BIOACCUMULATION OF CYANOTOXINS ON TERRESTRIAL FOOD PLANTS AND THE DEVELOPMENT OF A NOVEL SORBENT FOR MONITORING CYANOTOXINS IN IRRIGATION WATER

Report to the

WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

BACKGROUND

South Africa is known as a water-scarce country, thus management of this key resource is of paramount importance. As is the case in other parts of the world, eutrophication of freshwater resources is a serious problem in South Africa. 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 (Meneely & Elliott, 2013; Machado *et al.*, 2017). Among the impacts of eutrophication is the increased growth and dominance of cyanobacteria which produce metabolites posing a threat to aquatic ecosystems, animals and human health (Matthews *et al.*, 2010).

Many cyanobacterial species produce toxins and there are various types of cyanotoxins that pose a risk to public health, including nodularins, saxitoxins, cylindrospermopsin and microcystins. The most common cyanotoxins are microcystins (MCs) of which there are almost 100 different variants (Qi *et al.*, 2015). 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 1.0 μg L^{-1} for microcystin-LR (MCLR) in drinking water (Meneely & Elliott, 2013) and a tolerable daily intake (TDI) of 0.04 μg MC-LR kg^{-1} body weight (bw) in food (Miller & Russell, 2017). Recently, several studies have reported the toxic effects of MCs and cylindrospermopsin (CYN) on terrestrial plants including plants used for food. Ever since, the use of surface water contaminated with cyanobacteria and cyanotoxins for agricultural purposes has been receiving growing attention (Lee *et al.*, 2017a).

PROJECT SCOPE

The problems of eutrophication and cyanobacteria in South African reservoirs are well known and well documented (Newcombe, 2009; Matthews *et al.*, 2010; Department of Water Affairs, 2011; Turton, 2016). Levels of MCs in the range between 10 000 and 18 000 μg L^{-1} have been reported in South African reservoirs and rivers (Turton, 2016). These levels are way above the 1.0 μg L^{-1} recommended by the WHO in drinking water.

In light of the above, the project studied the bioavailability, toxicokinetics and effects of a range of cyanobacterial metabolites on terrestrial food plants and developed and evaluated a crosslinked chitosan (sorbent) passive sampler which was applied in the monitoring of the bioavailability of cyanotoxins in water intended for irrigation. This is particularly relevant since multiple classes of these metabolites are now being simultaneously detected in water bodies.

RESULTS AND DISCUSSION

The findings are 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) Occurrence of cyanobacteria, cyanotoxins, toxic metals and anionic surfactants in irrigation water and agricultural soils in the crocodile West & Marico Water Management Area, potential risk of transfer of cyanotoxins into food plants, human and health risks associated with consuming contaminated plants; 3) Bioaccumulation and elimination (toxicokinetics) of cyanotoxins by plants; 4) Accumulation of cyanotoxins and toxic metals in the presence of las on *Brassica oleracea* and *Solanum tuberosum;* 5) Development & application of a crosslinked chitosan-based Solid Phase Adsorption Toxin Tracking Technology (SPATT) adsorbent; 6) Field application of a crosslinked chitosan-based Solid Phase Adsorption Toxin Tracking Technology (SPATT) adsorbent.

The first section aimed at providing detailed literature on the impacts of cyanotoxins on food plants with emphasis on the South African context, the impact of multiple stressors, monitoring tools for cyanotoxins in irrigation water and application of the SPATT technology for monitoring irrigation water. The reviewed literature 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 as a result of poorly managed and -run municipal wastewater treatment plants, industrial activities and the changing climate, which seems to promote harmful cyanobacterial blooms.

With the prevailing eutrophication levels in the country and lack of monitoring and legislation to govern the use of such water for agricultural purposes, there could be an inherent chronic threat to human health as a result of the exposure of low levels of cyanotoxins via food crops irrigated with cyanotoxin-contaminated water. South Africa also lacks research on human exposure to cyanotoxins via irrigated crops and regulations to manage cyanotoxins 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.

The reviewed literature also showed many studies have been conducted to investigate the potential risk of transfer of cyanotoxins from irrigation water into edible parts of plants, but none of these have been conducted in South Africa. Considering the rising prevalence of harmful cyanobacterial blooms in South African waters, there is an urgent need to conduct such studies under local conditions to investigate the extent of this potential route of exposure to humans. Also, much of the work performed in this field has been conducted hydroponically and there is need for field-based data from experiments.

The second section aimed at investigating the occurrence of cyanotoxins, anionic surfactants and toxic metals in irrigation water, agricultural soils and plants to assess the human health risks associated with consuming cyanotoxins contaminated plants. This section also evaluated the applicability of a passive sampling technology (SPATT) to monitor and detect cyanotoxins using the DIAON HP20 resin as an adsorbent in the study area. Four field surveys were conducted, in June 2019, September 2019, February 2020 and March 2021. Parameters monitored included physicochemical parameters (pH, EC, TDS, DO, Turbidity, and Temperature), nutrients (nitrates and phosphates), metal elements (in water and soils), cyanobacterial biomass (as chlorophyll-a), microcystins (MCs) and anionic surfactants. Data analysis was done using Microsoft Excel (2013 version), IBM SPSS Statistics 26 and Instat Graphpad. Data analysis included univariate analysis, which included calculation of the mean, standard deviation and generation of tables and bar charts and bivariate analysis, which mainly involved the Pearson and Spearman correlation to test for the correlation between MCs, chlorophyll-a and other parameters monitored.

Our field sampling revealed the presence of cyanotoxins, metals and anionic surfactants in irrigation water (canals and farm dams), and agricultural soils in all the sampled sites during the sampling period. It also emerged that pH, turbidity, EC, and TDS have a correlation with levels of MCs in the irrigation water, thus suggesting that these could be used to predict MCs levels/presence in the irrigation water from the two dams. Findings also demonstrated the applicability of SPATT using the resin DION HP20 for the passive sampling of MCs in the irrigation water as it increased the likelihood of detecting MCs in instances where grab samples would miss or detect very low levels of the toxins. This is of significance, since grab sampling could miss some episodic HAB events, thus SPATT could be used to complement grab sampling and give early warnings in water intended for irrigation purposes. Among toxic cyanobacterial genus identified in the irrigation water from the two dams were *Microcystis,*

Anabaena and *Oscillatoria*, with *Microcystis* being the most dominant throughout the sampling sites.

The study also found that metals in irrigation water were below the DWAF (1996) recommended threshold while in agricultural soils, metals like Cr, Ni, Cu, Pb, and As were above the guideline values set for agricultural soils. The findings also showed that MCs and metals do accumulate in food crops when irrigated with contaminated water. The calculated estimated daily intake (EDI) for MCs in the collected food plants was below the WHO guideline threshold of 0.04 $\mu g\ kg^{-1}$. Thus, plants being irrigated by water from these two dams are still safe for human consumption. Among the metals of concern in the food plants, Cr, Fe, Cu, Zn, As, and Pb were found above the EU and FAO/WHO threshold for these metals in food crops. However, the calculated EDI for each of the metals detected were below the maximum tolerable daily intake (MTDI), thus implying that the plants being irrigated by water from the two dams are still safe for human consumption.

The presence of anionic surfactants, metals species and cyanotoxins in irrigation water and agricultural soils in the study area as reported in the study is of concern since the risk of indirect exposure of humans and animals via consumption of contaminated plants remains high. This is because anionic surfactants are known to promote the uptake of cyanotoxins and metals and thus increase the risk to humans.

The third section looked at the accumulation and elimination capacities of MCs in different parts of the plants *Brassica oleracea* and *Solanum tuberosum*. To investigate cyanotoxins uptake, accumulation capacities in edible parts of the plants in 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 experiments on accumulation capacity were determined in order to explain accumulation of cyanotoxins when plants are irrigated with water from the two dams and assess the human health risks associated with using such water for irrigating food crops.

Data gathered in this section was expected to contribute to further understanding of accumulation of different MCs congeners on *Brassica oleracea* and *Solanum tuberosum*.

Water used to irrigate the plants 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.

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 than the South African guideline and FAO limits for irrigation water. Findings indicated that the raw dam water did not have any effect on the germination of potato seeds, but severe effects were found on the germination of cabbage seeds, with 84.3% successful germination in the control trays and only 12.7% successful germination in the trial's trays. Such findings were in inconsistent with previous studies which have reported negative effects of cyanotoxins on seed germination and development and also highlight the possible impacts of irrigating crops with such water. The findings from this section also demonstrated that the two plants can bio-accumulate 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. Our findings, together with findings in other studies discussed here, demonstrate terrestrial food crops can accumulate cyanotoxins to levels that can pose human-health risks when exposed to naturally relevant levels of cyanotoxins.

The fourth part investigated the uptake and the accumulation of MCs and metal species in different parts of the plants *Brassica oleracea* and *Solanum tuberosum* in the presence of the anionic surfactant LAS.

To investigate if cyanotoxins and metal species uptake and accumulation in different parts of the plants is affected by the presence of LAS, pot-culture experiments with the two plant species were conducted. The two plant species were selected for the field experiment as they are commonly cultivated in the study area. The results obtained from the experiments on accumulation capacity of plants from the pot-culture experiments were determined in order to explain accumulation of cyanotoxins and metals when plants are irrigated with water from hypertrophic reservoirs which are also likely to be contaminated with LAS and other anionic surfactants. Data gathered in this section was expected to contribute to further understanding of uptake and accumulation of MCs in the presence of other pollutants such as LAS and metal species. This is of importance, since terrestrial food plants can be exposed to numerous anthropogenic pollutants and other stressors such as linear alkylbenzene sulfonate (LAS) and toxic metal species, which may enhance cyanotoxins accumulation from the surrounding environment after irrigation.

The findings indicated that the dam water which was used to irrigate the plants was alkaline, with a pH of 9.02, had high EC and TDS levels (228 mg L^{-1} and 380 µs cm^{-1} respectively), high cyanobacterial biomass (Chlorophyll-a 440.24±328.147 µg L^{-1}) and contained a significant load of anionic surfactants (1.64±0.163 mg L^{-1}). Water used to irrigate the plants 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. Based on the findings, the presence of anionic surfactants did not induce 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 different 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 here. 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 high 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 which 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 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 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 fifth part looked at synthesising a crosslinked chitosan sorbent that could be used in SPATT format to passively sample cyanotoxins. For the synthesis of the in-house adsorbent for potential use as an alternative to the commercial resins, chitosan was crosslinked with glutaraldehyde to form a chitosan-glutaraldehyde crosslinked copolymer, which was then further modified with the addition of multi-walled carbon nanotubes (CNTs). The synthesised crosslinked chitosan (ChGLA) and the ChMWCNTs sorbents were then characterized by BET, SEM-EDX and FTIR-ATR. Data gathered in this section is expected to contribute to further

understanding of the applicability of the SPATT technology for the monitoring and use as an early warning sign for the presence of microcystins in irrigation water and characterise the physicochemical properties of the modified/crosslinked chitosan sorbents for their potential/ability to adsorb a range of MCs and to determine the potential applicability of SPATT for passive sampling MCs in irrigation water.

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 also 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 compared to the HP20 resin, suggested better capacities for the ChMWCNT to adsorb MCs.

Both the glutaraldehyde crosslinked chitosan hydrogel (ChGLA) and the chitosan-multi-walled CNT (ChMWCNT) composite were applied for the adsorption and desorption of MCs. The findings indicate that both adsorbents are good sorbents for MC-LR and their adsorption capacities are much better compared to the commonly used aromatic resin HP20. The desorption efficiencies of the two synthesized chitosan sorbents were also much better compared to that of the HP20 resin, making them ideal candidates for application in the SPATT bag format for the passive sampling of MCs. Since chitosan can easily be made by deacetylation of chitin which occurs in abundance as a by-product in the food industry from crab and shrimp shells, this could be a cheaper and readily available alternative to the commercial resins currently applied in SPATT samplers.

The last part evaluated the developed chitosan-based sorbents, application as passive samplers in SPATT format in the study area. To investigate the applicability of a chitosan-based biopolymer as a sorbent to be used for the adsorption of MCs in SPATT bag format in the field, a glutaraldehyde crosslinked chitosan hydrogel (ChGLA) and a multi-walled carbon nanotube composite of the ChGLA (ChMWCNT) was synthesised. The two newly synthesized materials were packed in a SPATT bag format (in nylon cloth, 55 mm * 55 mm) and applied in a laboratory trial to determine the time required for the adsorbent to be saturated then later deployed in the field. The ChGLA and ChMWCNT sorbents were deployed in the field

together with the commonly used commercial DION HP20 resin. Grab samples were also collected upon deployment and retrieval of the SPATT samplers and physicochemical parameters such as pH, EC, DO, salinity, chlorophyll-a, temperature, nutrients, turbidity and TDS were also monitored.

Data gathered in this section was expected to contribute to further understanding of the applicability of the SPATT technology for the monitoring and use as an early warning sign for the presence of microcystins in irrigation water. The study also aimed to synthesise and assess the physicochemical properties of modified/crosslinked chitosan sorbents for their potential/ability to adsorb a range of MCs and to determine the potential applicability of SPATT for tracking MCs in irrigation water.

The findings showed a good correlation of the toxins detected by the 3 samplers compared to the grab samples, but strong positive correlation was recorded for grab samples vs the ChGLA samplers, whereas the ChMWCNT samplers showed a strong positive correlation with the HP20 samplers. Among physicochemical parameters monitored, there was a strong positive correlation between chlorophyll-*a* and MCs in grab samples and ChGLA. There was also a strong positive correlation between dissolved nitrate levels and toxin levels detected by HP20 and ChMWCNT.

Importantly, factors correlating to MC detection by HP20 also correlated to ChMWCNT and the physicochemical parameters such as conductivity, salinity and TDS had a correlation with the MC levels detected by ChMWCNT and HP20 in the field water. In addition, pH had a strong positive correlation with MC detection by ChGLA. Based on these findings, it was concluded that SPATT using the synthesized chitosan-based adsorbents has the potential to be integrated into current cyanobacterial monitoring programmes and would be a very useful and economical tool for early warning and monitoring of toxic cyanobacterial events in water intended for irrigation.

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 a very useful and economical tool for early warning and monitoring of toxic cyanobacterial events in water intended for irrigation.

INNOVATION

The use of chitosan (either on its own, in combination or modified) for MCs sorption has not been studied much, especially for passive sampling in SPATT format. In this study, multi-

walled carbon nanotubes were inserted into the crosslinked chitosan network and this had 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 to form a chitosan-multiwalled carbon nanotubes (ChMWCNT) composite with improved MC adsorption and desorption properties. The composite was applied in the passive sampling of MCs in SPATT bag format and also has potential to be applied in the solid phase extraction of MCs in water and other sample matrix. The hydroxyl groups (-OH), amine group ($-NH_2$) and the sulfhydryl group (-SH) groups in chitosan seemed to favour MC adsorption onto the ChMWCNT composite. The synthesised novel materials have the potential to be commercialized and will need to be protected by registration of a patent.

RECOMMENDATIONS

Based on the findings of the literature survey, it is recommended that further studies be conducted to provide policy-makers with local evidence-based data to guide the process of policy formulation with regards to 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 prioritizes research addressing issues specific to cyanotoxins in irrigation water and development of local guidelines/regulations for cyanotoxins in agricultural water.

Findings of the field survey indicated the presence of anionic surfactants, metals contaminants and cyanotoxins in irrigation water and agricultural soils in the study area and the risk of indirect exposure to humans and animals via consumption of contaminated plants. It is thus recommended that these pollutants be regularly monitored and managed in light of the synergic impacts of these pollutant on plants and eventually in human. This considering the prevailing poor quality of sewage effluent being discharged into the catchment and the impact of the mining and industrial activities taking place in and around the area.

