION HP20 resin.

The samplers also detected all the 3 MCs (-LR, -RR and –YR) tested here comparably well to DIAION HP20, further suggesting their suitability in an application for the SPATT monitoring of

MCs in irrigation water. Based on these findings, it can be concluded that SPATT using the synthesized chitosan-based adsorbents has the potential to be integrated into current cyanobacterial monitoring programmes and would be an especially useful and economical tool for early warning and monitoring of toxic cyanobacterial events in water intended for irrigation.

7.1 Introduction

Although passive sampling has been successfully used several times to monitor microalgal toxins using different bulk polymeric sorbents (Zendong et al., 2014) most of these sorbents are synthetic and relatively costly to buy. On the other hand, chitin and its de-acetylated product, chitosan, are the world's second most abundant natural polymers after cellulose (Gang et al., 2010) and also the most abundant amino polysaccharide (Crini, 2005). As a sorbent, it thus poses advantages due to its availability and less cost. Chitosan is a nontoxic, biocompatible and hydrophilic option to be an adsorbent since it is obtained from natural resources and has a high affinity for a variety of pollutants ranging from metal ions to organic compounds (Gonçalves et al., 2017).

The use of chitosan (either on its own, in combination or modified) for MCs sorption has recently been studied mainly for water purification studies for example studies by Gomez-Maldonado et al. (2022) and Tran et al. (2022) but not for passive sampling of MCs in SPATT format. Based on literature, the insertion of multi-walled carbon nanotubes into the network was hypothesized to have a synergistic effect, of improving the mechanical and adsorptive characteristics of the hydrogel.

In this way, the innovation of this work was to insert multi-walled carbon nanotubes into the hydrogel structure and evaluate the respective effects on the material characteristics, and in MC adsorption and desorption. This chapter aimed to assess the field applicability of the synthesized gluteraldehyde-crosslinked chitosan (ChGLA) hydrogel and the synthesized composite of glutaraldehyde-crosslinked chitosan and multiwalled carbon nanotubes (ChMWCNT) for MCs monitoring in irrigation water in SPATT samplers. The synthesized samplers' performance in this regard was compared to that of the commercially available resin DIAION HP20.

7.2 Materials and methods

7.2.1 Chemical and reagents

Chitosan was purchased from Rochelle Chemicals as a flaked material, with a deacetylation percentage of approximately 57.72%. Glutaraldehyde (GLA) and acetic acid were also purchased from Rochelle Chemicals and were all analytical-reagent grade. Hydroxyl functionalized Multiwalled Carbon Nanotubes (MWCNTs, Purity: >98%, Average diameter: 10-20 nm) were purchased from SabiNano (Pty) Ltd, Johannesburg, S.A. Synthetic aromatic adsorbent resins based on cross-linked polystyrene matrices (DIAION HP20 and SP700) were purchased from Rochelle Chemicals (Johannesburg, S.A). De-ionized water obtained from a milli-Q water purification system (Merck-Millipore Ltd., Germany) to 18mΩ quality was used to prepare all the solutions.

7.2.2 Preparation of glutaraldehyde crosslinked chitosan hydrogel

Chitosan (1 g) was dissolved overnight in 50 mL of 1% v/v acetic acid. After complete dissolution of chitosan, glutaraldehyde (2 % (v/v)) was used as a crosslinking agent and slowly added to the chitosan solution under mechanical stirring (50 rpm) until it formed the gel. After, the hydrogel was subjected to 48 h freeze drying at -48°C under a constant vacuum of 44 µmHg (constant vacuum of 44 µmHg using a Telstar Lyoquest Freeze Dryer, Terrassa, Spain). The freeze-dried material was then ground to powder using a mortar and pestle and sieved through a 250 µm sieve and only material with a diameter of ≥250 µm was used in the SPATT bags to avoid leaching of the material.

7.2.3 Preparation of chitosan/MWCNT composite

Chitosan (1 g) was dissolved overnight in 50 mL of 1% v/v acetic acid. After complete dissolution of chitosan, carbon nanotubes (CNT) (10% wt) were added to the solution. Then, glutaraldehyde (2 % (v/v)) was used as a crosslinking agent and slowly added to the carbon nanotube chitosan solution under mechanical stirring (50 rpm) until it formed the gel. After, the composite was subjected for 48 h and -48 °C under a constant vacuum of 44 µmHg to freeze-drying (under a constant vacuum of 44 µmHg to (Telstar Lyoquest Freeze Dryer, Terrassa, Spain). The freeze-dried material was then ground to powder using a mortar and pestle and sieved through a 250 µm sieve and only material with a diameter of ≥250 µm was used in the SPATT bags to avoid leaching of the material.

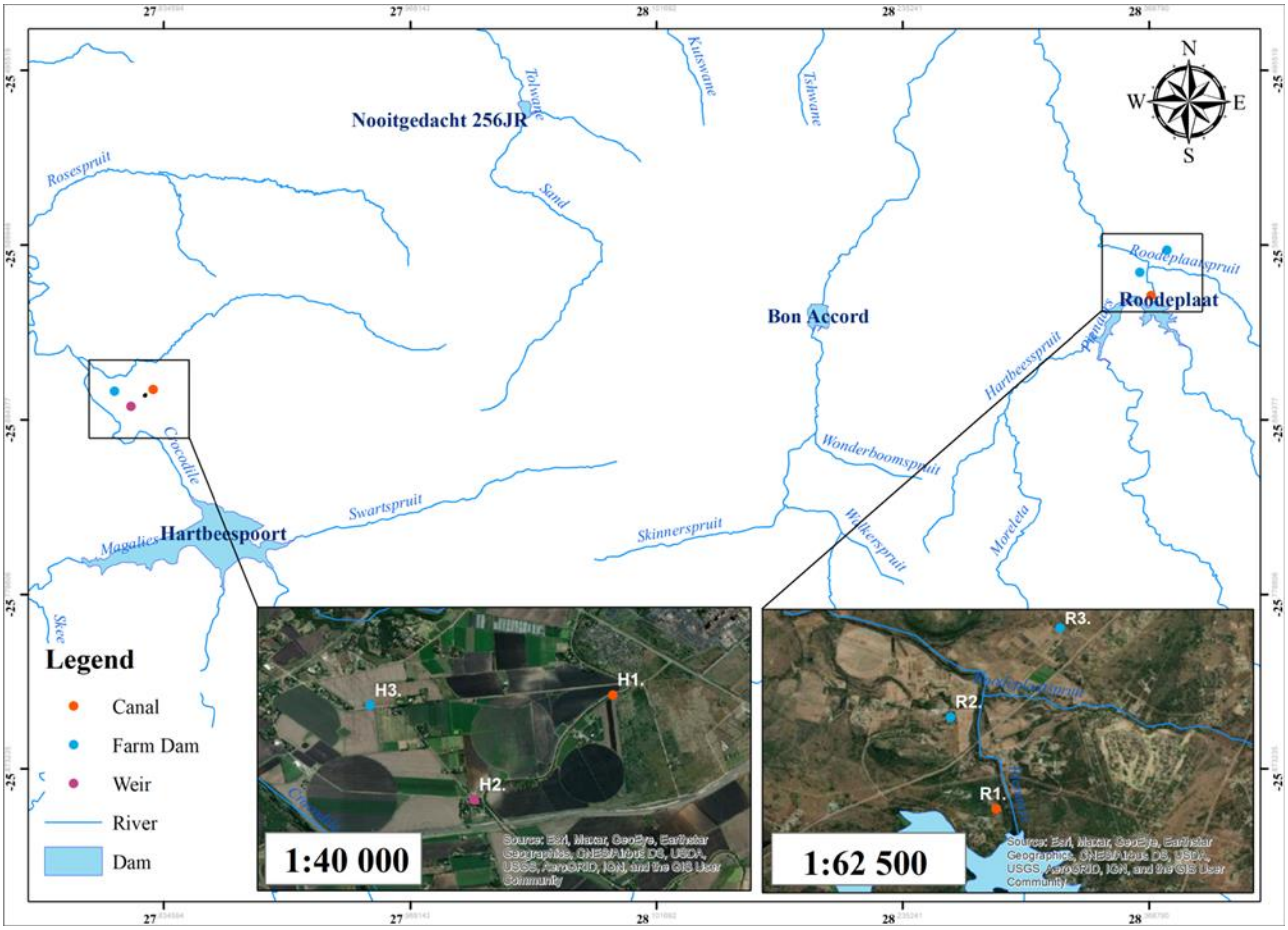
7.2.4 Construction of SPATT bags

The SPATT bags were constructed using nylon mesh with approximately 95–100-micron pore size, purchased from Ecotao Enterprises (Stanger, S.A). The nylon mesh cloth was sewn on 3 sides by an electric sewing machine and form an open bag of 60 mm width. The SPATT bags were filled with 3 g (dry weight) of DIAION HP20 and 0.2g of ChGLA and ChMWCNT per each bag, then sewn on the fourth side forming a 60 x 60 mm dimension bag (Figure 7.1). The SPATT bags were activated by soaking in 100 % methanol for 48 hours. The methanol was then rinsed off with deionized water by incubating the SPATT bags inside a beaker with 500 of mL deionized water (milli-Q). The SPATT bags were then placed in Zip-lock bags with deionized water covering the resin to prevent it from drying out and stored in a cooler box with ice and transported to the field for deployment.

Figure 7. 1. Sorbents in SPATT bags (a) commercial DIAION HP20 (b) synthesized ChGLA (c) synthesized ChMWCNT

7.2.6 Field deployment of SPATT samplers

The constructed SPATT samplers were deployed in the field at Roodeplaat and Hartbeespoort sites and points as indicated in Figure 7.3. The samplers were deployed at 6 points, 2 irrigation canals and farm dams. Three points (R1, R2, R3) were selected for the Roodeplaat site and three (H1, H2, H3) for the Hartbeespoort site (sampling points description is in Table 7.1). In all the selected sites, the water is being used to irrigate vegetables and other crops for human consumption, thus presenting an indirect route of exposure to MCs to humans through ingesting MC-contaminated food.



Source: Esri, Maxar, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community

Figure 7. 2. Location of the sampling points & sites for the SPATT samplers' deployment.

Table 7. 1. Description of sampling points

Site	Sampling Point	Description
Hartbeespoort	H1	Farm Dam
	H2	Canal
	H3	Farm Dam
Roodeplaat	R1	Canal
	R2	Farm Dam
	R3	Farm Dam

Six SPATT samplers (two containing each of the sorbent, ChGLA, ChMWCNT, and DIAION HP20) were deployed for 2 days (based on the findings of the laboratory exposure study in Chapter 6) in the field during the period 10 January 2022 to 13 January 2022 then again from the 12th of July to the 15th of July 2022. The SPATT were clamped onto plastic embroidery hoops and were protected by wire and plastic cages to prevent them from being damaged by fish and other aquatic organisms. The samplers were secured with a rope at a depth of 0.5 – 1 m (Figure 7.4) and were attached to weights in the form of mugs/metal bolts to give them weight and keep them suspended in the water column, Figure 7.4 shows the configuration of the samplers for deployment.

Grab samples were collected using 100 mL amber bottles at the time of samplers' deployment and retrieval. Upon retrieving, SPATT bags were unclamped from embroidery hoops and rinsed with field water then deionized water to remove silt and debris. The samplers were then placed in labelled zip lock bags and stored in a cooler box with ice for transportation to the laboratory and were stored in a fridge at 4 °C until toxin extraction and analysis.

Figure 7. 3. SPATT samplers' configuration for field deployment

7.2.7 Toxin extraction and analysis from the SPATT samplers

SPATT samplers were taken out from the fridge and rinsed with deionized water before extraction. The SPATT bags were cut open using a pair of scissors, and the material was decanted too into 15 mL glass centrifuge tubes for the extraction of MCs from the sorbents. MCs were extracted from the sorbents following a modification of the method used by Kudela (2011). Sequential extraction using 50% methanol was used to extract MCs from the respective sorbents. To extract the MCs from the sorbents, 10 mL of 50% methanol was added to the sorbent in a glass centrifuge tube and for sonicated 5 min. After sonication, the extracts were centrifuged (Hermle Z 366 centrifuge, Wehingen, Germany) at 1750 rpm for 15 min. After centrifugation, the resulting supernatant was collected. This entire process (i.e., adding 10 mL of the homogenization solvent through collecting the supernatant after centrifugation) was repeated three times with the two preceding extractions being 5 mL each for each sample and the resulting supernatants afterwards. The combined extracts were evaporated to dryness at 50˚C using an electric water bath, under a stream of nitrogen gas. The dried samples were then re-suspended in 4 mL of deionized water.

Both grab samples and samplers eluant were analyzed for total MCs using the commercial ELISA test kits supplied by EUROFINS (Kit Lot No: 19I1120:PN 520011) following the manufactures instructions. The total dissolved microcystins concentrations in SPATT bags were determined using Equation 7.1 below:

$$\text{Total dissolved MCs (µg g}^{-1}\text{ resin)} = \frac{(\text{MCs conc µg/L} - \text{extract})\ \text{x}\ (0.002\text{L extract} - \text{VOL})}{(3\ \text{g resin})} \qquad (7.1)$$

Where: MCs conc (µg L^{-1}) is the total concentration of microcystins extracted from the SPATT bags resin; Extract volume is the amount of solvent (4 mL) of deionized water used to re-suspend the dried samples, and 3 g resin is the dry weight of DIAION HP20 resin used in the SPATT bags (0.2 g for ChGLA and ChMWCNT).

To determine the levels of some of the congeners of MCs (LR, RR, and YR) adsorbed by the SPATT samplers and in the grab samples, a chromatographic method using a Shimadzu triple quadrupole LCC-MSS/MS system (model LCMS-8045, Shimadzu Corporation, Japan) was used.

Chromatographic Conditions

Levels of Microcystins (LR, RR, and YR) in the samples were determined on a triple quadrupole LC–MS/MS system (model LCMS-8045, Shimadzu Corporation, Japan) with a Shim-pack Velox SP-C18, 2.7 µm, with dimensions 2.1 * 100 mm (Shimadzu, Japan). The injection volume was set at 10 µL and the mobile phases used were 0.1 % formic acid (FA) in water (A) and 0.1 % FA in acetonitrile (B). A flow rate of 0.5 mL min^{-1} and a 5 min binary gradient was used with an elution profile of: 2 % B (0.4 min), linear gradient to 70% B (3.1 min), 100 % B (0.5 min), and, finally, 2% B (1 min).

The interface conditions of LCMS-8045 were set as follows: the ESI interface temperature at 300 ˚C, DL temperature at 235 ˚C, nebulizing gas flow at 3 L min^{-1}, drying gas flow at 10 L min^{-1} and heating gas flow at 10 L min^{-1}. The interface voltage was set at 3.0 kV for positive (ES+) electrospray.

The final concentration of the toxins in the samples was determined by using equation 7.2:

$$\text{Conc in sample (µg/L)} = \left(\frac{\text{Co}*\text{Vol of extract used (L)}}{\text{Volume of sample used (L)}}\right) \qquad (7.2)$$

Where: C_o = the conc of sample determined from the calibration curve (µg L^{-1})

7.2.8 Physicochemical parameters

Physicochemical parameters such as pH, TDS, EC, salinity, temperature, turbidity, and DO were recorded in situ from the irrigation canals/farm dams at each site. The pH, EC, TDS, Temperature, salinity and DO were monitored using the Rugged Dissolved Oxygen electrode (RDO) attached to a Thermo-scientific meter (Singapore). Turbidity was monitored using a TB200 portable turbidimeter model (#TB200-10). The instruments were calibrated following the manufacturers' instructions prior to use. Samples for nutrients (nitrates and dissolved orthophosphates), and chlorophyll-*a* were collected at the beginning of SPATT sampler deployment. Levels of nutrients were determined using Spectro-quant® Merck Pharo 100 model No: 07531-45 (Merck KGaA 64293 Darmstadt, Germany), and the photometric test kits supplied by Merck (Germany). Chlorophyll-*a* was used to estimate phytoplankton biomass in water samples according to Lawton et al. (1999).

7.3 Results and discussion

7.3.1 Physicochemical parameters monitored

Among the physicochemical parameters monitored in-situ and ex-situ during the sampling period were Electrical Conductivity (EC) (us/cm); TDS (ppm); pH; DO (mg L^{-1}); Temp ^{o}C; Turbidity (NTU); phosphates (mg L^{-1}); nitrates (mg L^{-1}) and chlorophyll-*a* (µg L^{-1}) (Table 7.2). With regards to EC and TDS, the water from the Hartbeespoort site was exceeding the guideline recommended for irrigation water, the pH of the water for both sites was slightly alkaline exceeding the DWAF (1996) thresholds in two sampling points from the Hartbeespoort site and in one sampling point from the Roodeplaat site. Table 7.2 shows that four of the six points (two from each site) exceeded the DWAF (1996) 5 mg L^{-1} threshold. The phosphate levels for all the sampling points were within the FAO guideline thresholds.

The concentration of chlorophyll-*a* present in the water is usually related to the number of algae living in the water. Generally, concentration levels of chlorophyll-*a* above 10 µg L^{-1} result in eutrophication which ultimately increases the likelihood, and rapid growth of cyanobacterial bloom in the aquatic ecosystem (Kansas Department of Health and Environment (KDHE), 2011). With regards to the chlorophyll-*a*, one sampling point (H3) from the Hartbeespoort site and all the three points from e Roodeplaat site had chlorophyll-*a* levels above the 10 ug L^{-1} threshold, implying the possibility of heavy presence of cyanobacteria in these two farm dams.

With regards to DO, healthy water generally has dissolved oxygen concentrations above 6.5-8 mg L^{-1}. When elevated levels of cyanobacteria are present in a water body, the biological condition of the water resource may also be degraded, as the condition that allows for cyanobacterial growth is typically high in nutrients and low in dissolved oxygen. Five of the six sampled sites (except R1) had DO levels below the 6.5 mg L^{-1} threshold for healthy waters thus implying conditions suitable for cyanobacterial dominance.

Table 7. 2. Summary statistics of the physicochemical parameters of the irrigation water monitored

Parameter		Sampling Point						DWAF (1996b)
		H1	**H2**	**H3**	**R1**	**R2**	**R3**	
EC (us/cm)	Min	434.8	414.1	395.2	325.1	330.1	262.7	*0-400.0
	Max	818.7	463.1	592.4	369.4	335.9	296.5	
	Mean ± SD	678.9*±212.2	445.0*±26.9	462.8*±112.3	352.3±23.8	332.8±2.9	274.9±18.8	
TDS (mg/L)	Min	213.6	203.4	194.2	159.8	162.3	129.2	*0-260.0
	Max	401.7	227.4	290.8	181.5	165.1	145.8	
	Mean ± SD	333.2*±103.9	218.5±13.2	227.3±55.0	173.1±11.7	163.6±1.4	135.2±9.2	
pH	Min	7.88	7.5	8.04	6.94	7.94	8.73	*6.5-8.4
	Max	8.93	8.65	9.03	8.13	8.59	9.23	
	Mean ± SD	8.5*±0.5	8.1±0.6	8.7*±0.5	7.4±0.7	8.3±0.3	8.9*±0.3	
DO (mg/L)	Min	8.9	5.44	9.65	2.35	7.34	13.49	n.a
	Max	10.27	11.39	14.37	9.42	11.95	14.9	
	Mean ± SD	9.7±0.7	8.6±3.0	12.5±2.5	7.0±4.0	10.3±2.6	14.4±0.8	
Temp °C	Min	16.04	16.4	16.7	13.8	14.6	14.7	n.a
	Max	36.5	26	30.1	20.2	29	29.6	
	Mean ± SD	23.3±11.4	19.7±5.4	21.2±7.7	16.0±3.6	19.5±8.2	20.3±8.1	
Turbidity (ntu)	Min	31.37	4.7	29.45	9.34	21.86	27.73	n.a
	Max	71.25	9.28	80	11.83	35.28	36.14	
	Mean ± SD	49.3±20.3	6.5±2.4	54.6±25.3	10.3±1.3	28.3±6.7	32.5±4.3	
Phosphates (mg/L)	Min	0	0.8	0	0.4	0.4	0	0 – 2**
	Max	0.68	1	1.05	1.08	1.2	0.73	
	Mean ± SD	0.2±0.4	0.9±0.1	0.4±0.6	0.8±0.4	0.9±0.4	0.2±0.4	
Nitrates (mg/L)	Min	3	8	0	7	5	0	< *5 (as inorganic N)
	Max	16	10	5	8	6	6	
	Mean ± SD	10.3±6.7*	9.0±1.0*	3.0±2.6	7.3±0.6*	5.7±0.6*	2.0±3.5	
Chlo a (mg/L)	Min	0.9627	0	21.252	4.665	22.807	6.665	n.a
	Max	18.735	19.253	56.204	21.326	108.85	35.84	
	Mean ± SD	8.0±9.4	6.6±10.9	40.5±17.7	11.3±8.8	54.3±47.4	20.1±14.7	

*Department of Water Affairs & Forestry (DWAF, 1996), **Food and Agriculture Organization (FAO) guidelines of 1985 (Ayers and Westcot, 1985)

7.3.2.1 Levels of MCs detected by the SPATT samplers

Despite the laboratory study has shown that the samplers were getting saturated after 24 hours, the samplers with the three sorbents (ChGLA, ChMWCNT and DIAION HP20) were deployed for 48 hours in the field. The assumption was that the field water had less MC load compared to the field water used for the laboratory trial since the study sites were receiving excessive rains before the first field visit. Findings of the field monitoring confirmed that the field water in both sites had traces of MCs (below 1 ug L^{-1} for 5 of the sampling points and approximately 4 µg L^{-1} at one sampling point) (Table 7.3).

Of importance to note was the fact that, despite the low levels of MCs found in the grab samples, all the three types of samplers were detecting significant levels of MCs with all the samples needing at least a 10 factor dilution to be within the 0-5 µg L^{-1} range for the ELISA test used for quantification. Figure 7.4 shows that DIAION HP20 and the ChMWCNT samplers detected MCs better than the ChGLA even though there were no statistically significant differences in the mean levels of MCs detected by the three samplers (ANOVA at .05 level of significance). What was apparent was the higher detection levels of ChMWCNT in highly alkaline sampling points (H3 and R3) (see Table 7.2) compared to the DIAION HP20 which detected MCs better in sampling points H2 and R1 which had slightly neutral mean pH of 8.1 and 7.4, respectively. Such findings suggest that pH could be a factor in the performance of the samplers and will thus need further investigation.