The pot culture experiments showed that the existing levels of anionic surfactants, metal contaminants and microcystins being found in hyper-eutrophicated reservoirs 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.

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 carried to further validate the findings and the impact of various physicochemical parameters on the performance of the samplers with the synthesized material.

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 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 and using a different method to detect and quantify the toxins are recommended, since the ELISA method does not differentiate the different congeners of MCs and is affected by a host of other external factors. The use of techniques such as LCMS will give valuable information on the effectiveness of the synthesized material in adsorbing and desorption of the different congeners of MCs and other cyanotoxins commonly 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 SPAT 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.

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List of abbreviations

ARC	Agricultural Research Council
BET	Brunauer Emmett and Teller
ChGLA	Gluteraldehyde crosslinked chitosan
ChMWCNT	glutaraldehyde crosslinked chitosan combined with multi-walled carbon nanotubes
CYN	Cylindrospermopsin
DAFF	Department Agriculture, Forestry and Fisheries
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
EC	Electrical conductivity
ED	Electrodialysis
EDI	estimated daily intake
FAO	Food and Agriculture Organization (United Nations, Rome, Italy)
FTIR	Fourier transform infrared
HP20	a unique rigid polystyrene/divinylbenzene matrix
ICP-MS	Inductively coupled plasma-Mass spectroscopy
LAS	Linear alkylbenzene sulfonate
MCs	Microcystins
MTDI	Maximum Tolerable Daily Intake
NOD	Nodularins
SA	South Africa
SEM-EDS	Scanning electron microscopy-Electron dispersive spectroscopy
SPATT	Solid Phase Adsorption Toxin Tracking
TDI	Tolerable Daily Intake
TDS	Total dissolved solids
TGA	Thermogravimetric analysis
WHO	World Health Organization
WMA	Water Management Area
WRC	Water Research Commission

List of Symbols & Units

$\mu g\ L^{-1}$	:	micrograms per litre
$mg\ L^{-1}$	:	milligrams per litre
$mg\ kg^{-1}$	:	milligrams per kilogram
$\mu g\ kg^{-1}$	:	micrograms per kilogram
$\mu S\ cm^{-1}$	:	micro Siemens per centimetre
°C	:	degrees Celsius

CHAPTER 1: INTRODUCTION

This chapter was prepared by Glynn K. Pindihama & Gitari W. Mugera

1.1 BACKGROUND

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).

Irrigation of edible plants with cyanobacteria-containing water may pose a threat of indirect exposure of human health to cyanotoxins via bioaccumulation of these toxins in plant tissues. Cyanotoxins have also been shown to inhibit plant growth and development (Purkayastha *et al.*, 2010). In South Africa, Turton (2016) has reported a possible human health risk when the food is grown on farms near dams and rivers polluted with cyanotoxins.

Besides all these concerns, cyanotoxins contaminant as microcystins (MCs) in water utilized to irrigate food crop plants have not yet been considered within any official monitoring program on water quality in South Africa. A survey of cyanobacterial water blooms carried out from 2000 to 2004 in South African reservoirs confirmed an average frequency of 95% toxicity in field samples tested by the ELISA method (Oberholster *et al.*, 2005) and yet no studies have been done on the impacts of cyanotoxins on agricultural crops locally.

On the other hand, the combined pollution of cyanotoxins, linear alkylbenzene sulfonate (LAS), an anionic surfactant and metal species (as is the case in the Crocodile (West) and Marico catchment) in eutrophic water bodies, has been reported to be common due to toxic cyanobacterial blooms and exogenous organic chemicals pollution (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 ecotoxicological risk of multiple classes of cyanotoxins in combination with other stressors such as LAS on terrestrial plants has not been investigated in South Africa.

Despite the predicted increase in incidents of harmful cyanobacterial blooms, strategies for monitoring and managing them in South Africa tend to be reactionary and there is still a lack of proactive 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 labour consuming clean-up prior to instrumental analyses. Further, cyanotoxin concentrations may vary over the time, and episodic peaks of high concentrations may be missed in the traditional monitoring scheme (Kohoutek *et al.*, 2010).

Cyanotoxins risk assessment and characterization in relation to human health requires the identification of common exposure routes, among which consumption of contaminated food has been recognized (M. do C. Bittencourt-Oliveira *et al.*, 2016). By understanding the fate of cyanobacterial cells and toxins during and after spray irrigation with water containing cyanobacteria and the development of an effective, reliable and efficient monitoring program, the current study will 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.

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). Drobac *et al.* (2013) also reiterates the need to improve sample preparation and detection methods for cyanotoxins, as well as the continuous monitoring of water and food products. The study is motivated by the need to fill in this gap by evaluating the threats posed by using eutrophicated waters for irrigating food crops and test passive sampling in the form of solid phase adsorption toxin tracking technology (SPATT) as a monitoring tool, based on the assumption that water-exposed passive samplers will better reflect the pollution situation in the water and also provide a better monitoring tool for the toxins. Findings of the study will thus be of importance to the water and agricultural sectors and will contribute to 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.

1.2 PROJECT AIM

The main aim of the project was to study the bioavailability, toxicokinetics and effects of a range of cyanobacterial metabolites on terrestrial food plants and to develop and evaluate the

use of crosslinked chitosan (sorbent) passive sampler to monitor the bioavailability of cyanotoxins in water intended for irrigation. This is particularly relevant since multiple classes of these metabolites are now being simultaneously detected in water bodies. The following were the specific aims of the project:

1. To conduct a detailed literature survey on impacts of cyanotoxins on food plants. Emphasis will be on the South African context, impact of multiple stressors, monitoring tools for cyanotoxins in irrigation water and application of the SPATT technology for monitoring irrigation water.
2. To investigate occurrence of cyanotoxins, anionic surfactants such as linear alkylbenzene sulfonate (LAS) and metals in irrigation water, agricultural soils and plants to assess the human health risks associated with consuming cyanotoxins contaminated plants.
3. Investigate the bioaccumulation of cyanotoxins in edible plant organs in cabbage (*Brassica oleracea*) and cultivated potato (*Solanum tuberosum*).
4. To investigate the impact of multiple stressors by examining the accumulation of cyanotoxins and toxic metals (Manganese, Aluminum & Strontium) in the presence of the anionic surfactant linear alkylbenzene sulfonate (LAS) in cabbage (*Brassica oleracea*) and cultivated potato (*Solanum tuberosum*).
5. To develop a crosslinked chitosan-based solid phase adsorption toxin tracking technology (SPATT) configuration suitable for monitoring bioavailable cyanotoxins in aquatic environments.
6. To evaluate the field application of the developed crosslinked chitosan-based resin in SPATT bags as a passive sampler for cyanotoxins in water intended for farming and irrigation.

1.3 SCOPE

The scope was limited to:

- The assessment of the bioavailability, toxicokinetics and effects of the three congeners of Microcystins (MC-LR, MC-RR and MC-YR) on terrestrial food plants and also developed and evaluated the use of a crosslinked chitosan (sorbent) passive sampler to monitor the bioavailability of cyanotoxins in water intended for irrigation.
- Use of Roodeplaat and Hartbeespoort dams as case studies since water from these dams is being used for irrigation and the dams are also hypertrophic.

- Use of commercially available seeds of cabbage (*Brassica oleracea*) and cultivated potato (*Solanum tuberosum*) and the plants were watered by field water from the two dams.
- Synthesis the chitosan based sorbent by crosslinking chitosan with glutaraldehyde then freeze drying and by adding Multiwalled Carbon Nanotubes (MWNCNTs) to the crosslinked chitosan.
- Use of commercially available resin DIAION® HP20 used in the SPATT bags for comparison purposes. The resin was purchased from Rochelle Chemicals, South Africa.
- Construction of the SPATT bags using nylon mesh (approximately 95-100 micron pore size). The mesh was purchased from Ecotao Enterprises (Stanger, South Africa).
- The anionic surfactants used for the experiments were done using commercially available Sodium Dodecyl Sulfate (SDS) salts which was purchased from BYMAZ (Pty) Ltd, South Africa.
- The SPATT bags were tested and evaluated in the laboratory using dam water from Roodeplaat and Hartbeespoort dams.
- The SPATT bags were deployed in canals channelling water to the farms and farm dams around Roodeplaat and Hartbeespoort dams.
- The evaluation of adsorption and desorption of microcystins only, and no other cyanotoxins due to the limited availability of analytical standards.

CHAPTER 2: IMPACTS OF CYANOTOXINS AND OTHER EUTROPHICATION-RELATED STRESSORS ON FOOD PLANTS AND APPLICATION OF SPATT FOR MONITORING IRRIGATION WATER: A LITERATURE SURVEY

This chapter was prepared by Glynn K. Pindihama & Mugera W. Gitari

2.1 INTRODUCTION

Many of 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 as result of rapid population growth, urbanization, industrial and agricultural activities (Lu, 2018). Rising usage of detergents, pharmaceuticals and other chemicals in homes and industries have led to high 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). Animals deaths as a result of cyanotoxins poisoning have also been reported for decades in the country (Harding, 2015).

According to Lu (2018), a lot of work assessing the levels and the impacts of eutrophication and the resulting HABs 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 thorough literature search was conducted to document the possibly existing long-term threat posed by the use of eutrophicated and cyanobacteria-infested waters for agricultural purposes in South Africa. In addition to cyanotoxins, the survey also looks 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 were used to inform this report. Literature was accessed through the 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 own structural analogues or 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, lipopolysaharide endotoxin), 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 also have different chemical structures, for instance, Anatoxin-a (ATX) and Cylindrospermopsin (CYN) are alkaloids, whereas Microcystins (MCs) and Nodularins (NOD) are polycyclopeptides (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 and 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, affects any exposed tissue

In South Africa, MCs 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 North West Province.

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). All in all, a total of 10 microcystin (MC) variants have been described from Hartbeespoort Dam in different studies: MC-RR, MC-LR, MC-FR, MC-YR, MC-LA, MC-YA, MC-LAba, 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 molluscs), 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.*, 2013).

Several studies have assessed the human health threats posed by the bioaccumulation of cyanotoxins (mainly MC-LR) in food plants. 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 the World Health Organization by a factor of two. The findings by Crush *et al.* (2008) indicated the potential for movement of microcystins into human and animal food chains via irrigation water. Crush *et al.* (2007) concluded that the implications for human health are unknown until the nature and toxicity of the immune-reactive components detected in the plants are determined.

There has 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 on human beings (Mashile & Nomngongo, 2017). Cases of fatalities in wildlife have been reported in South Africa, for example rhino fatalities due to cyanotoxin poisoning have been reported in the Kruger National Park (KNP), South Africa (Mashile & Nomngongo, 2017). Mass fatalities of Lesser Flamingos have also been reported in countries East African lakes, for example lake Bogora, 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, particularly microcystins are very common in the aquatic environments. They 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 disrupt the cyanobacterial cells by physical or chemical action (Metcalf & Codd, 2014; Babica, 2006; Li & Pan, 2015). However, active transport (via putative 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 a number of plant membrane transporters which are known to have an affinity for amino acids and peptides (Machado *et al.*, 2017a). Because 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 an important 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). There is no literature on the possible interactions of cyanotoxins with mycorrhizae, thus the need for research to fill this gap to fully understand the uptake of these toxins by plants.

Many studies have demonstrated that the accumulation of cyanotoxins in plants is dose dependent (Bouaïcha & Corbel, 2016; Lee *et al.*, 2017; Pham & Utsumi, 2018), 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 nor 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 than from rice, 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 the 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 have the ability to 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 agricultural crops (D'Anglada *et al.*, 2015) via oxidative stress and cell death (Lefebvre, 2013). Milligan (2009) documented data of 15 common food 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 environmentally relevant levels of MC-LR and accumulate the toxin. Meneely and Elliott (2013), also reported the uptake and accumulation of MCs in food plants having been irrigated by infested water in lettuce, rocket, dill, cabbage, runner beans, radish, rice, runner beans, parsley, soybean, pumpkin, sesame, mung bean and sweet potato.

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 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, it is clear that cyanotoxins and more so MCs have a negative impact on the productivity, yield and quality of food crops.

Based on the literature reviewed here, it is clear that plants can detoxify cyanotoxins and microcystins 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). Also based on available literature, a lot of work on effects of cyanotoxins on plants has been done with MCs and more so MC-LR than other cyanotoxins and other congeners of MCs. 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 cyanotoxins infested water does not necessarily always result to 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 (CLY) 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 CLY 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 & Corbel (2016) report relative 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 on 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). In fact, 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 like China could be posing threats to earthworms and other soil organisms.

Possible impacts of cyanotoxins on soil biota have not been getting much attention. However, Valdor and Aboal (2007) reported inhibitory effects of cyanobacterial extracts and purified MCs on the growth of cultured bacteria such as E. coli and Streptoverticillium sp. (Cao *et al.*, 2017). The inhibitory effects can be attributed to antibacterial substance released by cyanobacteria which alter the permeability of cell membranes and 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 such as earthworms, arthropods and molluscs 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 also play an important role in the study of cyanotoxin-mycorrhizae interactions and the possible effects of cyanotoxins on mycorrhizae and the possible uptake of nutrients and water by plants.

Lee *et al.* (2017b) reported MC persistence in agricultural soils long after harvesting, thus prompting the need 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 which 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. In fact, 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).

2.3.5 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 recommended by the WHO (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 recommended by the World Health Organization (WHO), assuming a body weight 60 kg and consumption of 150 or 40 g 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).

To date, more than 100 variants of MCs have been identified. However, the bioaccumulation of these 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 MCs 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.

Previous studies reported no MCs in milk or meat after administering toxic *M. aeroginosa* to cows through drinking water ((IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2010).

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 in particular 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 the 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 80 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).

However there were insufficient data to derive a guideline value for cyanotoxins other than microcystin-LR (Chorus & Bartram, 1999). The toxicity of this microcystin variant is representative of that of other variants and of nodularin, and therefore it is assumed to provide a reasonable

approximation of the toxicity of naturally occurring mixtures of these variants in water bodies. A number of countries have adopted similar guideline values for microcystins, some of which refer to the total concentration of microcystins present in water samples (du Preez & van Baalen, 2008). In South Africa, water quality is regulated by the South African National standard: Drinking Water (SANS 241, 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 (SANS 241, 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, 1996a). 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. Based on the scientific evidence of cyanotoxins accumulation in food crops, Meneely & Elliott (2013) stress the need for risk assessments to incorporate this exposure route and for regulatory authorities to initiate monitoring of cyanotoxin contamination of such foods.

In a country like 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) cite the lack of chronic toxicity data as a hurdle in improving cyanotoxin regulations via this route. In the absence of legislation, farmers are encouraged 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 of 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 has to be of acceptable quality and irrigating with poor 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 Sulfonates (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 (Z. 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, detergents, solubilizers and wetting agents in many pharmaceutical, personal care, home care and food products.

After use, LAS can enter into the aquatic environment (Z. 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 the 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 mal-functional 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 like 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 a number of 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 (Z. 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. In fact, Wang *et al.* (2012) reports that, the combined pollution of cyanotoxins and LAS in aquatic environments like eutrophic lakes is common due to toxic cyanobacterial blooms and exogenous organic chemicals pollution. According to Wang *et al.* (2012), the ecotoxicological risk of their combination in aquatic ecosystems is still unknown.

In another study, Wang *et al.* (2015) also demonstrated that low LAS (≤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 look into 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 also 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 as a result of human activities, especially mining. Metals such as mercury, beryllium, lead, cadmium, nickel and copper 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 alkylphenolethoxylates 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 the 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 as a result of a difference between chemical potentials of the analyte in the two media (Figure 4.1). Figure 4.1 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).