Table 7. 3. Summary statistics of MCs detected in the irrigation water monitored by grab sampling

Parameter		**Sampling Point**					
		H1	**H2**	**H3**	**R1**	**R2**	**R3**
MC Grab (µg L^{-1})	Min	0.112	0.144	0.178	0.099	0.126	3.316
	Max	0.597	0.173	0.910	0.130	0.493	4.037
	Mean ± SD	0.28±0.22	0.15±0.01	0.66±0.34	0.12±0.01	0.25±0.17	3.75±0.31

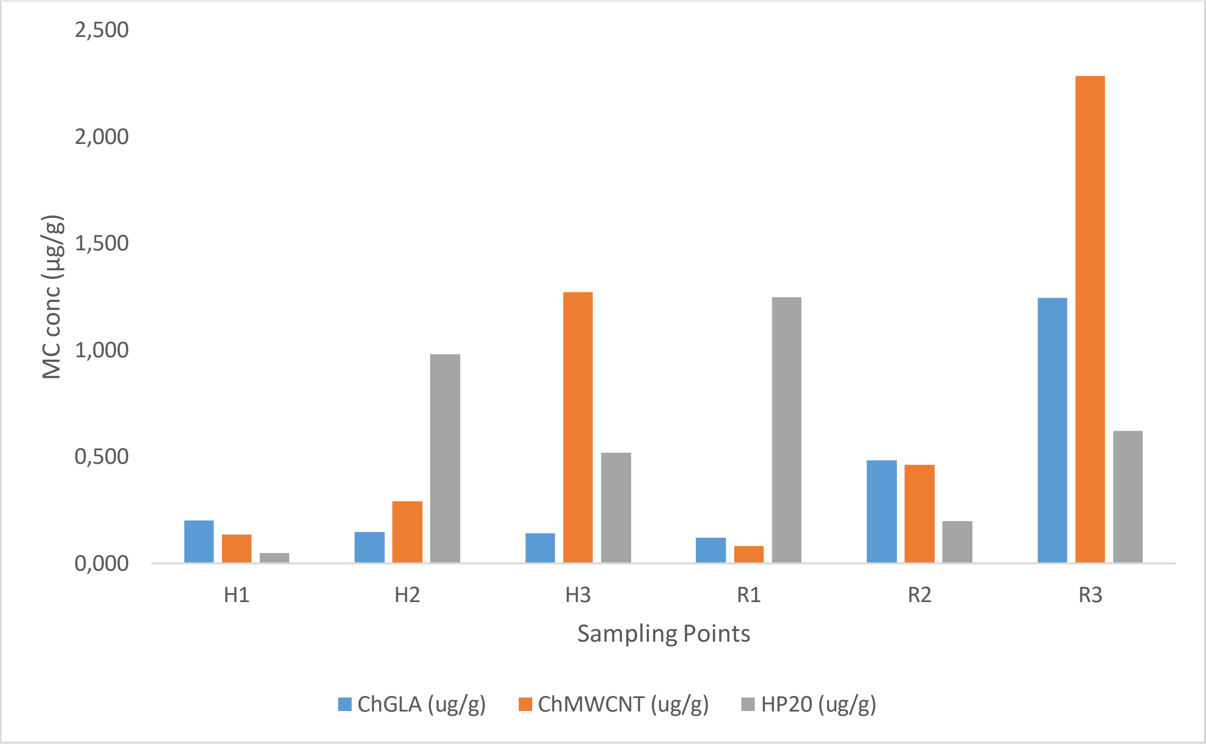


Figure 7. 4. Total MCs levels adsorbed by the three samplers in µg g-1-adsorbent over the 2 days
N.B: No significant differences were found among the three samplers across all sampling points (ANOVA at .05 level of significance)

Figure 7.5 shows the pattern of the MC detection by the samplers across the sampling points. A clear pattern/trend in the detection of MCs by ChGLA, ChMWCNT and grab sampling can be observed, thus inferring that the two samplers were depicting the MC levels in these water bodies better.

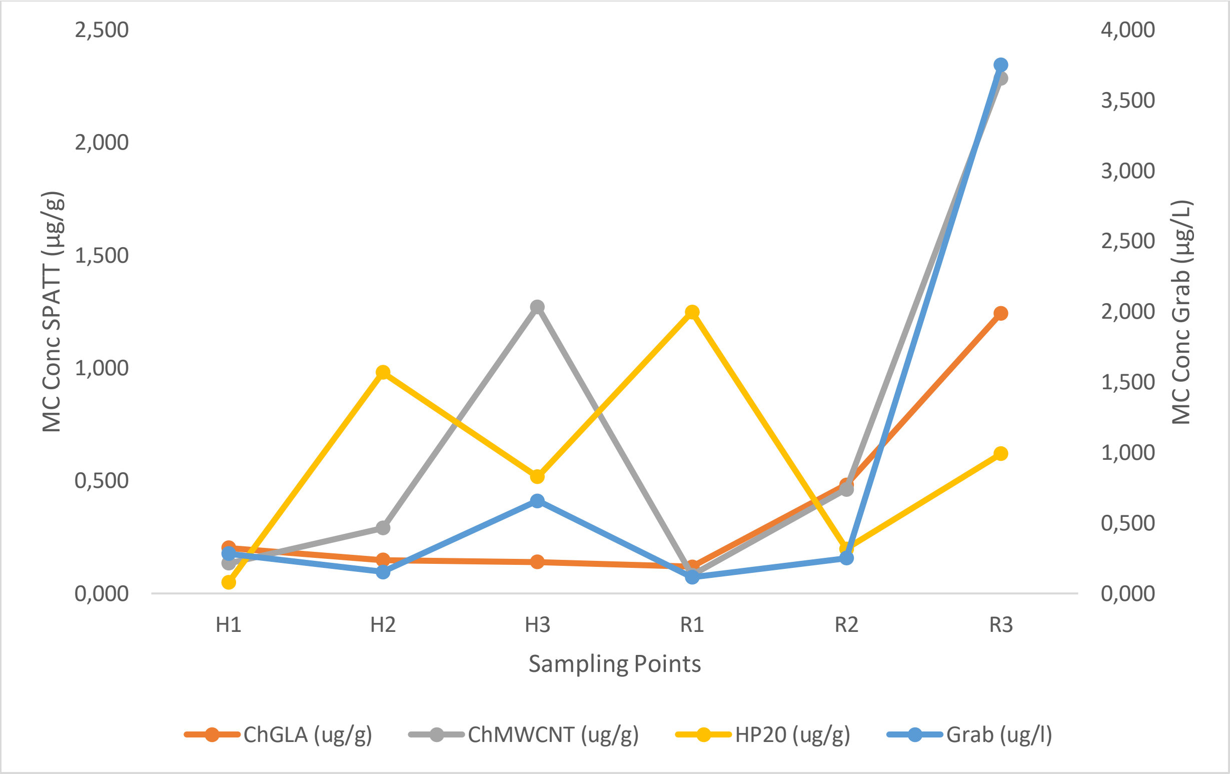


Figure 7. 5. Comparison of the levels of Total MCs adsorbed by the samplers vs grab samples

7.3.3 Samplers' Adsorption of different MCs

With regards to the adsorption and desorption of MC-LR, -RR and –YR, figure 7.6 (a, b & c) show the levels of the 3 MC congeners detected by the samplers. Findings indicate that, the ChMWCNT sampler was detecting MC-LR and –RR better even though no statistical differences were reported in most of the sampling points (one-way ANOVA at .05 level of significance). Statistically significant differences in the detection of MCs by the samplers were only reported for sampling point H2 for MC-LR and –RR, where DIAION HP20 detected much higher levels of the toxins compared to the other two. Figure 7.6 shows that the levels of detection of MCs by the samplers were in the order MC-YR>LR>RR. The DIAION HP20 resin seems to be detecting MC-YR better compared to the newly synthesised chitosan-based sorbents, but no statistically significant difference was reported one-way way ANOVA at .05 level of significance).

Figure 7.7 (a,b,c) shows the trends of MC-LR, -RR and YR detection across the sampling points by the 3 samplers and by grab sampling. No definitive trend/correlation could be observed for the detection of MC-LR and MC-YR, but a clear trend/correlation can be observed for the detection of MC-RR by the 3 samplers versus grab sampling. The differences in adsorption patterns of the different MCs can also be explained in terms of their polarities. Although these MCs may be similar in structure, they have different amino acids MC-RR has arginine and arginine whereas MC-LR has leucine and arginine (Pavagadhi et al., 2013). Arginine being a more polar amino acid compared to leucine, would make MC-RR more polar and a preferred adsorbate onto the samplers' active sites, thus a more regular pattern in its adsorption by the 3 samplers tested.

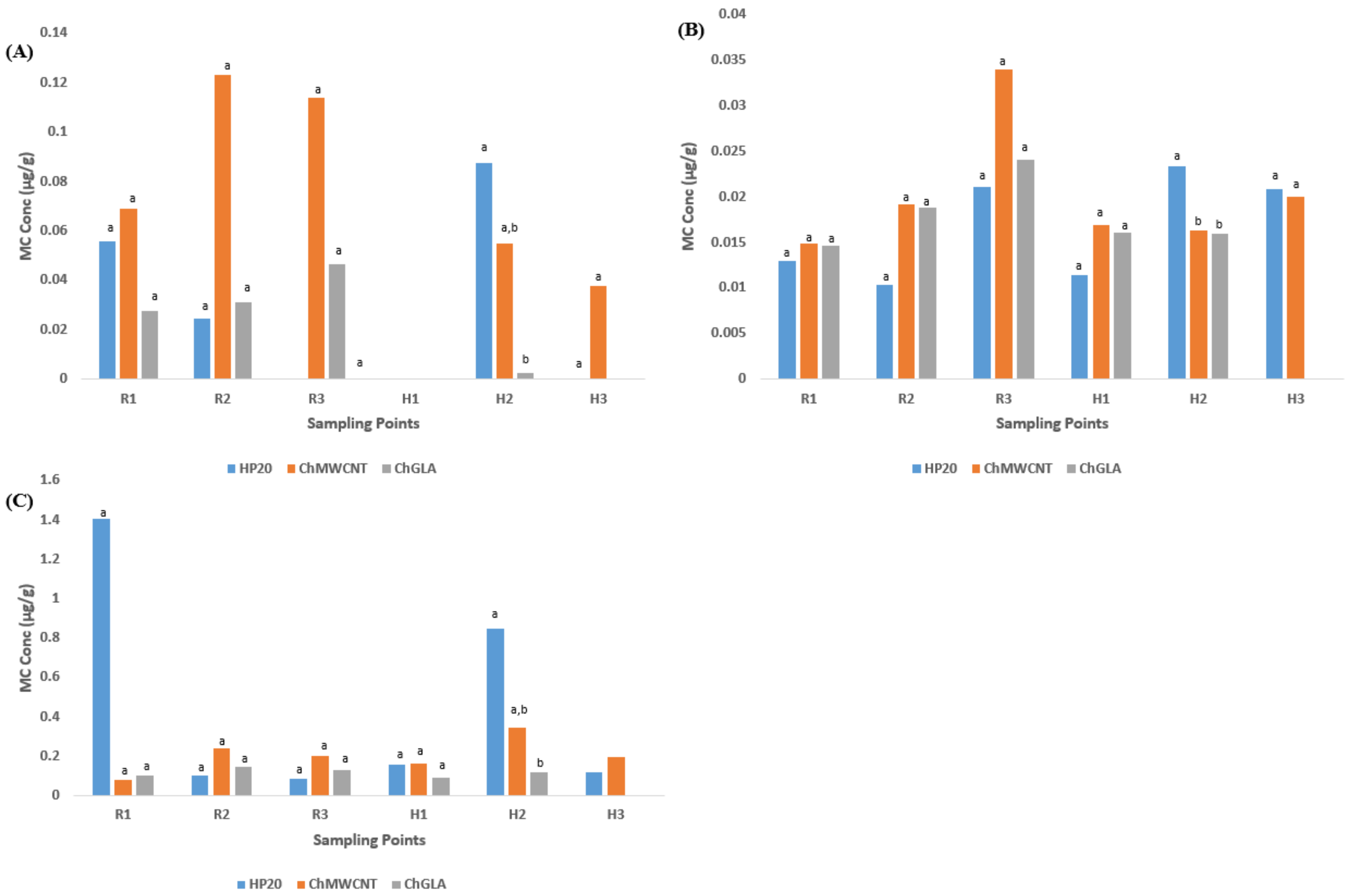


Figure 7. 6. MC levels were adsorbed by the three samplers in µg g-1-adsorbent over the 2 days. (A) MC-LR (B) MC-RR (C) MC-YR *N.B*: No significant differences were found among the three samplers across all sampling points (ANOVA at .05 level of significance) Data labelled with different small letters (a - c) differed significantly at $p < 0.05$ in each bar (n=3).

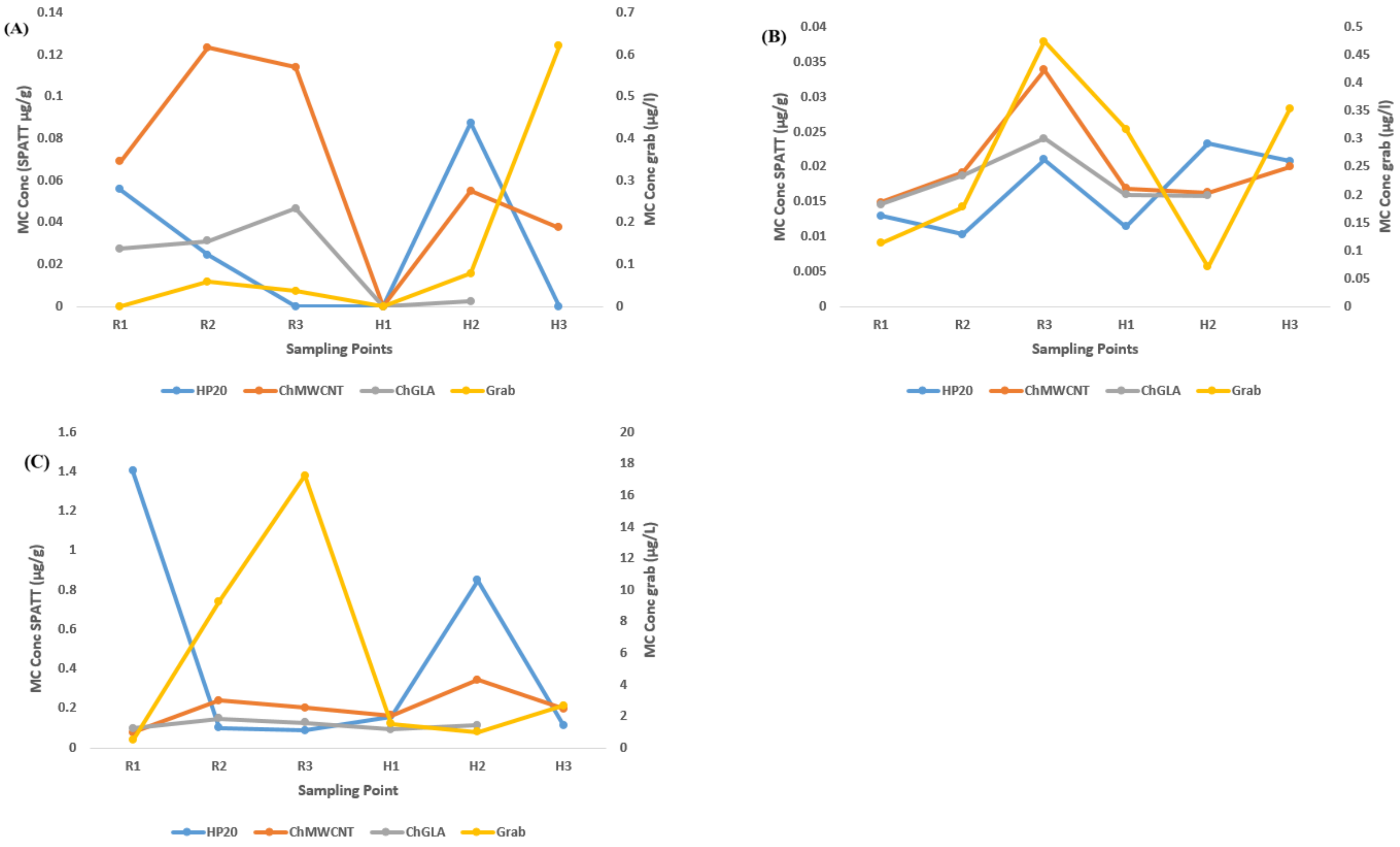


Figure 7. 7. Comparison of the levels of MCs adsorbed by the SPATT samplers' vs grab samples (A) MC-LR, (B) MC-RR and (C) MC-YR

7.3.4 Correlation of Physicochemical parameters and MCs levels

Table 7.4 shows the Pearson correlation coefficients between physicochemical parameters monitored and MCs in SPATT samplers and grab samples. Strong positive correlations were found between TDS and EC, pH and DO, phosphate levels and DO, phosphate levels and pH, nitrate levels and EC, and nitrate levels and TDS. Dissolved oxygen (DO) was the only parameter strongly positively correlated with MCs levels detected in grab samples. With regards to the gluteraldehyde crosslinked chitosan (ChGLA) and the crosslinked chitosan- multiwalled carbon nanotubes (ChMWCNT) composite samplers, DO and pH were the only parameters positively correlated with MCs levels detected by these samplers, whilst negative correlations were found between phosphate and nitrate levels and MC levels detected by the samplers. No physicochemical parameters positively correlated with MC levels detected by HP20, but a negative correlation was found between HP20 and temperature.

Much of the findings here can be explained in terms of the pH of the water. Previous studies (Zang et al., 2011; Li et al., 2013) found a significant positive correlation between pH and DO. Further, Li et al. (2013) reported high P release from sediments in both acidic and alkaline conditions. The significant positive correlations reported for DO, pH and the levels of MCs detected by the samplers, could be an indication that the newly developed material is affected by pH is adsorbs better in alkaline conditions. Most eutrophic water bodies have an alkaline pH thus suggesting better suitability of this material for monitoring MCs in such waters.

With regards to the MC levels detected by the samplers themselves, there was a correlation between MC levels detected by the ChGLA and ChMWCNT samplers, and the MC levels detected in grab samples also correlated with these two synthesized samplers. The MC levels detected in HP20 did not correlate with any of the other samplers or grab samples. Even though ChMWCNT showed more superiority and higher affinity for MCs as demonstrated in Figure 7.4 due to its superior pore sizes, the two (ChGLA and ChMWCNT) have more or less similar functional groups hence the positive correlation in their detection of MCs. In addition, the fact that the newly synthesized sorbents, correlated well with MCs in grab samples, also seems to suggest that they give a better indication of MCs in the sampled water bodies compared to HP20 which according to the findings, was affected by the water temperature. There are very few studies which used the DIAION HP20 resin for the monitoring of MCs and correlated the water physicochemical parameters with levels of MCs detected by the samplers. In one study by Kudela (2011), no correlation was reported between the water temperature and levels of MCs detected by DIAION HP20.

In this study, chlorophyll-*a* did not correlate to MC levels detected by any of the samplers or sampling methods used. Contrary to our findings, Howard et al. (2017), monitored lakes, reservoirs and coastal lagoons and found that chlorophyll-*a* was a statistically significant predictor of MC levels detected by SPATT samplers using the resin DIAION HP20. Kudela (2011) also found algae biomass as chlorophyll concentrations as the best single predictor for MC loads in Pinto Lake (U.S.A) in both grab and SPATT samplers using DIAION HP20.

In addition, negative correlations were found between MC levels in ChGLA and ChMWCNT and both nitrates and phosphates levels in this study. Both phosphates and nitrates did not positively correlate to any other physicochemical parameter or MC levels detected by the samplers or grab method. Howard et al. (2017) also did not find any statistically significant relation between MC levels detected by HP20 SPATT samplers and any of the environmental predictors they monitored such as alkalinity, nutrients, elevation, conductivity, and temperature. Contrary, Kudela (2011) found total dissolved nitrogen (TDN) to be significantly correlated to toxin concentrations in both grab and SPATT samplers. The negative correlation between nitrates and phosphates and MC levels detected by the newly synthesised sorbents will need further investigation as this might be an indication of these two anions competing with MCs for the same binding sites onto the adsorbents.

With regards to DO, it is one of the most important parameters indicating the health of a water body or system. Cyanobacterial blooms are normally associated with low DO levels, in this study even though some of the sites had recorded low DO levels, there was no correlation between DO levels and chlorophyll-*a* levels. Contrary to the findings here, Okogwu and Ugwumba (2009) found cyanobacteria biomass to be strongly negatively correlated to DO and attributed this to the high levels of degradation of cyanobacteria as the cells die following a bloom, resulting in depletion of oxygen and reduced pH.

Moderate to strong positive correlations reported for DO vs MC levels in ChGLA, ChMWCNT and grab samples. This could be attributed to the fact that two of our six sampled points were canals, were water was fast moving and the other four were farm dams were water was not really kept for long, thus promoting aeration. The MCs being detected in such points (canals) are likely to have been transported from other areas like the main reservoir/dams (Hartbeespoort and Roodeplaat Dams) were the water is stagnant and promoting proliferation of HABs and the resultant toxins.

Table 7. 4. Pearson correlation coefficients between physicochemical parameters monitored and MCs in SPATT samplers used

	EC (us/cm)	TDS (mg/l)	pH	DO (mg/l)	Temp °C	Turbidity (ntu)	PO$_4^{2-}$ (mg/l)	NO$_3^{2-}$ (mg/l)	[†]Chlo a (mg/l)	Grab (µg/l)	ChGLA (µg/g)	ChMWCNT (µg/g)	HP20 (µg/g)
EC (us/cm)	--												
TDS (mg/l)	1.000**	--											
pH	0.130	0.130	--										
DO (mg/l)	-0.213	-0.213	.755**	--									
Temp °C	0.014	0.014	-0.188	-0.237	--								
Turbidity (ntu)	0.117	0.117	0.434	0.439	0.326	--							
PO$_4^{2-}$ (mg/l)	-0.234	-0.234	-.723**	-.622**	.469*	-0.454	--						
NO$_3^{2-}$ (mg/l)	.692**	.692**	-0.122	-.496*	-0.179	-0.342	0.088	--					
Chlo a (mg/l)	-0.226	-0.226	0.377	0.383	-0.324	0.234	-0.329	-0.290	--				
Grab (µg/l)	-0.465	-0.465	.500*	.630**	0.023	0.197	-0.404	-.585*	0.005	--			
ChGLA (µg/g)	-0.366	-0.366	.540*	.692**	-0.403	0.103	-.605*	-.575*	0.313	.784**	--		
ChMWCNT (µg/g)	-0.406	-0.406	.551*	.690**	-0.356	0.296	-.638**	-.660**	0.308	.764**	.978**	--	
HP20 (µg/g)	-0.272	-0.272	-0.046	0.192	-.572*	-0.398	-0.108	-0.033	-0.054	0.017	0.090	0.159	--

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

[†]Chlorophyll a

7.4 Conclusions

A good correlation between the toxins detected by the two newly synthesized-grab grab samples and a strong positive correlation between the two newly synthesized materials (ChGLA and ChMWCNT) was reported. A strong/moderate positive correlation between DO and pH and MCs in grab samples, ChGLA and ChMWCNT, making DO and pH better predictors of toxin loads in the study area and also factors determining the suitability for the application of the newly synthesized samplers. The levels of MCs detected by the DIAION HP20 resin did not correlate to any of the physicochemical parameters monitored. There strong positive correlation between gab sampling and the ChGLA and ChMWCNT samplers, suggests a better prediction of toxin loads by the newly synthesized material compared to the DIAION HP20 resin.

The samplers also detected all the 3 MCs (-LR, -RR, and –YR) tested here comparably well to DIAION HP20, further suggesting their suitability in application for the SPATT monitoring of MCs in irrigation water. Based on these findings, it can be concluded that SPATT using the synthesized chitosan-based adsorbents has the potential to be integrated into current cyanobacterial monitoring programmes and would be an extremely useful and economical tool for early warning and monitoring of toxic cyanobacterial events in water intended for irrigation.