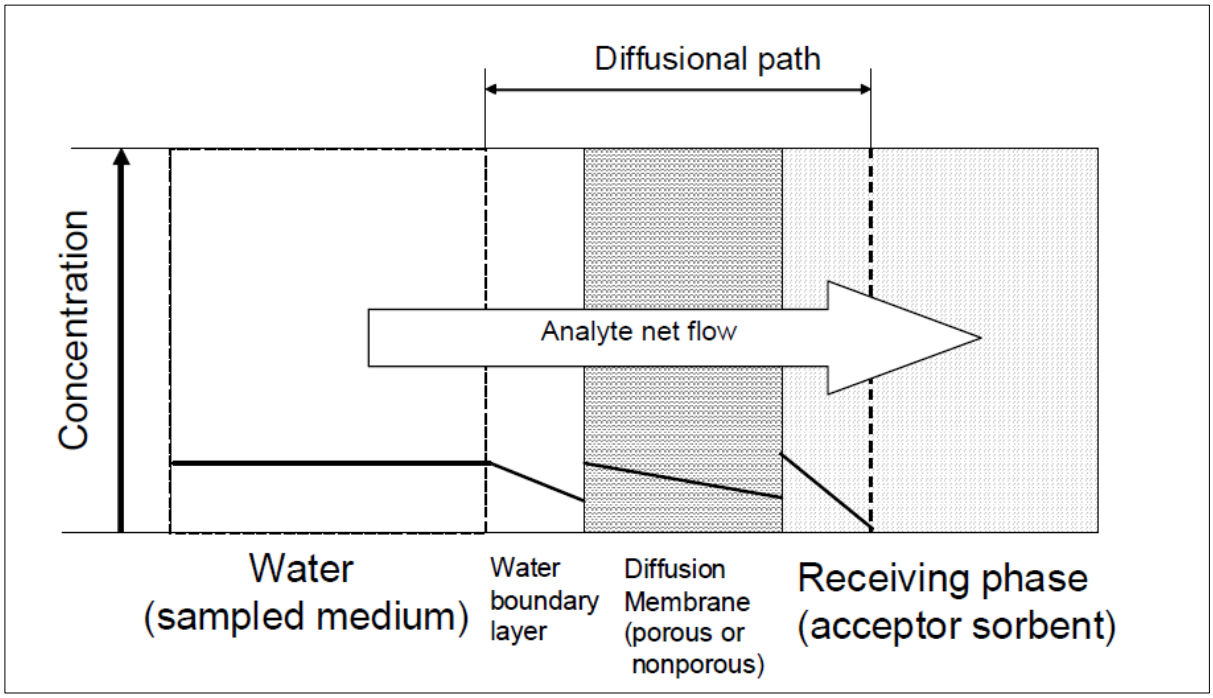

Figure 2.1. Functional principle of a passive sampling device (Adapted from Vrana *et al.*, 2010)

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).

$$NA = \frac{DS}{L}\ C \qquad [1]$$

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 4.2.

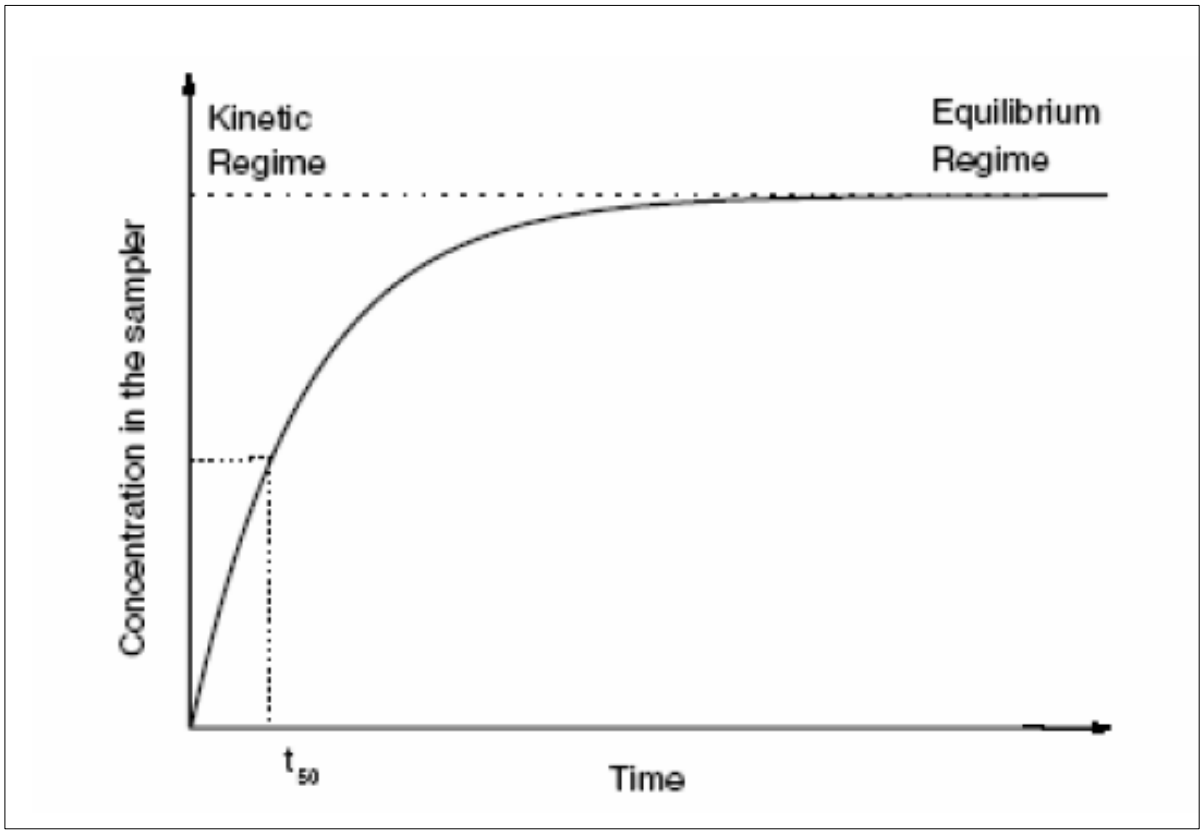

Figure 2.2. Uptake of analyte by passive sampling device (Kohoutek, 2010).

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:

$$Rs\frac{\mathrm{Dw}}{\mathrm{Lw}}\ A \qquad [2]$$

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:

$$Cw = \frac{\mathrm{CsMs}}{\mathrm{Rst}} \qquad [3]$$

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 has to be determined by performing calibration for particular sampling device. In theory, calibration in the kinetic regime requires the reproduction of conditions in the field. Therefore, mainly water-flow and also 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, 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 polystyrenedivinylbenzene 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 HP-20 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 HP-20 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 an important 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® HP-20, 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 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 HP-20 was found to be more appropriate for long 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 investigating 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 will be 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 will be evaluated.

2.7 SUMMARY

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 as a result of poorly managed and run municipal wastewater treatment plants, industrial activities and the changing climate, which seems to promote harmful cyanobacterial blooms.

Many studies have been conducted to investigate the potential risk of transfer of cyanotoxins from irrigation water into edible parts of plants, but to our knowledge none of these have been conducted in South Africa. Considering the rising prevalence of harmful cyanobacterial blooms in South African waters, there is an urgent need to conduct such studies under local conditions to investigate the extent of this potential route of exposure to humans.

Much of the work performed in this field was conducted hydroponically and field-based data from experiments carried out under real and natural conditions is still lacking. There is thus a need for studies conducted under natural conditions and in the South African context. This is very important because Corbel *et al.* (2014) reported different cyanotoxin adsorption properties exhibited by different soil types and differing biodegradation abilities of different soil bacteria.

To date, more than 100 variants of MCs have been identified and in South Africa, 10 congeners of MCs have been identified in the Hartbeespoort Dam only. However, the bioaccumulation of these congeners in plants has been poorly investigated. Under natural conditions, water used for irrigation may contain several MCs congeners, in addition to other cyanotoxins. Furthermore, cyanobacterial strains/species are known to produce more than one MC congener. This implies that crops irrigated with cyanobacterial and cyanotoxins-contaminated water may be exposed to more than one MCs congener per time. However, the simultaneous uptake and bioaccumulation of these congeners in vegetables and other crop plants is yet to be extensively studied. Studies on the simultaneous exposure and uptake of different MC congeners and other cyanotoxins, taking local conditions and cyanobacterial species into consideration will be of significant importance.

Based on the literature reviewed here, 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 major 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 gives is cheap, easy to use, sensitive and reliable for the monitoring of these toxins.

In addition to the above-mentioned research needs, there is also an urgent need for guidelines and policies on cyanotoxins in irrigation water, food plants and water used for livestock. These can only be guided by local evidence-based research and findings. Such research will need to look into factors such as the prevalent cyanotoxins, the country/region, climate, type of 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 (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2010). Such data cannot be gathered without government support through adequate resource allocation, training of personnel, investment into analytical and monitoring equipment for various cyanotoxins and prioritization of research in the subject.

With a huge burden of other diseases such as HIV and AIDs, Tuberculosis and diarrheal-related diseases, the South African population could be at high 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.

CHAPTER 3: OCCURRENCE OF CYANOBACTERIA, CYANOTOXINS, TOXIC METALS AND ANIONIC SURFACTANTS IN IRRIGATION WATER

This chapter was prepared by Salphina N. Sathekge, Glynn K. Pindihama, Gitari W. Mugera, Rabelani Mudzielwana, Shirley Mukangaya & Wasiu B. Ayinde

3.1 INTRODUCTION

Water resources in South Africa are limited, most farmers rely on untreated surface water for irrigation of their vegetable crop (Duhain, 2012). However, surface water can be contaminated by HABs and could contaminate fruits and vegetables with cyanotoxins because contaminated water is deposited onto the surface of the crops during irrigation. Such irrigated crops and vegetable plants might also accumulate cyanotoxins in their edible tissues, and therefore, these plants might contribute directly or indirectly to cyanotoxin transfer through the food chain, and thus constitute a potent health risk source.

Despite the predicted increase in incidents of harmful cyanobacterial blooms, strategies for monitoring and managing them tend to be reactionary and there is a lack of proactive early warning capabilities (Tyler *et al.*, 2009). In addition, cyanobacterial toxins do not occur in the aquatic environment separately but they naturally interact with other toxicants. Terrestrial food plants can be exposed to numerous anthropogenic pollutants and other stressors such as linear alkylbenzene sulfonate (LAS) and toxic metal species, which may enhance cyanotoxins accumulation from the surrounding environment after irrigation.

This part of the study aimed at studying the co-existence of cyanotoxins, toxic metals and anionic surfactants in irrigation water and agricultural soils and determine the health risks associated with consuming cyanotoxins contaminated plants in the Crocodile (West) Marico Water Management Area, which covers parts of Gauteng and Northwest Provinces. Hartbeespoort, Rietvlei, Roodeplaat and Bospoort Dams are all found within the WMA and these dams have been classified as hypertrophic for many decades (van Ginkel, 2004). The dams are of importance as they are used for irrigation, drinking water and recreational purposes (Mbiza, 2014). The area has a long history of mining and industrial activities and these activities and improperly treated domestic wastewater have had severe impacts on the integrity of the farms areas and reservoirs in the catchment.

The combination of these pollutants is very common in water bodies that are eutrophic, and edible terrestrial plants might be exposed through irrigation and thus posing human health risk via consumption of the contaminated food plants (Cao *et al.*, 2017; Pindihama & Gitari, 2020).

The aim of this part of the study was to investigate the occurrence of cyanotoxins, anionic surfactants and metal pollutants in irrigation water, agricultural soils and plants with the aim of assessing the human health risks associated with consuming cyanotoxins contaminated plants. To achieve this objective, field work and laboratory analysis was conducted. This section details the procedures and protocols which were followed to meet the objective.

3.2 MATERIALS AND METHODS

3.2.1 Study area description and the location of sampling points

The study was conducted around Hartbeespoort Dam site (25.7401° S, 27.8592° E) and Roodeplaat Dam site (25°37′15″S 28°22′17″ E / 25.62083° S) under the Crocodile (West) Marico Water Management Area (MWA). The MWA services Gauteng and parts of Northwest province. Hartbeespoort is 35 km west of Pretoria, south of Magaliesberg mountain range valley and north of the Witwatersberg mountain range. Roodeplaat is 20 km northeast of Pretoria and lies at the confluence of the Pienaar's river, Moreleta, and Edendale spruit (Conradie & Barnard, 2012). Both dams are situated in a temperate climate. The minimum and maximum surface water temperature of Hartbeespoort and Roodeplaat range from 14.4 to 25.7°C and 15.2 to 27.8°C, respectively (Mbiza, 2014). Both dams are considered hyper-eutrophic, and warm monomictic impoundment (van Ginkel, 2004).

Agricultural, industrial, and mining activities are common human activities taking place around the two dam sites. The purpose of these two water impoundments, includes livestock watering, irrigation, domestic and industrial activities, recreational activities, and fishing. The Hartbeespoort and Roodeplaat Dam distributes water via a long network of canals to farmlands. Figure 3.1 shows the study area, the green dots on the legend represents the agricultural site where soil samples were collected, the red dots represent canals and the blue dots represent farms where water samples were collected.

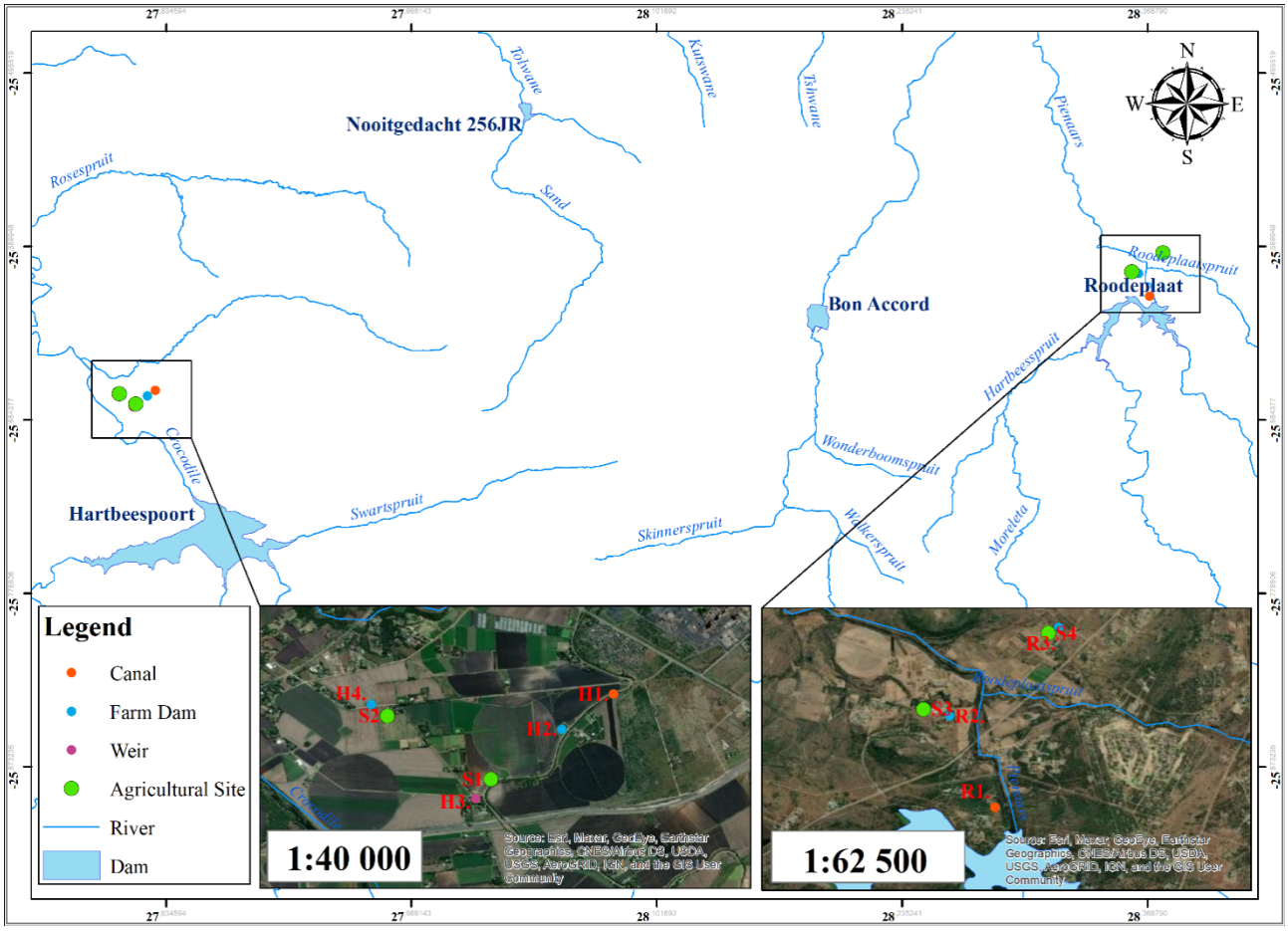

Figure 3.1. Map showing the study area and location of the sampling points

3.2.2 Sampling

Water and soil samples were collected in June 2019, September 2019, February 2020 and March 2021. The long breaks in between the sampling times were as result of the Covid-19 related lockdowns. Water samples were collected from the farm dams and irrigation canals, and soil samples were collected from cropping fields adjacent to the irrigation canals and being irrigated by water from the two dams. A total of 4 cropping sites (S1, S2 in Hartbeespoort, and S3 & S4 in Roodeplaat) were selected. While a total of 7 sampling sites (H1, H2, H3, H4 in Hartbeespoort), and R1, R2, R3 in for Roodeplaat) were selected for water sampling from irrigation canals and farm dams. Schott amber bottles, and HDPE bottles were used to collect water samples for analysis of cyanotoxins, anionic surfactants, and metal species, respectively. The sampling containers were cleaned in three stages prior to the sampling. Firstly, they were washed with Extran MA (phosphate-free) detergent and rinsed with deionized water and left to dry off. Secondly, 10% of hydrochloric acid (HCl) solution was used to wash before rinsing off with deionized water and left to dry. Thirdly, the sampling containers for MCs and anionic surfactants were rinsed again with 50 mL MeOH, then rinsed again with deionised water, and dried in the oven at temperature of 60°C for 10 minutes.

For anionic surfactants, 30 mL of 40% (v/v formalin) was added in each schott amber bottle to preserve water samples and prevent biodegradation of anionic surfactants by microorganisms. For total anionic surfactants in soil samples, grab samples from each chosen agricultural site were collected at a depth of 5 cm. The soil samples were then transferred into glass jars and preserved with 10% formalin. Methanol washed aluminium foil was placed over the mouth of a glass jar and then sealed with a lead to prevent sample contamination. Water samples collected for metals analysis were acidified with 3 drops of nitric acid to prevent precipitation of the metal species due to ingress of carbon dioxide gas as well as microbial growth. For agricultural soil samples, a clean plastic shovel was used to collect soil at depth of 5 cm. The soil samples were transferred into a polyester bag for further analysis of metal species. All samples were collected in duplicates, and immediately after sampling, they were properly labelled and stored in the cooler box with ice for preservation and transported to the laboratory for preparation and analysis.

3.2.3 Physicochemical parameters

The physicochemical parameters of the irrigation water monitored included; levels of chlorophyll-a; pH, Electrical Conductivity (EC); Total Dissolved Solids (TDS); Dissolved Oxygen and Turbidity. Physicochemical parameters such as pH, TDS, EC, temperature were determined in the field using a Jenway pH/Cond meter model (430). Turbidity was determined using TB200 portable turbidity meter model (#TB200-10) while dissolved oxygen (DO) was determined using a Thermo Scientific Orion RDO (Rugged Dissolved Oxygen) probe (087003). All instruments were calibrated following the manufacturers' instructions prior to analysis. Nutrients (nitrates and dissolved phosphates) in irrigation 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.

3.2.4 Determination of cyanobacterial biomass using chlorophyll-*a* analysis

Chlorophyll-*a* was used to estimate cyanobacterial biomass in the irrigation water. Chlorophyll-*a* concentration was determined according to the standard method adapted from Lawton *et al.* (1999). Briefly, 200 mL of water sample was filtered through a Whatman glass fibre filter membranes, 47 mm diameter to separate algal cell from water. The filter membrane with the algal cell was placed inside a 100 mL beaker with 2 mL of 90% boiling ethanol. The samples were sonicated for 10 minutes to break down the algal cells using ultrasonic cleaner model 705, manufactured in South Africa. The ethanol supernatant was decanted into 50 mL centrifuge tube and then centrifuged for 10 minutes at 3000 rpm. The total chlorophyll-*a* was measured using a spectrophotometer (BGM Labtech, 601-1106, Germany). The difference in absorbance of the extracted chlorophyll-*a* were determined at 665 and 750 nm wavelength against the 90% ethanol blank. The samples were measured on a Spectrophotometer 665a and 750a wavelength before acidification and corrected from turbidity, and 665b and 750b after acidification and corrected from turbidity. The same samples were then acidified with a drop of 1 mol/L of hydrochloric acid (HCl) and were determined at same wavelength after 2 minutes. The total chlorophyll-a was determined according to the following formula provided by Lawton *et al.* (1999):

Correction for turbidity: absorbance 665*a* -750*a* = corrected 665*a* absorbance
665*b*-750*b* = corrected 665*b* absorbance

$$Chlorophyll\text{-}a = \frac{29.62\ (665a - 665b\ )x\ Ve}{Vs\ x\ I}\ mg\ m^{-3} \quad [4]$$

$$Phaeophytin - a = \frac{20.73\ (665b\ x\ Ve)}{Vs\ x\ I}\ mg\ m^{-3} \quad [5]$$

$$Total\ Chlorophyll\text{-}a = Phaeophytin + Chlorophyll\text{-}a \quad [6]$$

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

3.2.5 Digestion of agricultural soils for metals analysis

Prior to the analysis of agricultural soil for toxic metals concentrations samples were prepared as follows: soil samples were oven dried at 100°C for 48 hours to eliminate moisture and then milled to obtain finer particles of ≤250 µm. Thereafter, samples were digested using the aqua-regia method described by Gaudino *et al.* (2007). Briefly, 10 g of finely milled samples were transferred into a 250 mL beakers and 10 mL of deionized water was added to hydrate the samples. Thereafter 15 mL of concentrated HNO_3 and 45 ml of concentrated HCl acids were added to each beaker, then the mixture was placed onto a hot plate for digestion at 100°C for 1 hour. Thereafter, the samples were removed from the hot plate and allowed to cool to room temperature. The cooled residues were then transferred into a 100 mL volumetric flasks and deionized water was used to fill the 100 mL flasks to the mark. Mixtures were then shaken vigorously for 1 minute, and then allowed to settle for 30 minutes. Samples were then filtered through 0.45 µm pore filter membranes. The filtered samples were then transferred into a 50 mL centrifuge tube and sent to the University of Stellenbosch for analysis of metals using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

3.2.6 Microcystins determination in irrigation water

The determination of MCs in natural water samples was performed using a commercially-available ELISA Microcystin Plate Kit (ENVIROLOGIX INC.). This assay uses antibodies against microcystin-LR.

Microcystins levels in irrigation water samples were determined using Elisa test kits supplied by Envirologix (Kit Lot: 071499 Cat No: EP 022) and EUROFINS (Kit Lot No: 19I1120:PN 520011) using Spectro-star Nano (BMG LABTECH, 601-1106, Germany) for quantification. Prior to analysis, 5 mL of each sample was filtered using the 0.20 µm glass fibre syringe filters.

3.2.7 Determination of anionic surfactants in water and soil samples

3.2.7.1 Sampling of anionic surfactants

Irrigation water and agricultural soils were sampled according to the sampling schedule at the three identified sites (as stated for metal species). On each sampling day, 1 L grab samples of irrigation water was collected in pre-washed glass Schott bottles, by opening the Schott bottles at a depth of 10 cm below the water surface. The samples were collected on the hour between 8 am and 5 pm at all the sites by employing three (triplicate) independent samplers for each site. In addition, three one-off soil samples (approximately 120 g per sample) were collected in glass jars from each site.

Prior to field sampling, the sample bottles and jars were pre-washed in the laboratory with 10% HCl, rinsed in deionised water, and left to dry. The sampling containers were then rinsed with 50-100 mL methanol, rinsed again in deionised water, and then dried. Prior to sampling, 30 mL of 37-40% formaldehyde (that is 3% v/v formalin) was placed in the Schott bottles to preserve the water sample and prevent further biological degradation of the anionic surfactants by micro-organisms. The Schott bottles were then sealed and packed for transport to the field.

Soil samples were obtained by taking three grab samples per site in-field agricultural soil to a depth of 5 cm. Excess water was allowed to drain out for 1 minute. Soils were then transferred to the glass jars and preserved with 10% formalin (enough volume was added to immerse the soils). Methanol-washed aluminium foil was placed over the mouth of the jar and then fastened with a lid to prevent sample contamination.

Samples were kept on ice. In the laboratory, water and soil samples were kept at approximately 4°C until required for analysis of anionic surfactants. The analysis of anionic surfactants was done using a Hanna HI96769 Anionic Surfactants Portable Photometer.

3.2.7.2 Extraction of soil samples

Overlying water will be removed prior to oven drying at 80°C for 16 hrs. The dry soil (10 g, large stones and grit not included) will be extracted by sonication with methanol at 50°C in a sonication bath. Three 10 min extractions (50 mL and 2 x 40 mL) will be carried out, with the soil separated from the extract by means of a centrifugation step. The combined extract will be concentrated to 2 mL to form the final extract for analysis.

3.2.7.3 Determination of anionic surfactants in soil and water samples

Anionic surfactants were determined using a Hanna HI96769 Anionic Surfactants Portable Photometer using the suppliers' instructions and provided reagents. In brief, the water sample to be analysed is treated with chloroform and an excess amount of azure **A** reagent. In the presence of the chloroform, the Azure A reacts with anionic surfactant and forms a chloroform-soluble blue-coloured complex. Such complexes can be designated as azure **A** active substances (AAAS). The intensity of blue colour in the vigorously shaken and subsequently settled chloroform layer is proportional to the concentration of the azure A-surfactant complex. The blue colour of the azure A-surfactant complex can be measured colorimetrically by making spectrophotometric readings in the chloroform. The measurements were made with a Hanna HI96769 Anionic Surfactants Portable Photometer.

3.2.8 Construction of SPATT bags

The resin DIAON HP20 was purchased from Rochelle chemicals, South Africa. SPATT bags for both laboratory trials and field trials were constructed using the 100 µm nitex bolting cloth, which was sewn into sachets bags. The bolting cloth was sewn on 3 sides to form an open bag of 55 mm width. The SPATT bags for both Laboratory trials and Field monitoring were filled with 3 g (dry weight) of the DIAON HP20 resin. The bags were then sewn on the fourth side to form a 55 x 55 mm dimension bag. The SPATT bags were activated by soaking in 100% methanol for 48 hours. The methanol was then rinsed off with deionised water by incubating the SPATT bags inside a beaker with 500 mL deionised water (Milli Q). The SPATT bags were then placed in Zip-lock bags with deionised 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 (Lane *et al.*, 2010; Kudela, 2011; Roué *et al.*, 2018).

3.2.9 SPATT laboratory trials

A laboratory trial was carried to evaluate the approximate saturation duration of the DIAON HP20 SPATT samplers. This was an attempt to assess the number of days to deploy the SPATT samplers in the field. The samplers with the resin were incubated for 9 days in 1L amber bottles filled with Roodeplaat Dam water and known to have MCS and agitated at 100 rpm using a reciprocal shaker (see Figure 3.2). Control bottles (with no samplers) were also run concurrently with the experimental bottles with the samplers. Individual SPATT samplers were retrieved daily from the bottles and rinsed off with deionized water and put kept in zip lock bags. Also, water sample treated as grab samples were collected daily concurrent to the SPATT samplers. All samples were stored at 4 ˚C until toxin extraction.

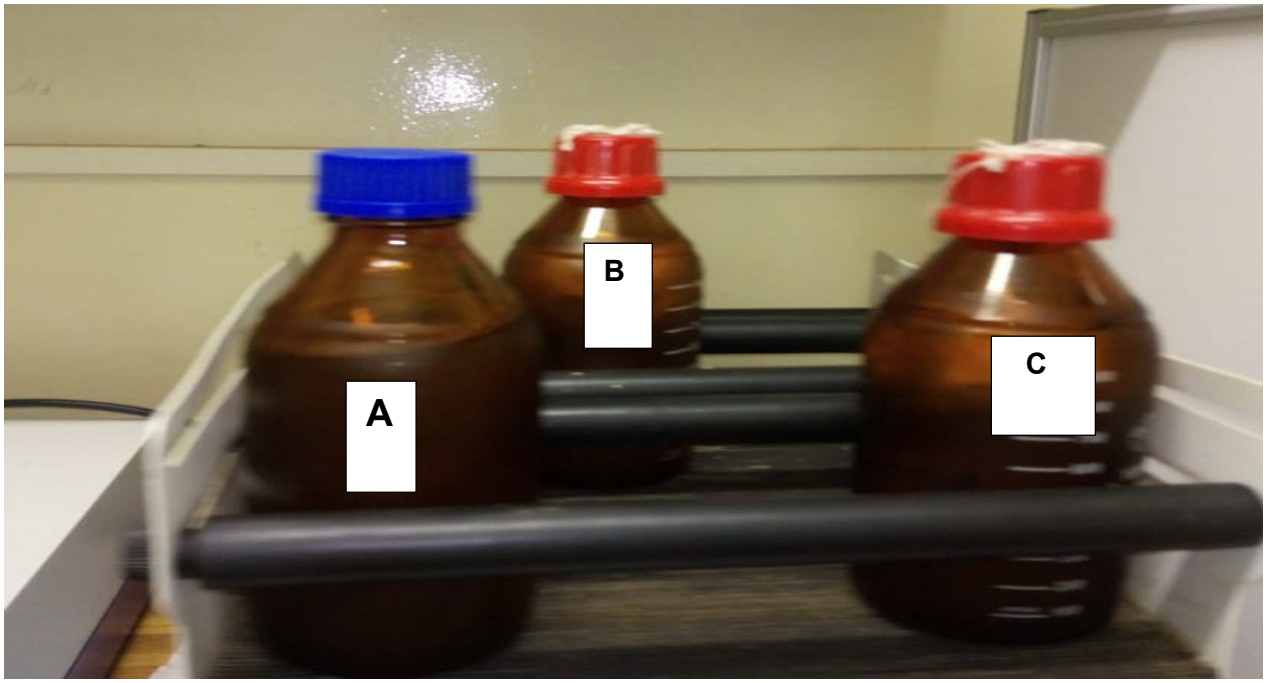

Figure 3.2. Laboratory trial for SPATT samplers (Bottle A control sample with no SPATT bags inside; Bottle B and C water samples with SPATT bags inside.