Based on the findings the following is recommended:

- Further monitoring of the sites with the synthesized sorbents to validate the findings and the impact of various physicochemical parameters on the performance of the samplers with the synthesized material.
- Optimize the adsorption and desorption conditions for the sorbents and find the optimum mass to pack in SPAT bags for field applications and investigate further the effect of DO, pH, phosphates, and nitrate levels, as factors that affect the field application of the samplers.

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CHAPTER EIGHT: CONCLUSIONS & RECOMMENDATIONS

8.1 Introduction

Challenges posed by eutrophication have been on the rise in the past few decades as a result of intensifying agriculture, industrial activities, and the changing global climate. The problems of eutrophication and cyanobacteria in South African reservoirs are well-known and well-documented. The project studied explored the bioavailability and effects of a range of cyanobacterial metabolites on terrestrial food plants (*Brassica oleracea* and *Solanum tuberosum*) Chitosan-based sorbents were also developed and evaluated for their sorption and desorption of MCs and applied in passive samplers (SPATT) to monitor the bioavailability of MCs in water intended for irrigation.

8.2 Summary of key findings

The findings were presented in six sections: 1) Impacts of cyanotoxins and other stressors on food plants irrigated with eutrophicated waters and application of the SPATT technology for monitoring irrigation water: A literature survey, 2) Bioaccumulation and elimination of MCs by plants, 3) Effect of LAS on the uptake and accumulation of MCs in *Brassica oleracea* and *tuberosum* 4) Effect of LAS on the uptake and accumulation of metals in *Brassica oleracea* and *Solanum tuberosum* 5) Development & application of a crosslinked chitosan-based Solid Phase Adsorption Toxin Tracking Technology (SPATT) adsorbent, and 6) Field application of a crosslinked chitosan-based Solid Phase Adsorption Toxin Tracking Technology (SPATT) adsorbent.

8.2.1 Impacts of cyanotoxins and other stressors on food plants irrigated

The first section provide an in-depth review of literature on the potential risks of transfer of cyanotoxins from irrigation water into edible parts of plants, the co-existence of cyanotoxins and other pollutants in the natural environment and the synergic impact of these pollutants and the monitoring tools available to monitor cyanotoxins and their pros and cons. The reviewed literature revealed that most rivers and reservoirs in highly industrialized and populated areas of South Africa such as the Vaal system and Crocodile-West Marico Water Management Area seem to be severely polluted with a range of other pollutants. High salinity, toxic metal species, and Persistent Organic Pollutants (POPs) have all been reported to be key issues of concern. With that in mind, it was thus important to investigate the synergism of multiple stressors in water being used for agricultural purposes and agricultural soils in South Africa and how this could be impacting productivity and the possible human and animal health impacts. In addition, the literature review highlighted the urgent need for guidelines and policies on cyanotoxins in irrigation water, food plants water used for livestock.

8.2.2 Bioaccumulation of MCs by plants

In the second section, the accumulation and elimination capacities of MCs in distinct parts of the plant's *Brassica oleracea* and *Solanum tuberosum* was studied. Water was used to irrigate the plant's mad Microcystins (MCs) levels ranging from 0.12 to 2.84 µg L^{-1}. The pH for the water was slightly alkaline (pH 7.29 ±0.71 to 10.03±0.29) but within the permissible limits according to the South African and FAO guidelines and the EC ranged from 296.67±13.87 to 878.67±42.44 µS cm^{-1} and was in most cases higher the South African guideline and FAO limits for irrigation water.

Based on the findings, the levels of MCs in the dam water collected from Roodeplaat Dam, have a significant impact on the seed germination of *Brassica oleracea* but did not show significant impact on the general plant growth, nor induce significant oxidative stress as demonstrated by comparable total chlorophyll between the trial plants and the controls. The findings demonstrated that the two plants can bio-accumulate MCs to concerning levels when irrigated with water derived from the Roodeplaat Dam. MC accumulation levels in the two tested plants ranged from 0.001415 to 0.135508 mg kg^{-1} DW for individual MC congeners and this was comparable to the concentrations reported in other studies. These findings, together with findings in previous studies, demonstrate terrestrial food crops can accumulate MCs to levels that can pose human-health risks when exposed to naturally relevant levels of MCs.

8.2.3 Effect of LAS on the uptake and accumulation of MCs in *Brassica oleracea* and *Solanum tuberosum*

In the fourth section, the effect of multiple stressors on food plants and uptake of MCs was investigated. The hypereutrophic conditions in major water reservoirs used for irrigation purposes in South Africa promotes the co-existence of MCs and other pollutants such as linear alkylbenzene sulfonate (LAS). LAS is known to alter the permeability of membranes and promote the uptake of other pollutants by plants. Here we investigated the combined effects of LAS and microcystins (MCs) when cyanobacteria-infested water is used to irrigate terrestrial crops in light of the potential human health risks and prevailing hypereutrophic conditions in some catchments in South Africa. To understand the potential risks, pot-culture experiments were conducted to assess the effect of LAS on the accumulation of MCs in *Brassica oleracea* (cabbage) and *Solanum tuberosum* (potato) plants. The plants were watered with dam water containing 3.48 mg L^{-1} of the LAS (sodium dodecyl sulfate) and MCs (MC-LR: 10.47 (±3.879); 6.158 (± 4.127) for MC-RR and 8.160 (± 2.544) for MC-YR µg L^{-1}), for 20 days.

The presence of LAS, at environmentally relevant concentrations in the irrigation water, did not enhance uptake of MCs in the two plants, as demonstrated by statistically insignificant differences in the means of the treatments (with and without LAS). In addition, the presence of the LAS, high pH, EC and presence of cyanotoxins in the water did not affect the total chlorophyll and the wellbeing of the plants. However, in some cases the levels of MCs bio-accumulated by the two plants exceeded the WHO recommended TDI. These findings imply that, the tested levels of LAS and MCs did not have any synergic effects on the two plant species, but irrigating food crops with such water still poses a human health risk.

8.2.4 Effect of LAS on the uptake and accumulation of metals in *Brassica oleracea* and *Solanum tuberosum*

In the third section, the effect of multiple stressors on food plants and uptake of metals Al, Mn, and Sr by food plants *Brassica oleracea* and *Solanum tuberosum* was investigated. Rapid population growth, urbanization, industrial and mining activities around South African major cities have promoted hypereutrophic conditions in impoundments used for irrigation and this promotes the co-existence of MCs, linear alkylbenzene sulfonate (LAS), and other pollutants such as metals. The combined effects of LAS and other con such as metals when such water is used to irrigate crops have not been thoroughly investigated in light of human health risks and prevailing local conditions.

To understand the potential risks, pot-culture experiments were conducted to assess the effect of LAS on the accumulation of aluminum (Al), manganese (Mn) and strontium (Sr) in *Brassica* *oleracea* (cabbage) and *Solanum tuberosum* (potato) plants. The plants were watered with dam water containing 3.48 mg L^{-1} of the LAS (sodium dodecyl sulfate) and Mn (0.257 mg L^{-1}), Al (0.6 mg L^{-1}) and Sr (0.16 mg L^{-1}) as determined by field surveys, for 20 days. The presence of LAS, at environmentally relevant concentrations in the irrigation water, did not enhance the uptake of Sr, Mn, Al by the two plants, as demonstrated by statistically insignificant differences in the means of the treatments (with and without LAS). In addition, the presence of the metals, high pH, EC, and presence of MCs in the water did not affect the total chlorophyll and the well-being of the plants. These findings imply that, the prevailing levels of LAS, metals ,and other contaminants in the hypereutrophic reservoirs poses minor risk to the crop yields, quality of the crops, and human health due to the possible accumulation of these contaminants in irrigated plants.

8.2.5 Development & application of a crosslinked chitosan-based Solid Phase Adsorption Toxin Tracking Technology (SPATT) adsorbent

The fifth section focused on synthesising a crosslinked chitosan sorbent that can be used in SPATT format to passively sample MCs. Successful crosslinking and addition of multi-walled CNTs onto the chitosan structure crosslinked with glutaraldehyde as was confirmed by the FITR results. The SEM images confirmed the successful crosslinking of chitosan by glutaraldehyde and confirmed the successful addition of multi-walled CNTs onto the crosslinked chitosan. The SEM images also confirmed the successful adsorption of MCs onto the surfaces of both ChGLA and ChMWCNT. Of importance, the crosslinking and addition of multi-walled CNTs improved the surface area, pore volume, and pore sizes of the chitosan. Greater pore sizes for the synthesized ChMWCNT composite compared to the DIAION HP20 resin, suggested better capacities for the ChMWCNT to adsorb MCs.

Batch adsorption experiments to assess the effect of contact time, adsorbent dosage and initial MC-LR concentration were conducted. For desorption efficiency, various methanol solutions were tested. The composite was found to be efficient in adsorbing MC-LR showing 97% removal and maximum adsorption capacity of 4.639 µg g^{-1} under optimized conditions of 5 µg L^{-1} of MC-LR, adsorbent dose of 0.03g 5 mL^{-1}, and contact time of 30 mins. The adsorption kinetics was better explained by the pseudo-second order model, meaning that the adsorption was occurring through chemisorption. The isotherm data was better explained by the Langmuir isotherm model, thus inferring a monolayer surface adsorption of MC-LR onto ChMWCNT. In terms of desorption, 100 % methanol was found to be most effective, with the highest mean desorption efficiency of 84.71 ± 6.47 %. When applied for the adsorption of microcystins in raw dam water, the composite was saturated within two days of exposure and effectively adsorbed and desorbed three congeners of MCs (-LR, -RR and –YR) tested. The developed composite showed potential for application in SPATT for monitoring of MCs in freshwater regardless of its lower maximum adsorption capacity compared to other adsorbents used for similar purposes.

8.2.6 Field application of a crosslinked chitosan-based Solid Phase Adsorption Toxin Tracking Technology (SPATT) adsorbent

The last part evaluated the developed chitosan-based sorbents, application as passive samplers in SPATT format in the study area. In the field, the 3 types of SPATT samplers (newly developed ChGLA and ChMWCNT, and the commercially available DIAION HP20) were deployed for 48 hours. The findings indicated that MCs were detectable in all two sites and all six sampling points in all three types of samplers and grab samples. The three samplers were easily detecting MCs even in areas where grab samples detected traces of the toxin.

The findings indicated that MCs were detectable in both sites (Roodeplaat and Hartbeespoort), in all the six sampling points, and by all three types of samplers and grab samples. The sites sampled in this study are of critical importance as the water is directly used for irrigation and other agricultural purposes, hence the likelihood of the toxins being taken up by crops consumed by humans. The three samplers were easily detecting MCs even in areas where grab samples detected traces of the toxin.

The findings showed a good correlation of the toxins detected by the two newly synthesized and in-grab samples, and a strong positive correlation was also reported between the two newly synthesized material (ChGLA and ChMWCNT). Among physicochemical parameters monitored, there was a strong positive correlation between DO and pH and MCs in grab samples, ChGLA and ChMWCNT, making DO and pH better predictors of toxin loads in the study area and also factors determining the suitability for the application of the newly synthesized samplers. The levels of MCs detected by the DIAION HP20 resin did not correlate to any of the physicochemical parameters monitored. The strong positive correlation between gab sampling and the ChGLA and

ChMWCNT samplers, suggests a better prediction of toxin loads by the newly synthesized material compared to the DIAION HP20 resin.

The samplers detected all the 3 MCs (-LR, -RR, and –YR) tested here comparably well to DIAION HP20, further suggesting their suitability in an application for the SPATT monitoring of MCs in irrigation water. Based on these findings, it can be concluded that SPATT using the synthesized chitosan-based adsorbents has the potential to be integrated into current cyanobacterial monitoring programmes and would be an extremely useful and economical tool for early warning and monitoring of toxic cyanobacterial events in water intended for irrigation.