3.2.10 SPATT field monitoring

For SPATT samplers, deployments, a total of 6 irrigation points (two canal and four farm dams) were selected, see Figure 3.3. Three points (R1, R2, R3) for Roodeplaat and (H1, H2, H3) for Hartbeespoort sites. The water in all these six points selected is being used to irrigate vegetables and other crops in the study area.

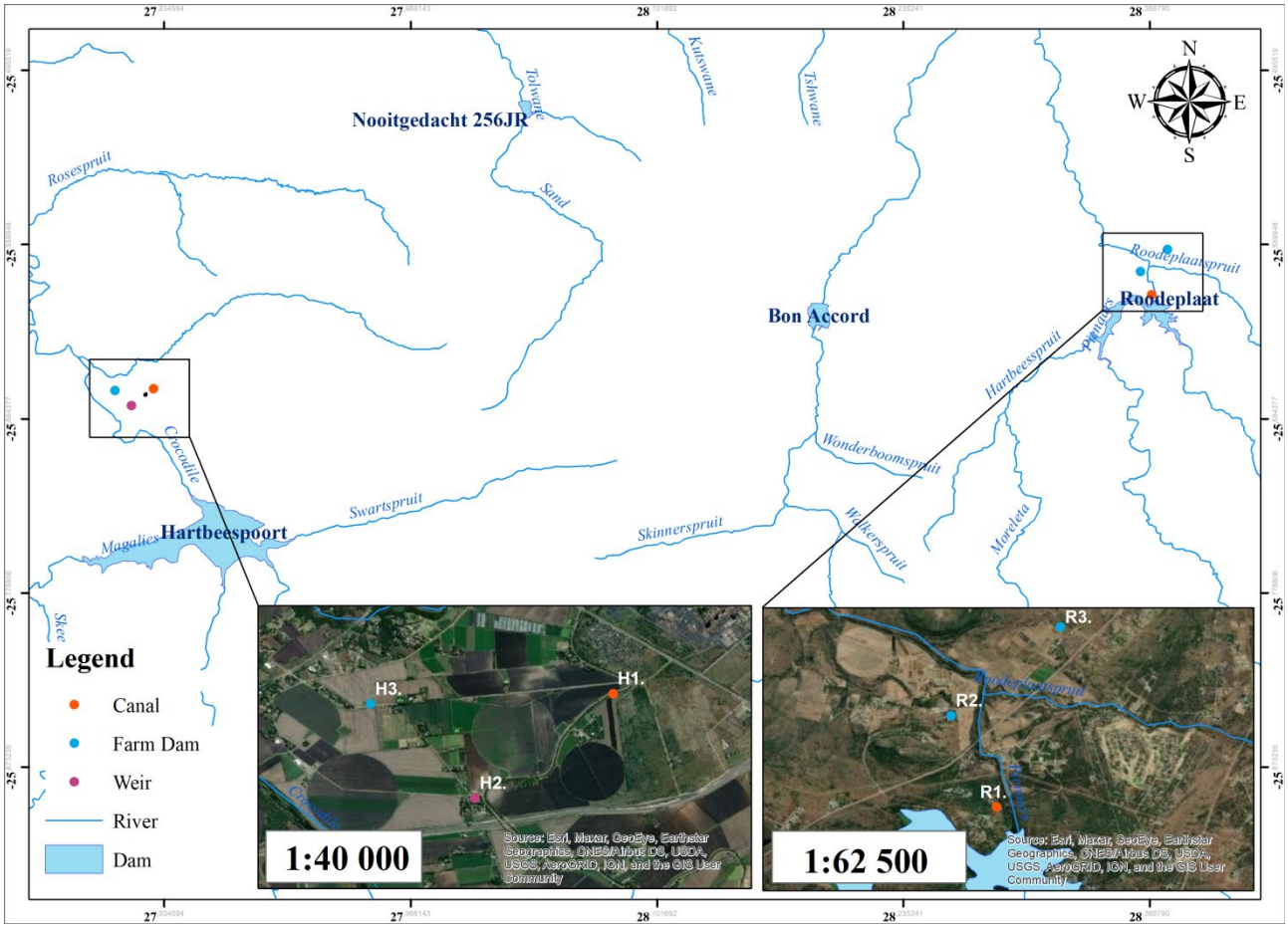

Figure 3.3. A Map showing sampling locations for cyanotoxins monitoring using SPATT samplers in irrigation canals and farm dams

3.3 RESULTS & DISCUSSION

3.3.1 Cyanotoxins, metals and anionic surfactants in irrigation water and agricultural soils

3.3.1.1 Physicochemical water quality parameters

The physicochemical characteristics of the irrigation water samples from the Hartbeespoort and Roodeplaat irrigation canals/farm dams collected during the sampling period are summarized in Table 3.1.

3.3.1.1.1 pH

The pH from selected irrigation canals/farm dam ranged from 6.3 to 10.59. This indicates that the water is slightly neutral to strongly alkaline. The highest pH value of 10.59 was observed in February (2020) at site R2, while the minimum 6.3 was observed in June (2019) at site H1 (Table 3.1). The observed pH levels throughout the sampling period revealed that three out of the seven sampled sites exceeded the recommended standard guideline for irrigation water of 6.5 to 8.4 (DWAF, 1996a),

whereas four were below the threshold. Irrigating with water with pH above 8.4 is known to have significant impact on plants' growth, yield, and quality (Hopkinson & Harris, 2019). Edokpayi *et al.,* (2014) also states that alkaline pH influence accumulation of algae blooms in the water columns. In addition, Mbiza (2014) showed that cyanobacteria favour pH between 6 to 9 and anything, above or below this value, significantly decreases the cyanobacterial biomass. Throughout the sampling months, the pH in all sites ranged between 6 and 10 and clearly those conditions favour the cyanobacterial growth.

3.3.1.1.2 TDS and EC

The TDS and EC values ranged from 169.2 to 974.0 mg L^{-1} and 285.0 to 1545.0 µS/cm, respectively as shown in (Table 3.1). The highest TDS value of 974.0 mg L^{-1} was observed in June 2019 at site H4, whereas the lowest TDS value 169.20 mg L^{-1}, was observed in February at site R1. The highest EC value 1545 µS/cm was observed in June 2019 at site H4, and the lowest value 285 µS/cm in February 2020 at site R1. The observed TDS and EC levels revealed that two out of three canals and three out of four farm dams sampled exceeded the recommended thresholds for water intended for irrigation water (DWAF, 1996b).

A pattern of high TDS and EC in June month (winter), moderate in March month (autumn), and low in September month (spring), and February month (summer) was observed (Table 3.1). Lower TDS and EC values were observed in September month (spring) and February month (summer) and this could be due to the dilution factor from precipitation during those seasons. The high levels of TDS and EC in June month (winter) in the irrigation water samples might be because of irrigation runoff from the agricultural lands containing dissolved ionic matters. Thus, irrigation canals receive high concentrations of inorganic salts and minerals such as carbonate, bicarbonate, chloride, sulphate, nitrate, sodium, calcium, magnesium, and potassium which might degrade the quality and health of the irrigated produce. Also, it may result in soil sodicity (DWAF, 1996b; Edokpayi *et al.*, 2014). Higher concentrations of EC reduce water available for plant up take even in wet soils (Mezgebe *et al.*, 2015).

3.3.1.1.3 Temperature

The temperature of the irrigation water samples ranged from 11.9°C to 32.8°C throughout the sampling months (Table 3.1). The highest water temperature of 32.8°C was recorded in February (2020) at site R2, whereas the lowest 11.9°C was recorded in June (2019) winter month at site H2. A pattern of low temperature levels in June month, moderate in September and high in February was

observed over the sampling period, and this was obviously due to the seasonal temperature variations. Findings from previous studies show that temperatures above 20°C (O'Neil *et al.*, 2012) or 23°C (Conradie & Barnard, 2012) promotes cyanobacterial blooms and cyanobacteria in the water column. Wang *et al.* (2016) also found temperature to be directly proportional to the biomass of the *Microcystis* genus in water bodies. The water temperature in the two study sites can thus be said to be ideal for the growth of cyanobacterial blooms. There are no set thresholds/guidelines for water temperature in water intended for irrigation.

3.3.1.1.4 Turbidity

The turbidity levels of the water ranged between 0.77 to 588.9 NTU (Table 3.1). The highest turbidity level was recorded in February (2020) at site R2, while the lowest 0.77 NTU was recorded at site R1 in September (2019) (Table 3.1). The high level of turbidity in site R2 in February month could be because of the presence of silt, suspended algal, clay, micro-algal, and fine organic matter suspended in the irrigation canals due to run-off as a result of the rains during this time of the year. The presence of silt and clay in all sites in February month could be because of agricultural activities, soil erosion from the cultivated land into the irrigation canals during heavy rainfall since the cropping field are adjacent to the canals. A pattern of high turbidity in February, moderate in March, and low in September was observed throughout the sampling period. There are no guideline standards for turbidity in South Africa for water intended for irrigation.

3.3.1.1.5 Dissolved oxygen

The dissolved oxygen (DO) in the water ranged from 2.4 to 21.1 mg L^{-1} (Table 3.1). The highest dissolved oxygen level of 21.1 mg L^{-1} was recorded in September (2019) at site R3, while the lowest 2.4 mg L^{-1} was recorded in February (2020) at site H2 (Table 3.1). The low DO in site H2 in February could be due to high temperature and nitrates levels which promotes cyanobacterial proliferation and when dying these consume dissolved oxygen in the water column (Vos & Roos, 2005).

Table 3.1. Summary statistics of the physicochemical parameters of the irrigation water monitored

Parameter	Points	R1	R2	R3	H1	H2	H3	H4	DWAF (1996b)
pH	Min	7.5	7.48	8.9	6.3	7.2	6.6	7.8	6.5-8.4
	Max	9.92	10.6	10.6	7.6	8.2	8.2	9.3	
	Mean ± SD	8.5 ±1.1	9.4 ±1.3	9.7±0.9	7.2 ± 0.6	8.0 ± 0.5	7.5 ± 0.7	8.8 ± 0.7	
TDS (mg L^{-1})	Min	169.2	190.0	176.8	230.0	270.0	242.0	265.0	0-260.0
	Max	549.0	540.0	498.0	663.0	604.0	558.0	974.0	
	Mean ± SD	303.05 ±168.7	297.3 ±162.9	280.9±146.5	378 ± 197.5	434.5 ± 167.0	352.3 ± 148.7	453.5 ± 347.2	
EC (µS/cm)	Min	285.0	312.0	293.0	378.0	453.0	395.0	440.0	0-400.0
	Max	918.0	890.0	828.0	990.0	987.0	947.0	1545.0	
	Mean ± SD	506.3 ± 281.7	493.0 ± 267.1	466.8 ± 243.8	601.8 ± 275.1	713.5 ± 218.2	590.8 ± 256.1	732.5 ± 541.9	
TEMP (°C)	Min	15.1	16.0	15.0	14.1	11.9	15.8	16.4	n. a
	Max	23.3	32.8	29.9	23.0	27.3	22.6	29.3	
	Mean ± SD	18.9 ± 4.14	22.95 ± 7.08	21.8 ± 6.2	18.95 ± 4.94	20 ± 6.49	19.73 ± 3.43	21.93 ± 5.51	
Turbidity (NTU)	Min	0.8	3.8	8.3	0.8	12.9	0.9	11.0	n. a
	Max	8.6	588.9	57.6	2.5	75.8	3.0	50.3	
	Mean ± SD	4.6 ± 3.9	245.3 ± 305.6	29.6 ± 25.3	1.6 ± 0.9	37.0± 33.9	1.97 ± 1.47	32.3 ± 19.8	
DO (mg L^{-1})	Min	7.9	8.3	15.5	3.6	2.4	3.0	8.9	
	Max	8.9	16.2	21.1	9.0	14.2	9.5	13.1	
	Mean ± SD	8.4 ± 0.7	12.2 ± 5.6	18.3 ± 3.9	6.3 ± 3.8	8.3 ± 2.0	6.3 ± 4.6	11.0 ± 3.0	

According to the EPA (Environmental Protection Agency, 2002), dissolved oxygen less than 2 mg L^{-1} in the aquatic system is considered hypoxic, and with the results obtained from all sites the DO levels was above 2 mg L^{-1}. A pattern of high DO in September month (Spring) and low in February month (Summer) was observed at site (R1, R3, H1, H2, & H3), except for site R2 and H4 which had moderate DO in the September month and high DO in the February month (Table 3.1). There is no standard guideline for DO for irrigation water use.

3.3.1.2 Nutrients & Cyanobacterial biomass

3.3.1.2.1 Nitrates

The levels of total nitrates in the irrigation water from the two dams ranged from 0.00 to 28.43 mg L^{-1} (Table 3.2). The month of February (2020) had the highest nitrate levels (27.50 mg L^{-1}) at site H2. The lowest nitrate levels were below the 1 mg L^{-1} detection limit for sites R1, R3 and H2 in June sampling month (Table 3.2). Three out of the seven sampled sites had nitrate levels above the recommended threshold for irrigation waters of 5 mg L^{-1} (DWAF, 1996a) and FAO (Ayers and Westcot, 1985). The high level of nitrates observed in February (2020) might be due to fertiliser from runoff from the cropping sites since it was still the rainy season.

With regards to promoting algal growth Shabalala *et al.* (2013) observed that a range of 2.5 to 10 mg L^{-1} of nitrate concentration induced eutrophication and resulted in algae and cyanobacterial blooms that favoured the *Microcystis* species. In all the sampling months, nitrates levels in most of the sampled sites fell within 2.5 to 10 mg L^{-1} range, while other sites had nitrates levels which were above the mentioned range particularly in February 2020. A pattern of low nitrates in June, moderate in September and March, and high in February was observed for all sites. Algae take up nitrates in winter for photosynthesis and for their growth, and this might explain the low levels of nitrates in the month of June (2019).

Table 3.2. Summary statistics of nutrients and chlorophyll-a monitored in the irrigation water between June & September 2019, to February 2020.

Nutrients		**Sampling sites**						
		R1	**R2**	**R3**	**H1**	**H2**	**H3**	**H4**
Phosphates (mg L^{-1})	Mean ± SD	1.0 ± 0.6	0.6 ± 0.5	0.5 ± 0.3	1.0 ± 0.2	0.4 ± 0.3	0.9 ± 0.2	0.4 ± 0.4
	MAX	1.7	1.5	0.9	1.1	0.7	1.1	0.9
	MIN	0.44	0.2	0.2	0.8	0.1	0.7	0.1
Nitrates (mg L^{-1})	Mean ± SD	3.6 ± 3.1	3.2 ± 2.9	8.9 ± 3.3	5.4 ± 2.3	12.3 ± 10.9	4.3± 1.7	4.0 ± 2.3
	MAX	8.4	7.5	8.7	9.1	28.43	5.9	8.1
	MIN	0.0	0.4	0.0	2.7	0.0	2.1	0.9
Chlorophyll-*a* (µg L^{-1})	Mean ± SD	58.01 ± 70.13	176.13 ± 237.86	250.81 ± 120.79	31.97 ± 39.72	265.47 ± 172.92	19.83 ± 16.23	422.61 ± 575.75
	MAX	208.2	672.27	373.90	115.20	441.41	46.35	1408.9
	MIN	0.00	1.78	109.72	5.92	10.37	0.00	1.48

SD: Standard deviation. (< 5 mg L^{-1}) Nitrates (DWAF, 1996); FAO, 1985 (0-2 mg L^{-1}) Phosphates', (DWAF, 2002) Chlorophyll-a (0<x<10 Oligotrophic); (10<x<20 mesotrophic); (20<x<30 Eutrophic); (> 30 hypertrophic); R (Roodeplaat samples); H (Hartbeespoort samples)

3.3.1.2.2 Phosphates

The total phosphates levels ranged between 0.1 to 1.7 mg L^{-1} as shown in Table 3.2. September (2019) recorded the highest phosphate levels at site R1, and June recorded the lowest recorded value at site H2 (Table 3.2). The phosphate levels in all the sampling months fell within the recommended FAO (1985) guideline of 0-2 mg L^{-1} for irrigation water. Low levels of phosphates in the wet season (February 2020 and March 2021) are associated with phytoplankton and bacteria that uses dissolved phosphate for growth and photosynthesis. Moreover, the wet season accelerate phosphate adsorption to sediments (Balcıoğlu, 2019). At phosphates levels between 0.025-0.25 mg L^{-1}, the water body reaches the eutrophic level which supports toxic algae growth or formation (Balcıoğlu, 2019). In this study, phosphates levels were above the 0.025 to 0.25 mg L^{-1} threshold in all the sites in the sampled months. Thus, sampling sites can be described as eutrophic and can sustain/promote HABs.