8.3 Concluding remarks

South Africa is known for having scarce and extremely limited water resources and depends mainly on surface water resources for most of its urban, industrial and irrigation requirements. The country largely depends on water stored in man-made reservoirs for the sustained supply of raw potable and irrigation water. Irrigation is a common agricultural practice that involves the use of water from public supply reservoirs, rivers and ponds to irrigate farms/crops. Unfortunately, these surface water sources are sometimes contaminated with cyanobacteria and cyanotoxins, which may be taken up and bio-accumulated in plants tissue. This makes the consumption of crops and vegetables irrigated with the contaminated water a potentially dangerous route for human exposure to different cyanotoxins, including microcystins (MCs). The study also assessed the risk posed by other pollutants such as metals and anionic surfactant in hyper-eutrophicated water bodies such as those found in the Crocodile (West) and Marico catchment.

Based on the pot-culture experiments conducted here, the levels of MCs in the dam water collected from Roodeplaat Dam, was shown to have a significant impact on the seed germination of *Brassica oleracea* but did not show significant impact on the general plant growth, nor induce significant oxidative stress as demonstrated by comparable total chlorophyll between the trial plants and the controls. The findings demonstrated that the two plants can bio-accumulate MCs to concerning levels when irrigated with water derived from the Roodeplaat Dam. MC accumulation levels in the two tested plants ranged from 0.001415 to 0.135508 mg kg^{-1} DW for individual MC congeners and this was comparable to the concentrations reported in other studies. Findings from this study, together with findings in other studies discussed here, demonstrate that terrestrial food crops can accumulate MCs to levels that can pose human-health risks when exposed to naturally relevant levels of MCs.

The findings of the study showed that the presence of anionic surfactants did not induce the uptake of Mn, Sr, and Al and the same could also be said for the uptake of other major and trace cations. The presence of LAS did not induce synergic effects of metals and MCs on the plants, as demonstrated by the comparable levels of total chlorophyll in both plant species upon exposure to contaminated water containing various levels of LAS. Similar to the uptake of metals in presence of LAS, the presence of LAS did not alter the uptake of MCs by the two plant species tested MC MCs uptake was in most cases significantly higher in plants exposed to raw dam water compared to the plants exposed to raw dam water spiked with environmentally relevant levels of LAS.

Except for MC-LR, the levels of MCs accumulated by the tubers did not reach prominent levels to exceed the TDI of 0.04 mg kg^{-1} of body weight recommended by the WHO. Since MC-LR is normally the dominant congener in many waters dominated by the *Microcystis* and *Aeruginosa* genus, the raw dam water used was dominated by MC-LR hence it was the only congener that exceeded the recommended TDI. Even though MC-RR was in lower concentration in the raw dam water compared to MC-LR, the findings of the current study have shown that it can accumulate in cabbage leaves to levels that can exceed the 0.04 mg day^{-1} kg^{-1} of body weight when plants are watered with contaminated dam water. This is of concern since this limit was reached after only 5 days of exposure to the dam water. However, the fact that the TDI was not exceeded in the cabbage leaves after 20 days of exposure to the same dam water could be a reflection that the plants were finding ways of copying and bio-transforming the toxins as the exposure was prolonged.

The last two Chapters focused on the development and application of a chitosan-based sorbent to be used in a SPATT format for the passive sampling of MCs and possibly other cyanotoxins. The developed chitosan-based sorbents showed a lot of promise in both the laboratory and field trial when applied either free or in SPATT bag format. The use of SPATT using the newly synthesized chitosan-based sorbents showed a lot of promise for the monitoring of MCs and possible use as an early warning sign for the presence of MCs in irrigation water derived from eutrophicated water bodies in South Africa.

The findings of this study are relevant to the water and agricultural sectors and intended to contribute to the development of policies in South Africa on the use of such water and the acceptability of plants for human consumption after irrigation with contaminated water.

8.4 Limitations

Among the limitations of the study were:

- The use of the University of Venda School of Agriculture nursery instead of a location around the study area (Crocodile (West) and Marico catchment area) for the pot-culture experiments. Since the type of soils and environmental conditions affect MCs' half lives in the environment and their eventual uptake by plants.
- The use of pot-culture instead of open fields since pot cultures do not exactly mimic the open field scenario.
- Monitoring of three congeners of MCs (MC-LR, MC-RR and MC-YR) only, since there are more than 100 congeners of MCs and other metabolites simultaneously found in the aquatic environment, all of which could be affecting the plants and available for uptake. The limiting factor was the cost and availability of the reference material and techniques required for their identification and quantification.
- The evaluation of the sorption and desorption of MCs by the chitosan-based sorbents only looked at MC-LR, -RR, and –YR and did not investigate adsorption and desorption of other cyanotoxins and other congeners of Microcystins due to the limited availability of analytical standards.

8.5 Recommendations

Based on the findings of the literature survey, it is recommended that further studies be conducted to provide policymakers with local evidence-based data to guide the process of policy formulation about cyanotoxins in irrigation water. The major risk of exposure to cyanotoxins in both drinking water and diet was deemed to be via long-term exposure to low levels of the toxins. It is thus recommended that the South African water sector, industry and authorities prioritize research addressing issues specific to cyanotoxins in irrigation water and development of local guidelines/regulations for cyanotoxins in agricultural water.

The pot culture experiments showed that the existing levels of anionic surfactants, metal contaminants, and microcystins being found in hypereutrophic reservoirs such as Roodeplaat and Hartbeespoort Dams in South Africa, pose minor risk to the crop yields, quality of the crops and human health due to the possible accumulation of these contaminants in irrigated plants. The pot-culture experiments demonstrated that irrigating plants with water derived from the two dams may pose a risk to human health through the ingestion of accumulated MCs, since in some cases the accumulated MCs exceeded the recommended TDI set by the WHO. In as much as there might not be an immediate inherent risk to the plants and human health, continuous monitoring of the contaminants in water, soil, and irrigated plants is recommended since the conditions, the concentrations, and other factors can quickly change if the management of the catchment does not improve shortly.

The synthesised chitosan-multiwalled carbon nanotubes (ChMWCNT) composite and the glutaraldehyde crosslinked chitosan (ChGLA) hydrogel were shown to have the potential to adsorb and desorb MCs and be applied for the passive sampling of MCs in a SPATT bag format. Based on these findings it is recommended that further monitoring of the sites using the synthesized sorbents be conducted to further validate the findings and the impact of various physicochemical parameters on the performance of the samplers with the synthesized material.

8.6 Future research areas

Based on the findings of the study, there is an urgent need for local guidelines and policies on cyanotoxins in irrigation water, food plants and water used for livestock. Future studies need to look at the local prevailing factors such as the prevalent cyanotoxins and their levels, the climatic region, type of the irrigation involved and local agriculture and aquaculture practices, local population, eating habits and importantly the socioeconomic status of the population under consideration among other factors to assist in the possible formulation of policies and guidelines.

Further pot-culture experiments using locally available soils and environmental conditions in the Crocodile (West) and Marico catchment area, looking into the possible uptake of MCs by the locally grown plants in different seasons are also recommended. Such studies will be of significance since cyanotoxins' half-lives in the soil are known to be affected by the soil types and the local biota and environmental conditions.

Further studies using the synthesised sorbents will need to investigate the adsorption and desorption of the different congeners of MCs and other secondary metabolites (such as β-Methylamino-L-alanine (BMAA)) commonly produced by cyanobacteria and found in the catchment under study. Further optimization of the adsorption and desorption conditions for the sorbents to find the optimum mass to pack in SPATT bags for field applications and the optimum duration of exposure in the field will also give valuable scientific information important for the future applications of the material.

Appendices

Appendix 3.1: ANOVA results for MC-RR in *B. oleracea* after 20 day exposure

One-way Analysis of Variance (ANOVA)

The P value is 0.1784, considered not significant.
Variation among column means is not significantly greater than expected
by chance.

Post tests
Post tests were not calculated because the P value was greater than 0.05.

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical
SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 14.725
The P value is 0.0006.
Bartlett's test suggests that the differences among the SDs is extremely significant.
Since ANOVA assumes populations with equal SDs, you should consider
transforming your data (reciprocal or log) or selecting a nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?

```
ANOVA assumes that the data are sampled from populations that
follow
Gaussian distributions. This assumption is tested using the method
Kolmogorov and Smirnov:

    Group       KS     P Value  Passed normality test?
=============== ======   ========
=======================
          Exp   0.1683 >0.10     Yes
        50/50   0.2616 >0.10     Yes
      Control   Too few values to test.

Intermediate calculations. ANOVA table

      Source of            Degrees of   Sum of     Mean
      variation            freedom    squares    square
============================  ==========  ========
========
Treatments (between columns)          2  0.0001085 5.425E-05
Residuals (within columns)           16  0.0004513 2.821E-05
---------------------------  ----------  --------
Total                              18  0.0005598

F = 1.923  =(MStreatment/MSresidual)


                    Summary of Data

             Number                Standard
               of          Standard   Error of
    Group      Points     Mean   Deviation    Mean     Median
=============== ======   ======== =========
========  ========
```

```
      Exp      8     0.005622 0.004796    0.001696  0.004969
    50/50      7     0.006718 0.006953    0.002628  0.005928
  Control      8     0.00039480.0002664  0.0001332 0.0004975

                             95% Confidence Interval
     Group       Minimum  Maximum       From        To
=============== ======== ======== ==========
==========
      Exp     0.000  0.01465   0.001612   0.009632
    50/50     0.000  0.01598  0.0002876    0.01315
  Control     0.000 0.0005841-2.915E-05  0.0008187
```

Appendix 3.2: ANOVA results for MC-YR in *B. oleracea* after 20 day exposure

```
          One-way Analysis of Variance (ANOVA)

 The P value is < 0.0001, considered extremely significant.
 Variation among column means is significantly greater than expected
 by chance.


 Tukey-Kramer Multiple Comparisons Test
 If the value of q is greater than 3.628 then the P value is less
 than 0.05.


                     Mean
       Comparison         Difference    q      P value
==================================== ==========
======= ===========
       Exp vs 50/50         0.001646   4.570  *   P<0.05
       Exp vs Control        0.003894   8.825 *** P<0.001
      50/50 vs Control        0.002247   5.094  **  P<0.01

                     Mean    95% Confidence Interval
       Difference         Difference  From     To
==================================== ==========
======= =======
       Exp - 50/50          0.001646 0.00033940.002953
       Exp - Control         0.003894 0.0022930.005494
      50/50 - Control         0.002247 0.00064670.003848
```

Appendix 3.3: ANOVA results for MC-LR in *B. oleracea* after 20 day exposure

```
          Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.3371, considered not significant.
Variation among column medians is not significantly greater than
expected
by chance.

The P value is approximate (from chi-square distribution) because
at least one column has two or more identical values.

Calculation detail

             Number    Sum     Mean
               of       of      of
    Group       Points    Ranks    Ranks
=============== ======= ======= =======
          Exp        7   66.000    9.429
         50/50       7   86.000   12.286
       Control       5   38.000    7.600

Kruskal-Wallis Statistic KW = 2.175 (corrected for ties)

Post tests were not calculated because the P value was greater
than 0.05.
                    Summary of Data

             Number
               of
    Group       Points    Median  Minimum    Maximum
```

```
=============== ======   ======== ========
========
     Exp     7      0.000    0.000   0.008202
    50/50     7    0.001162 -8.875E-05 0.004631
   Control    5    2.191E-06   0.000   1.980E-05

           *     *     *
```

Appendix 3.4: Mann-Whitney Test results for MC-RR in *S. tuberosum* after 20 day exposure

Mann-Whitney Test

Do the medians of Exp and Control differ significantly?

The two-tailed P value is 0.8361, considered not significant.

The P value is an estimate based on a normal approximation.

The 'exact' method would not be exact, due to tied ranks.

Calculation details

Mann-Whitney U-statistic = 30.000

U' = 34.000

Sum of ranks in Exp = 70.000. Sum of ranks in Control = 66.000.

Summary of Data

Parameter:	Exp	Control
Mean:	0.001415	0.0001398
# of points:	8	8
Std deviation:	0.002621	0.0002588
Std error:	0.0009266	9.150E-05
Minimum:	0.000	0.000
Maximum:	0.005697	0.0005596
Median:	0.000	0.000
Lower 95% CI:	-0.0007760	-7.663E-05
Upper 95% CI:	0.003607	0.0003562

* * *

Appendix 3.5: Unpaired t test results for MC-YR in *S. tuberosum* after 20 day exposure

Unpaired t test

Do the means of Exp and Control differ significantly?

P value

The two-tailed P value is < 0.0001, considered extremely significant.

t = 8.363 with 14 degrees of freedom.

95% confidence interval

Mean difference = -0.008254 (Mean of Control minus mean of Exp)

The 95% confidence interval of the difference: -0.01037 to -0.006137

Assumption test: Are the standard deviations equal?

The t test assumes that the columns come from populations with equal SDs.

The following calculations test that assumption.

F = 283.79

The P value is < 0.0001.

This test suggests that the difference between the two SDs is extremely significant. Since the t test assumes populations with equal

SDs, you should consider transforming your data (reciprocal or log), selecting a nonparametric test, or selecting the alternate (Welch) t test.

Assumption test: Are the data sampled from Gaussian distributions?

The t test assumes that the data are sampled from populations that follow

Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

```
   Group       KS     P Value  Passed normality test?
=============== ======   ========
======================
          Exp   0.2526 >0.10     Yes
       Control  0.1766 >0.10     Yes

                      Summary of Data

    Parameter:            Exp        Control
        Mean:        0.008694      0.0004408
  # of points:              8              8
Std deviation:        0.002786      0.0001654
    Std error:     0.0009851      5.848E-05
      Minimum:        0.005988      0.0001591
      Maximum:         0.01398      0.0006963
       Median:      0.008058      0.0004635
 Lower 95% CI:        0.006365      0.0003025
 Upper 95% CI:         0.01102     0.0005791
```

* * *

Appendix 3.6: Mann-Whitney Test results for MC-YR in *S. tuberosum* after 20 day exposure

```
                  Mann-Whitney Test
Do the medians of Exp and Control differ significantly?

The two-tailed P value is 0.0003, considered extremely
significant.
The P value is exact.


Calculation details
Mann-Whitney U-statistic = 1.000
U' = 63.000
Sum of ranks in Exp = 99.000.  Sum of ranks in Control =
37.000.

                  Summary of Data

    Parameter:        Exp       Control
       Mean:       0.1355      0.01413
  # of points:          8            8
Std deviation:      0.09443     0.009997
    Std error:     0.03338     0.003535
      Minimum:      0.03083     0.006106
      Maximum:       0.3209      0.03818
       Median:     0.1299      0.01171
 Lower 95% CI:       0.05655     0.005768
 Upper 95% CI:        0.2145      0.02249

                  *    *    *
```

Appendix 5.1: Kruskal-Wallis Test results for MC-RR in *S. tuberosum* after 20 day exposure

```
          Kruskal-Wallis Test (Nonparametric ANOVA)

 The P value is 0.0793, considered not quite significant.
 Variation among column medians is not significantly greater than
 expected
 by chance.

 The P value is approximate (from chi-square distribution) because
 at least one column has two or more identical values.

 Calculation detail

             Number    Sum     Mean
               of       of      of
     Group      Points   Ranks   Ranks
 =============== ======= ======= =======
          T1        5   35.000    7.000
          T2        6   99.000   16.500
          T3        6   58.000    9.667
          T4        6   84.000   14.000

 Kruskal-Wallis Statistic KW = 6.778 (corrected for ties)

 Post tests were not calculated because the P value was greater
 than 0.05.
                    Summary of Data

             Number
               of
     Group      Points   Median  Minimum    Maximum
```

```
=============== ======   ======== ========
========
          T1    5      0.000    0.000   0.0006611
          T2    6    0.005677    0.000   0.006119
          T3    6    0.0006046   0.000   0.0007773
          T4    6    0.005629    0.000   0.005862
```

* * *

Appendix 5.2: Kruskal-Wallis Test results for MC-YR in *S. tuberosum* after 20 day exposure

```
        Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.0007, considered extremely significant.
Variation among column medians is significantly greater than
expected
by chance.

The P value is approximate (from chi-square distribution)
because
exact calculations would have taken too long.

Calculation detail

          Number    Sum     Mean
            of       of      of
   Group      Points   Ranks   Ranks
=============== ======= ======= =======
       T1       5   23.000    4.600
       T2       6   108.00   18.000
       T3       6   43.000    7.167
       T4       6   102.00   17.000

Kruskal-Wallis Statistic KW = 16.956

Dunn's Multiple Comparisons Test

                    Mean Rank
      Comparison          Difference   P value
==================================== ==========
===========
       T1 vs. T2           -13.400  **  P<0.01
```

```
     T1 vs. T3             -2.567  ns  P>0.05
     T1 vs. T4            -12.400  *   P<0.05
     T2 vs. T3             10.833  *   P<0.05
     T2 vs. T4              1.000  ns  P>0.05
     T3 vs. T4             -9.833  ns  P>0.05


              Summary of Data

          Number
            of
   Group    Points   Median  Minimum    Maximum
=============== ======   ======== ========
========
     T1    5    0.00050960.0003888  0.0007417
     T2    6    0.006337 0.003462    0.01059
     T3    6    0.00071510.0005379  0.002687
     T4    6    0.004643 0.003054   0.008006
```

* * *

Appendix 5.3: ANOVA Test results for MC-LR in *S. tuberosum* after 20 day exposure

```
          One-way Analysis of Variance (ANOVA)

The P value is 0.0013, considered very significant.
Variation among column means is significantly greater than expected
by chance.


Tukey-Kramer Multiple Comparisons Test
If the value of q is greater than 3.901 then the P value is less
than 0.05.


                             Mean
         Comparison          Difference    q      P value
==================================== ==========
======= ===========
          T1 vs T2            -0.1843   5.342  **  P<0.01
          T1 vs T3          -0.006667  0.1932  ns  P>0.05
          T1 vs T4            -0.1348   3.907  *   P<0.05
          T2 vs T3             0.1777   5.149  **  P<0.01
          T2 vs T4            0.04952   1.435  ns  P>0.05
          T3 vs T4            -0.1282   3.714  ns  P>0.05

                             Mean    95% Confidence Interval
         Difference          Difference  From     To
==================================== ==========
======= =======
          T1 - T2            -0.1843 -0.3190 -0.04973
          T1 - T3          -0.006667 -0.1413  0.1279
          T1 - T4            -0.1348 -0.2694 -0.0002110
          T2 - T3             0.1777 0.04306  0.3123
```

```
        T2 - T4             0.04952 -0.08510 0.1841
        T3 - T4             -0.1282 -0.2628 0.006456
```

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical
SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 22.798
The P value is < 0.0001.
Bartlett's test suggests that the differences among the SDs is extremely significant.
Since ANOVA assumes populations with equal SDs, you should consider
transforming your data (reciprocal or log) or selecting a nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow
Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

```
   Group       KS     P Value  Passed normality test?
=============== ======   ========
=======================
        T1   0.2969  0.0622   Yes
        T2   0.1695 >0.10     Yes
```

```
     T3   0.2829  0.0952   Yes
     T4   0.1791 >0.10     Yes

Intermediate calculations. ANOVA table

      Source of            Degrees of   Sum of     Mean
      variation            freedom    squares    square
============================  ==========  ========
========
Treatments (between columns)          3    0.1796   0.05988
Residuals (within columns)          24    0.2001  0.008336
----------------------------  ----------  --------
Total                               27    0.3797

F = 7.184  =(MStreatment/MSresidual)


                    Summary of Data

             Number                  Standard
               of            Standard   Error of
    Group      Points     Mean  Deviation    Mean     Median
=============== ======   ======== =========  ========
========
          T1     7     0.01604  0.02380   0.008997   0.01014
          T2     7      0.2004   0.1324    0.05003    0.1853
          T3     7     0.02271  0.02386   0.009017   0.01213
          T4     7      0.1509   0.1212    0.04580    0.1592

                           95% Confidence Interval
    Group      Minimum  Maximum     From        To
=============== ======== ======== ==========
==========
```

T1	0.000	0.06683	-0.005975	0.03806
T2	0.000	0.3496	0.07796	0.3228
T3	0.000	0.06805	0.0006436	0.04477
T4	0.000	0.3206	0.03879	0.2629

* * *

Appendix 5.4: Kruskal-Wallis Test results for MC-RR in *B. oleracea* after 5 day exposure

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.5427, considered not significant.
Variation among column medians is not significantly greater than
expected
by chance.

The P value is approximate (from chi-square distribution) because
at least one column has two or more identical values.

Calculation detail

```
              Number     Sum     Mean
                of       of       of
    Group      Points    Ranks    Ranks
===============  =======  =======  =======
           T1        4   42.500   10.625
           T2        6   86.500   14.417
           T3        6   60.500   10.083
           T4        7   86.500   12.357
```

Kruskal-Wallis Statistic KW = 2.146 (corrected for ties)

Post tests were not calculated because the P value was greater
than 0.05.

Summary of Data

```
             Number
               of
    Group     Points    Median  Minimum    Maximum
=============== ======    ======== ========
========
```

T1	4	0.000	0.000	0.002332
T2	6	0.005524	0.000	0.04253
T3	6	0.000	0.000	0.005304
T4	7	0.000	0.000	0.05730

* * *

Appendix 5.5: ANOVA Test results for MC-YR in *B. oleracea* after 5 day exposure

One-way Analysis of Variance (ANOVA)

The P value is < 0.0001, considered extremely significant.
Variation among column means is significantly greater than expected
by chance.

Tukey-Kramer Multiple Comparisons Test
If the value of q is greater than 4.020 then the P value is less
than 0.05.

```
                         Mean
         Comparison           Difference    q      P value
================================== ==========
======= ===========
          T1 vs T2            -0.005195  23.255 *** P<0.001
          T1 vs T3           -1.283E-05 0.05917  ns  P>0.05
          T1 vs T4            -0.004882  19.949 *** P<0.001
          T2 vs T3             0.005183  26.915 *** P<0.001
          T2 vs T4            0.0003133   1.402  ns  P>0.05
          T3 vs T4            -0.004869  22.446 *** P<0.001

                         Mean    95% Confidence Interval
         Difference           Difference  From     To
================================== ==========
======= =======
          T1 - T2            -0.005195 -0.006094-0.004297
          T1 - T3           -1.283E-05 -0.00088490.0008592
          T1 - T4            -0.004882 -0.005866-0.003898
          T2 - T3             0.005183 0.0044090.005957
```

```
          T2 - T4           0.0003133 -0.00058480.001211
          T3 - T4           -0.004869 -0.005741-0.003997
```