3.3.1.2.3 Chlorophyll-a

Chlorophyll-*a* is normally used to estimate the algal biomass in water samples (Ramaraj *et al.*, 2013). South Africa like many other countries across the globe, have no regulations or policies on cyanotoxins in water intended for crop irrigation (Pindihama & Gitari, 2020). Chlorophyll-*a* was measured in this study to determine the trophic state and phytoplankton biomass. Chlorophyll-*a* ranged between 0.7 to 402.4 µg L^{-1}. The highest chlorophyll-*a* was observed in June 2019 at site H2, whereas low chlorophyll-a was observed at site R2 in month of September 2019 (Table 3.2). In June (2019), four out of the seven sampling sites fell within the hypereutrophic state and in September (2019), two of the seven sampling sites fell within the hypereutrophic state, while the other five could be classified as oligotrophic. In February 2020, all sites 100% were within the hypertrophic state > 30 µg L^{-1} and in the March (2021) sampling month, six of the seven sampling sites fell within the hypereutrophic state, while one could be classified as mesotrophic.

The low chlorophyll-*a* levels in the September month indicate low photosynthetic activities in the sampled sites and this could be attributed to lack of nutrients, since spring comes long after winter dry spells, thus there is no rainfall to wash nutrients into the water bodies. According to Kansas Department of Health and Environment (KDHE, 2011) levels of chlorophyll-*a* above 10 µg L^{-1} indicate the likelihood, of rapid growth of cyanobacterial blooms in an aquatic ecosystem. The high levels of chlorophyll-*a* in the February month might be due to intensive photosynthetic activities. The levels of chlorophyll-*a* in all sampling sites throughout the sampling months were above the Target Water Quality Range (TWQR) value 0-1 µg L^{-1} for aquatic ecosystem health, except for site R1 in September 2019. High temperature and high levels of nitrates in February month, could be the reason behind the high levels of chlorophyll-*a* because organisms such as algae take up the nutrients as they

photosynthesize and grow, resulting in an increase in chlorophyll-*a*. The high levels of chlorophyll-*a* in February and March month could explain high levels of microcystins in the sampling sites during those periods (see Figure 3.4).

As chlorophyll-*a* increases, so do cyanobacterial blooms and the likelihood of microcystins formation in the water column (Table 3.2 and Figure 3.4). The chlorophyll-*a* levels observed in Hartbeespoort sites (H1, H2, H3, and H4) were way much lower throughout the sampling months compared to the one **reported** by Ololo (2013) for the same dam (0.14 µg L^{-1} to 8693 µg L^{-1}), thus implying, a decrease in the cyanobacterial biomass in the dam.

3.3.1.3 Cyanotoxins

The total concentration of MCs in the irrigation water samples ranged from 0.12 ± 0.00 to 15.57 ± 3.60 µg L^{-1}. The highest mean concentration of total MCs was recorded in March 2021 (autumn) at site R2, while the lowest was recorded at site H3, and H2 in June 2019 (winter) (Figure 3.4). Low levels of MCs were recorded in the water samples in September 2019 in all the sampling sites. This could be explained by the high pH which was above the optimal pH 7.5-9 for the growth of cyanobacteria. The low MC levels in the September month also correlated with low chlorophyll-*a* reported in the same month (Figure 3.4). The high MC levels in February and March month could be explained by favourable pH (within the 7.5-9 range) and warmer water temperatures (above 20°C) in those months which promoted the growth of HABs such as *Microcystis* and the resultant production and release of MCs.

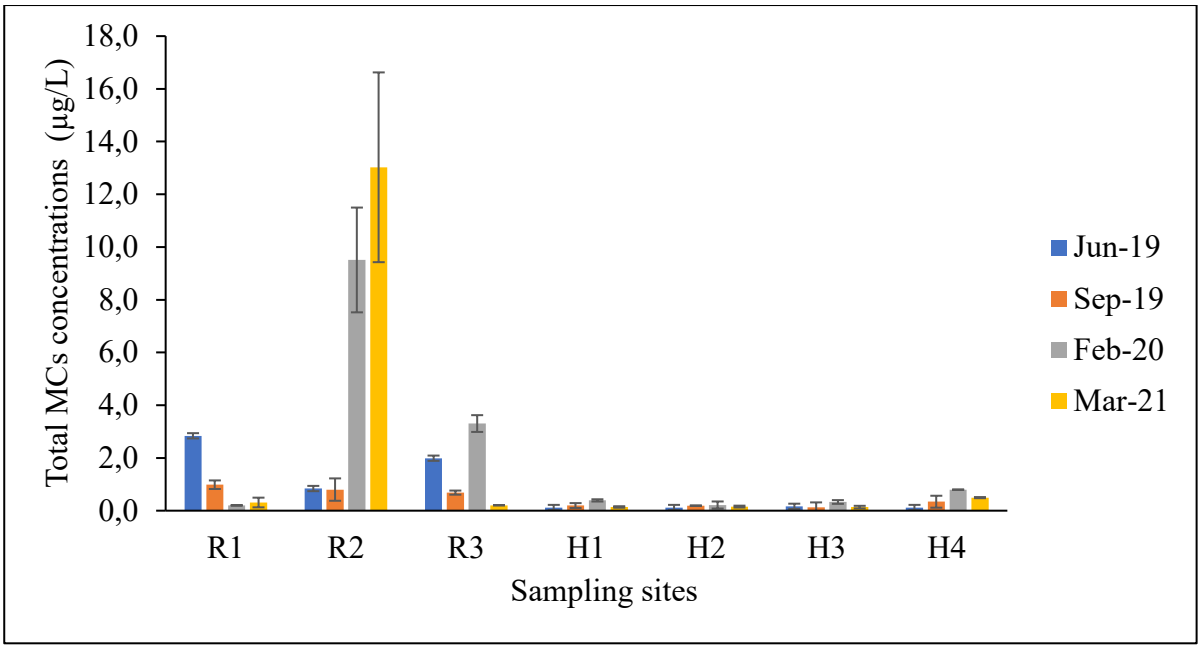

Figure 3.4. Total Concentrations of MCs in irrigation water collected from Hartbeespoort and Roodeplaat irrigation canals

These finding implies a risk to food crops irrigated with water from these two dams, regardless of lack of guidelines or standards for cyanobacteria and their toxins in irrigation water. The concentrations of MCs reported from all sites were very low compared to the findings of previous studies (Van Ginkel, 2004; Conradie and Barnard, 2012). Previous studies have shown median concentrations of MCs of 580 µg L^{-1} and a maximum level of 14 400 µg L^{-1}, with the lowest consistently exceeding 10 µg L^{-1} (Van Ginkel, 2004; Turton, 2015). Mbiza (2014) found total MCs level at Roodeplaat Dam and Hartbeespoort Dam to be as high as 2.5 µg L^{-1} in wet season. In the current study, only sites R2 and R3 in the February (2019) and March (2021) sampling month, showed total MCs concentrations above the 2.5 µg L^{-1} reported by Mbiza (2014). In line with our findings, Mbiza (2014) also reported high MCs levels in the summer season. Th low MC levels in Hartbeespoort sites throughout the sampling periods might be because of mechanical removal of algae from the main dam as part of the Hartbeespoort Dam "*maetsi a me*" rehabilitation project by the department of water affairs (Mbiza, 2014; Carroll & Curtis, 2021).

3.3.1.4 Anionic surfactants

3.3.1.4.1 Anionic surfactants in irrigation water

Artificial anionic surfactants are the active ingredients used in producing detergents with linear alkylbenzene sulfonate (LAS) being the primary anionic surfactants used in laundry detergents worldwide (Nomura *et al.*, 1998; Gordon, 2011). Figure 2 shows the total mean concentration of anionic surfactants monitored in selected irrigation canals and farm dams from Hartbeespoort and

Roodeplaat sites. The mean levels of anionic surfactants ranged from 0.01 ± 0.00 to 3.49 ± 0.00 mg L^{-1}. The highest mean level of anionic surfactant was recorded at site H4 3.49 mg L^{-1} in March sampling month, while the lowest concentration 0.01 mg L^{-1} was observed at site H3 in September month (Figure 3.5).

The high concentration of anionic surfactants at site H4 in March month, might come from domestic wastewater released into the main dams from the tributaries. The low mean concentration of anionic surfactants at site H3 might be due to moderate dissolved oxygen, DO is required by bacteria which degrade the surfactants. Worldwide there are no regulations governing the anionic surfactants concentrations in domestic wastewater and consequently, river water is polluted by high concentrations of surfactants (Wang *et al.*, 2012). After use, most of surfactants are ultimately discharged into aquatic ecosystems through treated or untreated wastewater. High levels of surfactants in aquatic ecosystems result in bloom of toxic cyanobacteria in the water column. Anionic surfactants are usually eliminated from water column via biodegradation and absorption.

The anionic surfactants degradation occurs very slowly under anoxic and anaerobic conditions; as a result, surfactants end up accumulating in the aquatic ecosystem. (Wang *et al.*, 2015) reported that the anionic surfactant linear alkylbenzene sulfonate concentration in surface waters normally vary between 0.001 and 20 mg L^{-1}, and the total anionic surfactants levels observed from the current study throughout the sampling months were within this range. Even though the surfactants level in all sampling sites seemed to be low, Wang *et al.* (2015) indicated that anionic surfactant linear alkylbenzene sulfonate levels as low as 0.02 to 1.0 mg L^{-1} may still cause significant impact in the aquatic ecosystems, such as damaging the cell membrane of organisms, enhancing bioaccumulation of other pollutants such as metals and cyanotoxins.

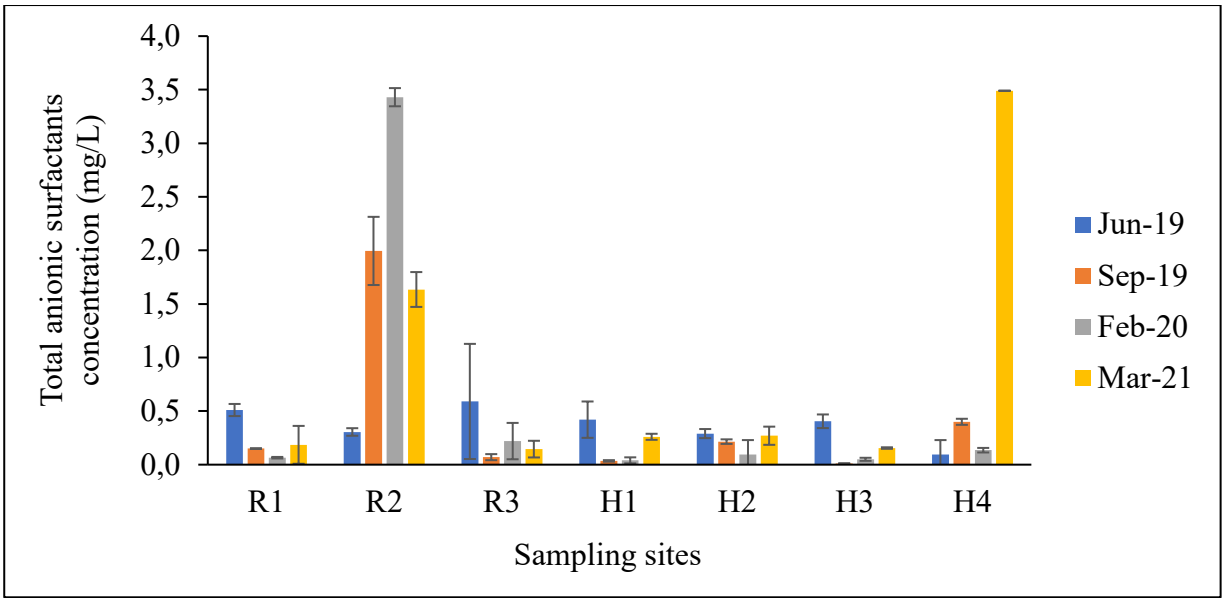

Figure 3.5. Total concentrations of Anionic surfactants in irrigation water from Roodeplaat and Hartbeespoort irrigation canals

3.3.1.4.2 Anionic surfactants in agricultural soils

The levels of anionic surfactants in agricultural soils are shown in Figure 3.6. The mean anionic surfactants levels in agricultural soils ranged from 0.91 ± 0.44 to 8.73 ± 0.00 mg kg^{-1}. The highest anionic surfactant level 8.73 mg kg^{-1} was observed at site S2 in March month, while the lowest mean level 0.91 mg/kg was observed at site S2 in September month. The source of anionic surfactants in the agricultural soils may be via irrigating with water infested with anionic surfactants, or application of pesticides. Boluda-Botella *et al.* (2010) found that the anionic surfactant linear alkylbenzene sulfonate sorption capacity levels were high in agricultural soils compared to commercial sand soils which have >90% of particle size (0.100-0.315 mm). Such soils have high concentration of organic matter and fine particulate materials in agricultural soils, that enhance the sorption of anionic surfactant linear alkylbenzene sulfonate into soil.

In this study, surfactants were found in all agricultural soils sampled even though they were in low concentrations in most of the soils. Unlike the current study, Ekmekyapar & Celtikli (2014), found the anionic surfactant linear alkylbenzene sulfonate ranging from 5.84 to 19.6 mg kg^{-1} in agricultural soils.

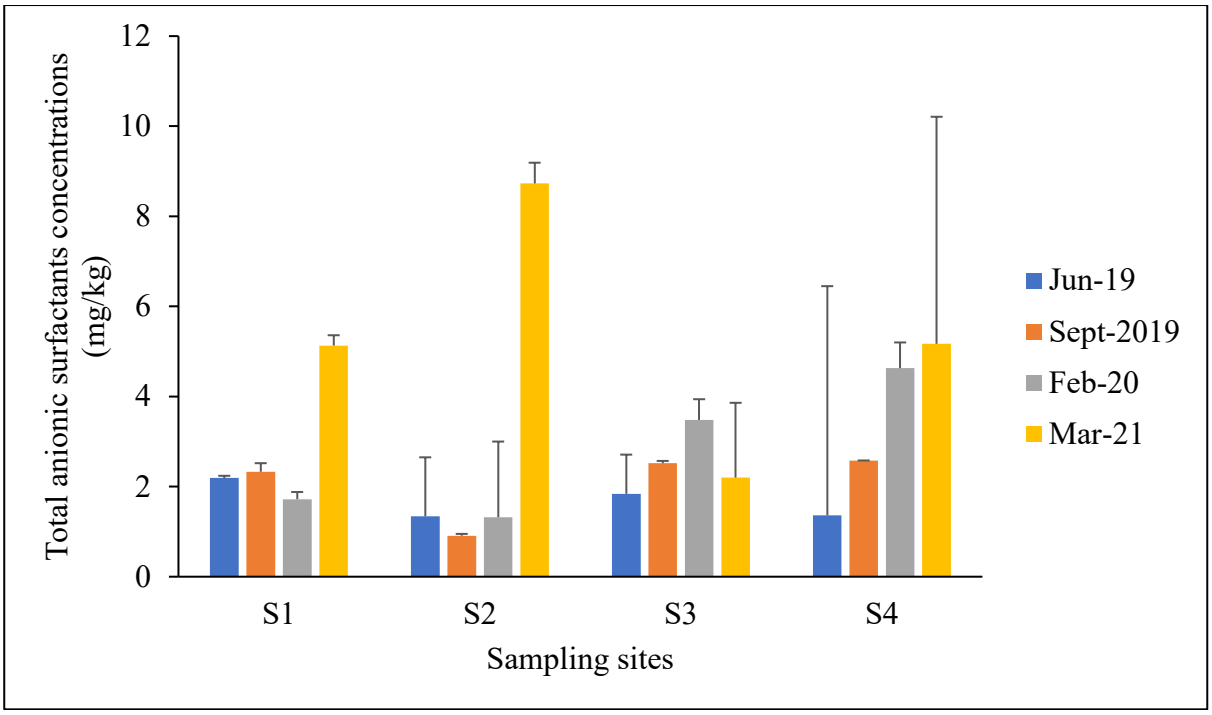

Figure 3.6. Mean levels of anionic surfactants in agricultural soils of Roodeplaat and Hartbeespoort agricultural farmlands

3.3.1.5 Factors influencing MC levels and cyanobacterial biomass

3.3.1.5.1 Physicochemical parameters influencing MC levels in irrigation water

Physicochemical parameters, nutrients (phosphates and nitrates) and anionic surfactants were monitored in the irrigation water to identify which of these parameters influence or better predict the risk of cyanotoxins and cyanobacterial biomass in the irrigation water. A spearman (non-parametric) correlation matrix was performed for physicochemical parameters and MCs in the irrigation water and the results are presented in (Figure 3.6: A, B and C). The findings showed that there was a strong positive correlation between *microcystins* and pH, moderate positive correlation between MCs and turbidity, and negative correlation between MCs and TDS and EC, while other physical parameters such as Temperature, and DO did not have any correlation with MC levels in the water. These findings contradicted with findings by Idroos and Manage (2014) who found that water temperature had a strong positive relationship with *MC-LR* concentrations, but were consisted with our finding in terms of MCs and pH, as they found pH as a moderate predictor of *MC-LR* in Beira Lake, in the city of Colombo, Sri Lanka. Also contrary to our findings, Subbiah *et al.* (2019), found a direct correlation between MCs and anatoxin concentrations with turbidity in a reservoir in the Southwest of the U.S.

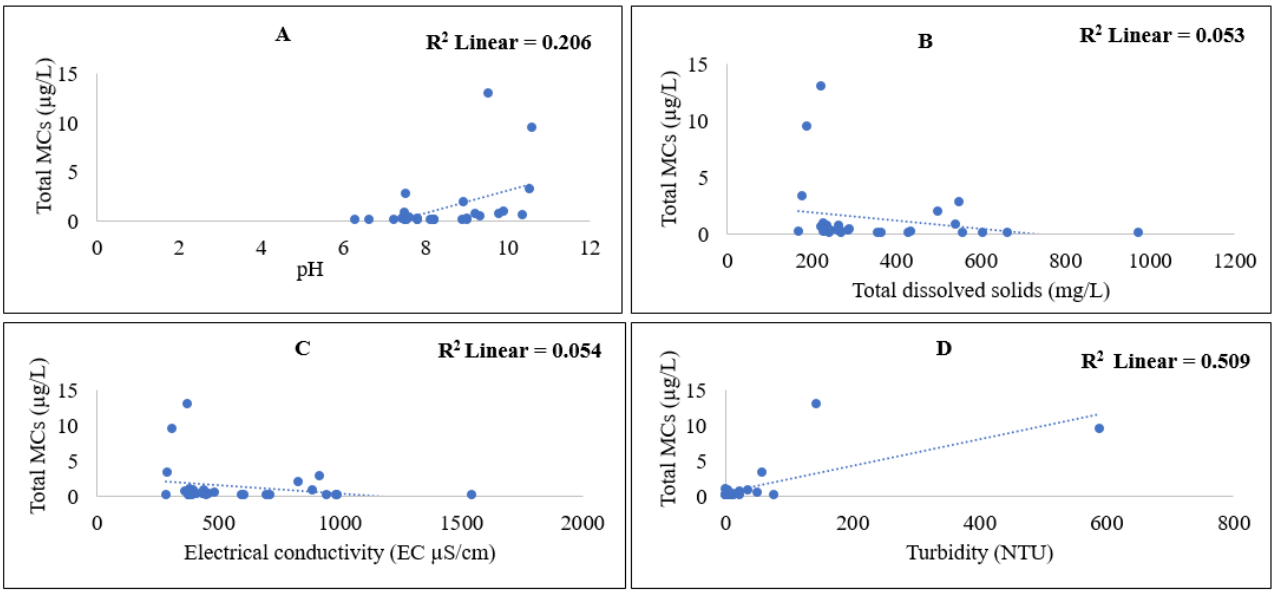

Figure 3.7. (A, B, C, D) Correlation matrix for MCs vs pH, TDS, EC, and turbidity.

Table 3.3. Spearman correlation coefficients for MC levels vs physicochemical parameter in irrigation water

Physical parameters	Microcystins (MCs µg L^{-1})
pH	0.624**
TDS (mg L^{-1})	-0.466*
EC (µS cm^{-1})	-0.445*
Temperature	0.220
Turbidity (NTU)	0.521*
DO (mg L^{-1})	0.326

**Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at 0.05 level (2-tailed)

3.3.1.5.2 Factors influencing cyanobacterial biomass in irrigation water

A Spearman correlation was conducted to determine if there is correlation between cyanobacterial biomass (estimated via chlorophyll-*a*) and physicochemical parameters to determine which physicochemical parameters better predict cyanobacterial biomass in the irrigation water (Table 3.4). A strong negative correlation was found between chlorophyll-*a* and dissolved phosphates. This could be due to the phytoplankton taking up the phosphates for their growth. A strong positive correlation between chlorophyll-*a* and turbidity was found. No relationships between Chlorophyll-*a* was and pH, TDS, EC, Temperature, DO and nitrates were found.

The findings from this current study were consistent with (Alcântara *et al.*, 2011) who found chlorophyll-*a* to be positively correlated with turbidity, while other parameters showed no correlation. Bbalali *et al.* (2013) also found a positive correlation between chlorophyll-*a* and nitrates, and no correlation between chlorophyll-*a* and dissolved phosphates. In lentic regions, Pan *et al.* (2009) found that total phosphorous was a major factor influencing chlorophyll-*a*. Higher nutrients levels do not necessarily translate into a large phytoplankton biomass under lotic conditions (Pan *et al.*, 2009). This probably explains the strong negative correlation between chlorophyll-*a* and phosphates in this current study since canals and farm dams are lotic in most cases. Also similar to this study, Balcıoğlu (2019) found a negative correlation between chlorophyll-*a* and phosphates indicating that phytoplankton used the phosphates.

Table 3.4. Spearman correlation coefficients for Chlorophyll-a levels vs physicochemical parameters in irrigation water

	Chlorophyll-*a* (µg L^{-1})
pH	0.227
TDS (mg L^{-1})	0.025
EC (µs cm^{-1})	0.030
Temperature (°C)	0.264
Turbidity (NTU)	0.777**
DO (mg L^{-1})	0.187
Phosphates (mg L^{-1})	-0.718**
Nitrates (mg L^{-1})	0.152

**Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at 0.05 level (2-tailed)

3.3.1.5.3 Nutrients and chlorophyll-a as predictors of MCs

The spearman correlation was conducted to test the association between chlorophyll-*a* and MCs levels, and nutrients with MCs levels, to determine if nutrient levels and chlorophyll-*a* can be used to predict the MC levels in the irrigation water from the two dams. High concentrations of nutrients are known to favour the rapid growth of harmful cyanobacteria in the aquatic ecosystems, including MCs producing species *Microcystis* and *Anabaena*, which are very dominant in the two dams (Conradie and Barnard, 2012; Mbiza, 2014). The Spearman correlation showed no relationship between MC levels with nitrates, phosphates, and chlorophyll-*a* (Table 3.5).

Table 3.5. Spearman correlation coefficients for MCs levels vs nutrients and chlorophyll-a in irrigation water

	Microcystins (MCs µg L^{-1})
Nitrate (mg L^{-1})	-0.225
Phosphate (mg L^{-1})	-0.110
Chlorophyll-*a* (µg L^{-1})	0.178

The findings of this study are consistent with Howard *et al.,* (2017) who conducted a screening assessment survey of lakes, reservoirs, and coastal lagoons in the U.S in 2013 and found that in depressional wetlands, chlorophyll-*a* was not a significant predictor of MCs concentrations. In addition, Howard *et al.* (2017) also found no correlation between total nitrogen and phosphorus with MC levels. However, findings of this study contradicted with findings from other similar studies such as Kudela (2011), who found that chlorophyll-*a* was the best single predictor for toxin loads in Pinto Lake for both grab and SPATT samples. Kim *et al.* (2021), also found that MCs were significantly correlated with chlorophyll-*a* levels ($R^2 = 0.44$, $P < 0.05$).

3.3.1.5.4 Anionic surfactants as predictor of MCs and cyanobacterial biomass

Anionic surfactants have been reported to be more common in eutrophic water bodies. Wang *et al.* (2012) and Wang *et al.* (2015) reported the co-existence of *Microcystins Aeruginosa* and anionic surfactants linear alkylbenzene sulfonate (LAS) under hypereutrophic condition. Based on that, we correlated total anionic surfactants with MCs and chlorophyll-*a*. Table 3.6 and Figure 3.8 shows that a moderate positive significant correlation was found between MC levels and total anionic surfactants concentrations in irrigation water, but no correlation with chlorophyll-*a* levels. Wang *et al.,* (2015) reported that low LAS (< 10 mg L^{-1}) concentration improved the growth of *M. aeruginosa*, and similar levels were observed in this current study (Figure 3.5). These findings suggests that anionic surfactants maybe a having a moderate influence on MC levels in the irrigation water and could be used as a predictor of MCs levels in the irrigation water from these two dams.

Table 3.6. Spearman correlation coefficients between anionic surfactants levels vs MCs and chlorophyll-a in irrigation water

	Anionic surfactants (mg L^{-1})
Chlorophyll-*a* (µg L^{-1})	0.216
Microcystins (µg L^{-1})	0.342*

*Correlation is significant at 0.05 level (2-tailed); **Correlation is significant at 0.01 level (2-tailed)

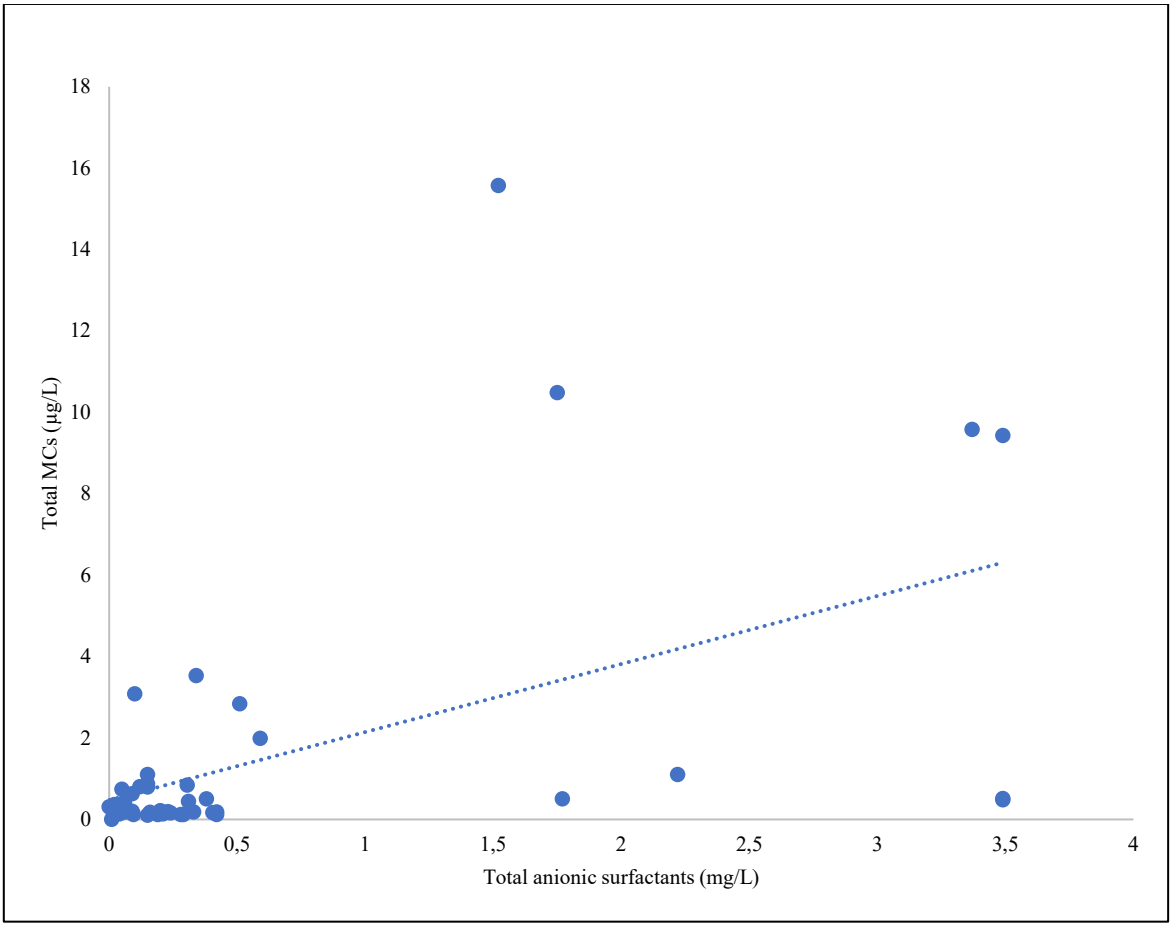

Figure 3.8. Correlation matrix between anionic surfactants vs MCs levels (Spearman correlation, P<0.05)

3.3.2 Solid Phase Adsorption Toxin Tracking (SPATT) technology for monitoring cyanotoxins in irrigation canals and farm dams of Roodeplaat and Hartbeespoort sites

3.3.2.1 Laboratory trial

Figure 3.9 (A) shows the levels of MCs adsorbed by the SPATT samplers over the duration of the experiment. The findings show that levels of the toxins adsorbed by the samplers were comparable over the 72 hr duration of the trail (0.025 µg g^{-1} after 24 hrs, 0.026 µg g^{-1} after 48 hrs and 0.025 µg g^{-1} resin after 72 hrs). With regards to the residual MCs levels in the water upon exposure to the samplers, Figure 3.9 (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. This indicated that SPATT samplers loaded with 3 g of the resin HP20 in 1 L of raw dam water with initial concentration of ±9 µg L^{-1}, the samplers either took up all the available MCs in solution and/or got saturated within 24 hrs.