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 21.846
The P value is < 0.0001.
Bartlett's test suggests that the differences among the SDs is extremely significant.
Since ANOVA assumes populations with equal SDs, you should consider transforming your data (reciprocal or log) or selecting a nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

```
    Group       KS     P Value  Passed normality test?
=============== ======   ========
=======================
          T1   Too few values to test.
          T2   0.2378 >0.10     Yes
```

```
     T3   0.1959 >0.10     Yes
     T4   Too few values to test.

Intermediate calculations. ANOVA table

      Source of           Degrees of   Sum of     Mean
      variation           freedom    squares    square
===========================  ==========  ========
========
Treatments (between columns)          3  0.0001345 4.482E-05
Residuals (within columns)          17  4.073E-06 2.396E-07
---------------------------  ----------  --------
Total                              20  0.0001385

F = 187.07  =(MStreatment/MSresidual)


                    Summary of Data

             Number                  Standard
               of           Standard   Error of
    Group      Points     Mean   Deviation    Mean     Median
=============== ======   ======== =========  ========
========
          T1     4    0.00059030.0001160  5.802E-05 0.0006170
          T2     6    0.005786 0.0007116  0.0002905 0.005988
          T3     7    0.00060328.197E-05  3.098E-05 0.0005910
          T4     4    0.005473 0.0006977  0.0003488 0.005658

                           95% Confidence Interval
    Group      Minimum  Maximum    From       To
=============== ======== ======== ==========
==========
```

T1	0.0004373	0.0006901	0.0004057	0.0007750
T2	0.004649	0.006482	0.005039	0.006533
T3	0.0004786	0.0007423	0.0005274	0.0006790
T4	0.004536	0.006038	0.004363	0.006582

* * *

Appendix 5.6: ANOVA Test results for MC-LR in *B. oleracea* after 5 day exposure

```
          One-way Analysis of Variance (ANOVA)

 The P value is < 0.0001, considered extremely significant.
 Variation among column means is significantly greater than expected
 by chance.


 Tukey-Kramer Multiple Comparisons Test
 If the value of q is greater than 3.958 then the P value is less
 than 0.05.


                         Mean
        Comparison          Difference    q      P value
 =================================== ==========
 ======= ===========
         T1 vs T2           -0.003260   8.165 *** P<0.001
         T1 vs T3          -1.521E-05 0.03940  ns  P>0.05
         T1 vs T4           -0.001065   2.668  ns  P>0.05
         T2 vs T3            0.003244   8.845 *** P<0.001
         T2 vs T4            0.002195   5.765  **  P<0.01
         T3 vs T4           -0.001050   2.862  ns  P>0.05

                         Mean    95% Confidence Interval
        Difference          Difference  From     To
 =================================== ==========
 ======= =======
         T1 - T2           -0.003260 -0.004840-0.001680
         T1 - T3          -1.521E-05 -0.0015430.001513
         T1 - T4           -0.001065 -0.0026450.0005151
         T2 - T3            0.003244 0.0017930.004696
```

```
          T2 - T4             0.002195 0.00068800.003701
          T3 - T4            -0.001050 -0.0025020.0004019
```

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with
identical
SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 69.209
The P value is < 0.0001.
Bartlett's test suggests that the differences among the SDs is
extremely significant.
Since ANOVA assumes populations with equal SDs, you should
consider
transforming your data (reciprocal or log) or selecting a
nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that
follow
Gaussian distributions. This assumption is tested using the method
Kolmogorov and Smirnov:

```
    Group       KS     P Value  Passed normality test?
=============== ======   ========
=======================
          T1   0.3267  0.0869   Yes
          T2   0.1641 >0.10     Yes
```

```
          T3   0.1820 >0.10     Yes
          T4   0.1774 >0.10     Yes

Intermediate calculations. ANOVA table

      Source of           Degrees of   Sum of     Mean
      variation           freedom    squares    square
============================  ==========  ========
========
Treatments (between columns)          3  4.228E-05 1.409E-05
Residuals (within columns)          20  1.739E-05 8.694E-07
----------------------------  ----------  --------
Total                             23  5.966E-05

F = 16.209  =(MStreatment/MSresidual)


                   Summary of Data

             Number                 Standard
               of            Standard   Error of
    Group      Points      Mean  Deviation    Mean      Median
=============== ======   ======== =========  ========
========
          T1     5    1.140E-051.526E-05  6.824E-06 2.209E-06
          T2     6    0.003271 0.001531   0.0006250 0.003321
          T3     7    2.662E-051.640E-05  6.198E-06 2.653E-05
          T4     6    0.001076 0.001064   0.0004346 0.0009624

                          95% Confidence Interval
    Group      Minimum  Maximum     From        To
=============== ======== ======== ==========
==========
```

```
T1    0.000 3.350E-05-7.536E-06  3.035E-05
T2 0.001180 0.005226   0.001664   0.004878
T3    0.000 4.995E-05 1.145E-05  4.179E-05
T4    0.000 0.002707 -4.075E-05   0.002194
```

* * *

Appendix 5.7: Kruskal-Wallis Test results for MC-RR in *B. oleracea* after 20 day exposure

```
        Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.0143, considered significant.
Variation among column medians is significantly greater than
expected
by chance.

The P value is approximate (from chi-square distribution)
because
at least one column has two or more identical values.

Calculation detail

           Number   Sum    Mean
             of      of     of
   Group    Points   Ranks   Ranks
=============== ======= ======= =======
       T1       5   42.000    8.400
       T2       4   75.000   18.750
       T3       7   57.000    8.143
       T4       6   79.000   13.167

Kruskal-Wallis Statistic KW = 10.563 (corrected for ties)

Dunn's Multiple Comparisons Test

                       Mean Rank
        Comparison          Difference   P value
=================================== ==========
===========
       T1 vs. T2            -10.350  *   P<0.05
       T1 vs. T3             0.2571  ns  P>0.05
```

```
          T1 vs. T4             -4.767  ns  P>0.05
          T2 vs. T3             10.607  *   P<0.05
          T2 vs. T4              5.583  ns  P>0.05
          T3 vs. T4             -5.024  ns  P>0.05



                    Summary of Data

              Number
                of
    Group      Points    Median  Minimum    Maximum
=============== ======   ======== ========
========
          T1     5      0.000    0.000   0.0007758
          T2     4     0.07183  0.02593     0.1661
          T3     7      0.000    0.000   0.004783
          T4     6     0.01746    0.000    0.09085

                  *     *     *
```

Appendix 5.8: ANOVA Test results for MC-YR in *B. oleracea* after 20 day exposure

```
          One-way Analysis of Variance (ANOVA)

 The P value is < 0.0001, considered extremely significant.
 Variation among column means is significantly greater than expected
 by chance.


 Tukey-Kramer Multiple Comparisons Test
 If the value of q is greater than 3.997 then the P value is less
 than 0.05.


                          Mean
         Comparison            Difference    q      P value
 ===================================== ==========
 ======= ===========
          T1 vs T2            -0.006308  25.262 *** P<0.001
          T1 vs T3            0.0001089  0.5036  ns  P>0.05
          T1 vs T4            -0.004504  19.757 *** P<0.001
          T2 vs T3             0.006417  29.673 *** P<0.001
          T2 vs T4             0.001804   7.915 *** P<0.001
          T3 vs T4            -0.004612  24.185 *** P<0.001

                          Mean    95% Confidence Interval
         Difference           Difference  From      To
 ===================================== ==========
 ======= =======
          T1 - T2            -0.006308 -0.007306-0.005310
          T1 - T3            0.0001089 -0.00075540.0009732
          T1 - T4            -0.004504 -0.005415-0.003592
          T2 - T3             0.006417 0.0055520.007281
```

```
        T2 - T4             0.001804 0.00089320.002715
        T3 - T4            -0.004612 -0.005375-0.003850
```

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical
SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 34.652
The P value is < 0.0001.
Bartlett's test suggests that the differences among the SDs is extremely significant.
Since ANOVA assumes populations with equal SDs, you should consider
transforming your data (reciprocal or log) or selecting a nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow
Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

```
    Group       KS     P Value  Passed normality test?
=============== ======   ========
======================
        T1   Too few values to test.
        T2   Too few values to test.
```

```
          T3   0.2470 >0.10     Yes
          T4   0.1823 >0.10     Yes

Intermediate calculations. ANOVA table

      Source of            Degrees of   Sum of     Mean
      variation            freedom    squares    square
===========================  ==========  ========
========
Treatments (between columns)          3  0.0001609 5.365E-05
Residuals (within columns)          18  4.489E-06 2.494E-07
----------------------------  ----------  --------
Total                              21  0.0001654

F = 215.10  =(MStreatment/MSresidual)


                    Summary of Data

            Number                 Standard
              of           Standard   Error of
    Group      Points     Mean  Deviation    Mean     Median
=============== ======   ======== =========  ========
========
          T1     4    0.00063010.0001164  5.818E-05 0.0006603
          T2     4    0.006938 0.0005466  0.0002733 0.007035
          T3     8    0.00052123.999E-05  1.414E-05 0.0005254
          T4     6    0.005134 0.0008415  0.0003436 0.005081

                           95% Confidence Interval
    Group      Minimum  Maximum     From       To
=============== ======== ======== ==========
==========
```

T1	0.0004639	0.0007358	0.0004450	0.0008152
T2	0.006217	0.007464	0.006068	0.007808
T3	0.0004481	0.0005744	0.0004877	0.0005546
T4	0.004185	0.006321	0.004250	0.006017

* * *

Appendix 5.9: ANOVA Test results for MC-LR in *B. oleracea* after 20 day exposure

```
          One-way Analysis of Variance (ANOVA)

The P value is < 0.0001, considered extremely significant.
Variation among column means is significantly greater than expected
by chance.


Tukey-Kramer Multiple Comparisons Test
If the value of q is greater than 4.199 then the P value is less
than 0.05.


                        Mean
       Comparison          Difference    q      P value
================================== ==========
======= ===========
        T1 vs T2          -0.005719   9.794 *** P<0.001
        T1 vs T3          7.598E-06 0.01301  ns  P>0.05
        T1 vs T4          -0.002510   4.298  *   P<0.05
        T2 vs T3           0.005726   9.807 *** P<0.001
        T2 vs T4           0.003209   5.496  *   P<0.05
        T3 vs T4          -0.002517   4.311  *   P<0.05

                        Mean    95% Confidence Interval
       Difference          Difference  From     To
================================== ==========
======= =======
        T1 - T2          -0.005719 -0.008171-0.003267
        T1 - T3          7.598E-06 -0.0024440.002459
        T1 - T4          -0.002510 -0.004962-5.786E-05
        T2 - T3           0.005726 0.0032750.008178
```

```
T2 - T4          0.003209 0.00075730.005661
T3 - T4         -0.002517 -0.004969-6.546E-05
```

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 41.444
The P value is < 0.0001.
Bartlett's test suggests that the differences among the SDs is extremely significant.
Since ANOVA assumes populations with equal SDs, you should consider transforming your data (reciprocal or log) or selecting a nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

```
   Group      KS    P Value  Passed normality test?
=============== ======   ========
=======================
         T1   Too few values to test.
         T2   Too few values to test.
```

```
          T3   Too few values to test.
          T4   Too few values to test.

Intermediate calculations. ANOVA table

      Source of          Degrees of   Sum of     Mean
      variation          freedom    squares    square
============================  ==========  ========
========
Treatments (between columns)         3  8.843E-05 2.948E-05
Residuals (within columns)         12  1.637E-05 1.363E-06
----------------------------  ----------  --------
Total                            15  0.0001048

F = 21.614  =(MStreatment/MSresidual)


                   Summary of Data

            Number                Standard
              of         Standard   Error of
    Group     Points     Mean   Deviation    Mean     Median
=============== ======   ======== =========  ========
========
          T1     4    2.172E-051.728E-05  8.639E-06 2.255E-05
          T2     4    0.005741 0.001303   0.0006513 0.005741
          T3     4    1.412E-051.634E-05  8.170E-06 1.047E-05
          T4     4    0.002531 0.001939   0.0009693 0.002527

                          95% Confidence Interval
    Group     Minimum  Maximum     From       To
=============== ======== ======== ==========
==========
```

```
T1    0.000 4.179E-05-5.767E-06  4.921E-05
T2 0.004229 0.007252   0.003668   0.007813
T3    0.000 3.556E-05-1.187E-05  4.012E-05
T4 0.00019150.004881 -0.0005527   0.005616
```

* * *