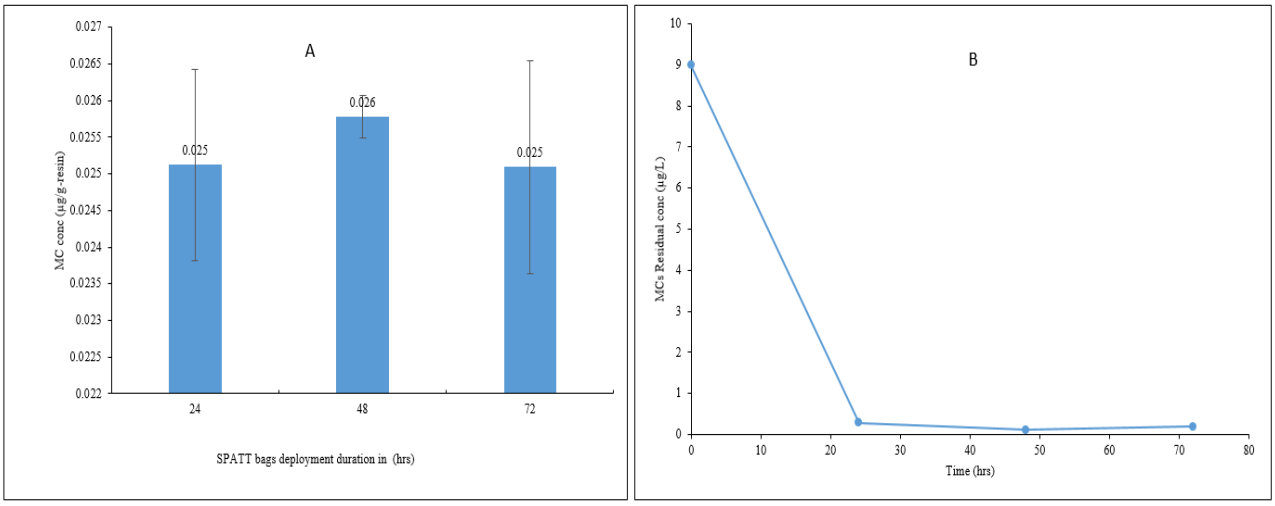

Figure 3.9. A) Variation of MCs (µg g^{-1}) adsorbed by resin with exposure time of SPATT bags in field water during laboratory exposure. B) The residual concentrations of microcystins (µg L^{-1}) after retrieval of SPATT bags over time

3.3.2.2 SPATT field deployment

3.3.2.2.1 Physicochemical parameters

Upon deployment of the SPATT samplers in the canals and farm dams, physicochemical parameters were also monitored. Table 3.7 shows the physicochemical characteristics of the water sampled at each sampling point (canals/farm dam) where the SPATT samplers were deployed at the Roodeplaat and Hartbeespoort sites. The pH of the water samples ranged from 7.04 ± 0.59 to 10.06 ± 0.75, indicating that the water was ranging from neutral to strong alkaline. The pH of the water at sampling

points R2, R3 and H3 were above the DWAF (1996) guideline for irrigation. Total dissolved solids (TDS) and electrical conductivity (EC) also varied between the sampling sites and sampling months and ranged from 202.4 ± 36.20 to 309.5 ± 67.78 mg L^{-1}, and 336.5 ± 61.52 to 519.5 ± 112.53 µS cm^{-1}, respectively. The total dissolved solids and electrical conductivity were above the 0-260 mg L^{-1} and 0-400 µS cm^{-1} DWAF guideline value set for irrigation water at site H1, H2, and H3, respectively.

Temperature did not vary much among sampling sites and throughout sampling months and ranged from 22.4 ± 1.27 to 29.95 ± 5.59 ˚C. In addition, the cyanobacterial biomass as chlorophyll-*a* ranged from 31.17 ± 14.79 to 526.1 ± 655.80 µg L^{-1}. According to Kansas Department of Health and Environment (KDHE) (2011) these levels of chlorophyll-*a* above the 10 µg L^{-1} threshold, indicates the likelihood, of rapid growth of HABs in the water system. The chlorophyll-*a* recorded in all sampling sites indicate that the irrigation canals and farm dams were falling under hyper-eutrophic category (chlorophyll-*a* > 30 µg L^{-1} (Kansas Department of Health and Environment (KDHE), 2011).

The levels of nitrates and phosphates in the water ranged from 4.25 ± 1.91 to 6.47 ± 3.28 mg L^{-1} and 0.2 ± 0.03 to 1.01 ± 0.06 mg L^{-1} respectively. Turbidity and DO ranged from 1.66 ± 1.23 to 366.03 ± 315.12 NTU and 3.03 to 16.2 mg L^{-1}, respectively for the sampling duration. The phosphates levels in all sampling sites during the sampling months were above the 0.25 mg L^{-1} threshold, indicating the likelihood of HABs (Balcıoğlu, 2019).

Table 3.7. Physicochemical characteristics of irrigation water recorded from Roodeplaat and Hartbeespoort irrigation canals and farm dams

Water Quality parameters	R1	R2	R3	H1	H2	H3	DWAF (1996) for irrigation water
pH	8.37 ± 0.77	10.06 ± 0.75	9.79 ± 1.08	7.58 ± 0.04	7.04 ± 0.59	9.28 ± 0.09	6.5-8.4
TDS (mg L^{-1})	217.6 ± 68.45	206.5 ± 23.33	202.4 ± 36.20	309.5 ± 67.78	304.5 ± 85.56	277 ± 16.97	0-260
EC (µS cm^{-1})	361.5 ± 108.19	341.5 ± 41.72	336.5 ± 61.52	519.5 ± 112.43	510.5 ± 136.47	461.5 ± 30.41	0.400
Temperature (°C)	22.4 ± 1.27	26.8 ± 8.49	29.95 ± 5.59	23.2 ± 0.28	22.6 ± 0.0	25.9 ± 4.81	n. a
Nitrates (mg L^{-1})	6.15 ± 2.97	5.52 ± 3.23	6.16 ± 2.22	6.47 ± 3.28	4.25 ± 1.91	4.85 ± 2.98	< 5
Phosphates (mg L^{-1})	0.55 ± 0.19	0.2 ± 0.03	0.29 ± 0.14	0.99 ± 0.10	1.01 ± 0.06	0.38 ± 0.12	n. a
Chlorophyll-*a* (µg L^{-1})	49.01 ± 5.73	183.96 ± 39.44	326.41 ± 79.83	58.24 ± 53.06	31.17 ± 14.79	526.1± 655.80	n. a
Turbidity (NTU)	6.56 ± 2.91	366.03 ± 315.12	32.94 ± 34.80	1.66 ± 1.23	2.00 ± 1.42	42.9 ± 10.49	n. a
DO (mg L^{-1})	7.89	16.2	15.51	3.64	3.03	13.08	n. a

Total mean MCs detected from grab samples (µg L^{-1}) and SPATT samplers resin (ng/g resin) in Roodeplaat and Hartbeespoort irrigation canals and farm dams collected in February 2020 and March 2021 are shown in Figure 3.10 (A & B). The total MC levels in grab samples and SPATT samples ranged from 0.14 to 13.03 µg L^{-1} and 0.99 to 2.28 ng g^{-1} resin as shown in (Figure 3.10 (A & B), respectively. Both Grab and SPATT sampling method revealed presence of toxins MCs in all sampling sites of Roodeplaat and Hartbeespoort throughout the sampling months. Grab samples showed high levels of MCs in the month of February, except for sites R2 and R3 where the levels of MCs were higher in March. The SPATT samplers detected higher MCs in February and compared to March, except for site H2 and H4 where the levels of MCs were higher in March.

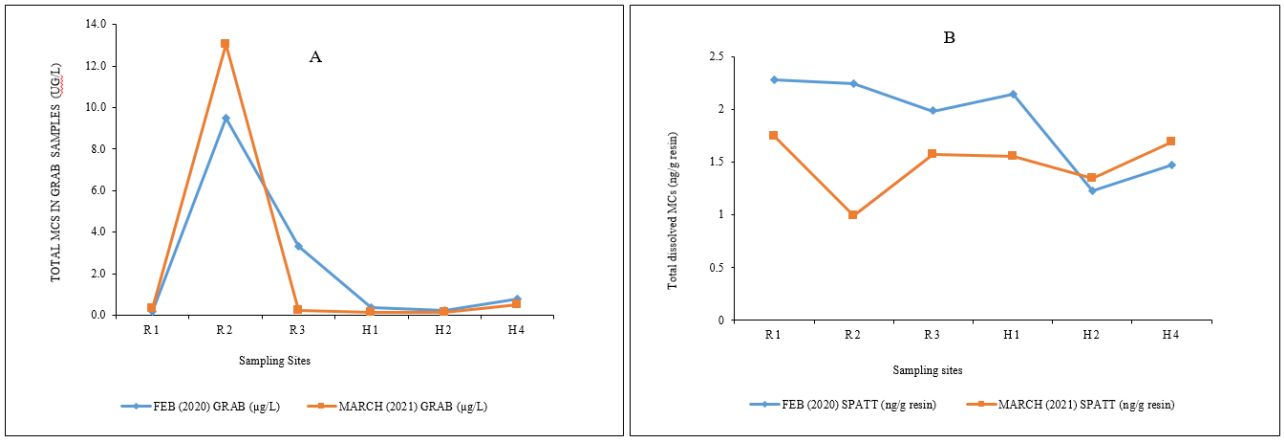

Figure 3.10. The mean total MCs concentrations in Grab samples (A) and SPATT bags collected (B) from Roodeplaat and Hartbeespoort irrigation canals and farm dam's sites

In most cases (particularly for sampling points R1 and all the Hartbeespoort points), grab samples detected very low levels of MCs compared to SPATT samplers which easily detected MCs in all the sampling points. This is because grab sampling can underestimate levels of toxins in the water column or miss peak episodic events of HABs and toxin release in water since it gives a snapshot of the levels of toxins in a water column at that point and that time.

In comparison, SPATT samplers detect MCs in the water over a period of time, giving an integrated reflection of toxins over space and time in water bodies. Our findings were supported by previous studies, for example Davis and Hansen (2013) found that SPATT samplers could detect MCs even where grab samples could not detect. The persistence of low levels to sometimes high levels of MCs in the irrigation water derived from the two dams is a call for concern since MCs can end up accumulating in edible parts of the plants being irrigated and eventually posing health risks to humans when they consume these plants.

3.3.2.2.2 Correlation between the environmental parameters and MCs

Table 3.8 shows the Spearman correlation coefficients MCs detected by grab and SPATT samples vs physicochemical variables monitored**.** No correlation was found between cyanobacterial biomass as chlorophyll-*a* and total MCs detected by Grab ($r = 0.208$, $P > 0.05$) and SPATT samplers ($r = -0.441$, $P > 0.05$). Unlike our findings, Kudela (2011), found chlorophyll-*a* as the best predictor for toxins in both Grab and SPATT samples in Pinto Lake. Lehman *et al.* (2010) also found a positive correlation between chlorophyll-*a* and *Microcystis* abundance and total MCs in San Francisco Estuary.

Out of all the monitored physicochemical parameters, pH (r = 0.776 **), turbidity (r = 0.699*) and dissolved oxygen (r = 0.829*) had a strong positive correlation with MCs levels in Grab samples. This suggests that these three physicochemical parameters could be used to try and predict/estimate MC levels in the irrigation water monitored. With regards to total dissolved toxins in SPATT samplers, only TDS and EC had a moderate negative association with the toxins detected in SPATT samplers. In addition, there was no correlation between total dissolved MCs in SPATT samplers and total MCs in grab samples over the sampling method. Unlike our findings, Kudela (2011) found the total MCs in grab and SPATT samplers to be having a good correlation (Spearman $r = 0.735$, $p < 0.001$) in Pinto Lake.

Table 3.8. Spearman correlation coefficient (r) for MCs in Grab and SPATT vs physicochemical parameters

	SPATT (ng g^{-1})		Grab (µg L^{-1})	
Physicochemical variables	Correlation (r)	P-Value	Correlation	p-Value
pH	0.098	0.762	0.776**	0.003
TDS (mg L^{-1})	-0.615*	0.033	-0.462	0.131
EC (µs cm^{-1})	-0.602*	0.038	-0.462	0.130
Temperature (˚C)	0.308	0.331	0.133	0.681
Turbidity (NTU)	-0.196	0.542	0.699*	0.011
Microcystins SPATT (ng g^{-1} resin day^{-1})			0.049	0.880
Microcystins Grab (µg L^{-1})	0.049	0.880		
Nitrate (mg L^{-1})	0.280	0.379	-0.231	0.471
Phosphate (mg L^{-1})	-0.402	0.195	-0.049	0.879
Chlorophyll-*a* (µg L^{-1})	-0.441	0.152	0.392	0.208
DO (mg L^{-1})	0.371	0.468	0.829*	0.042

3.3.2.2 Composition of cyanobacteria

The composition of various cyanobacterial species in the irrigation water from the irrigation canals and farm dams in the month of March (2021) was determined using a Benchtop Flow-Cam (Model US-IV). The following harmful cyanobacterial species were found across the sampling sites:

Microcystis, *Anabaena* and *Oscillatoria* (Appendix C and D). The *Microcystis genus* was the most abundant and the most frequently observed genus across all sampling sites, demonstrating that this specie is the most widespread in freshwater ecosystems.

This finding is of concern since the M*icrocystis* genus is the most common bloom forming, more poisonous and have potential to produce high levels of MCs which accumulate in food crops via irrigation (Bouaïcha & Corbel, 2016; Machado *et al.*, 2017a; Preece *et al.*, 2017). Acute and chronic exposure to MCs can promote tumour, and cause liver failure (Huo *et al.*, 2018; Greer *et al.*, 2018).

3.3.3 Bioaccumulation of microcystins and metal species in vegetable crops and their potential human health risk

3.3.3.1 MCs in edible crops

The levels of MCs detected in edible crops are presented in Table 3.11. The results for MCs accumulated in Beetroot, Corn flower, Onion leaves, Onion bulb, Wheat grains, Soybean H3, and Soybean plant H4 were found to be 0.002 µg kg^{-1}; 0.006 µg kg^{-1}; 0.017 µg kg^{-1}; 0.019 µg kg^{-1}; 0.013 µg kg^{-1}; 0.047 µg kg^{-1} and 0.122 µg kg^{-1}, respectively (Table 3.11). The highest level of MCs accumulated was observed in plants collected in February 2020, while the lowest was observed in plants collected in June 2019. A pattern of low MCs accumulation in June, moderate in September and high in February was observed throughout the sampling period. The seasonal variation of MCs bioaccumulation might be due to the different plant species taking up MCs at different rates and also the exposure levels to MCs, time, and the congener type that they were exposed to as suggested by (Bittencourt-Oliveira *et al.*, 2016).

The levels of MCs accumulated in plants throughout the sampling periods were lower compared to the ones reported by Bittencourt-Oliveira *et al.* (2016).). The calculated EDI values based on the levels of MCs found in the food crops were below the 0.04 µg kg^{-1} guideline set by the World Health Organization (WHO, 1998), for both adults and kids throughout the sampling period. This implied that the plants do not pose any health risk to people consuming them. The MCs levels, and EDI values observed in all food crops throughout the sampling months in this current study were way much lower than those reported by (Chen *et al.*, 2012; Zhu *et al.*, 2018).

Table 3. 9. Accumulated MCs (µg kg^{-1}) and calculated EDI in sampled food crops

	Sampling sites	Plant type	MCs (µg kg^{-1})	Adults (µg kg^{-1})	Children (µg kg^{-1})
Roodeplaat					
June (2019)	S5	beetroot bulb	0.002	0.000003	0.000008
	S5	cauliflower	0.006	0.000009	0.000024
Sept (2019)	S5	Onion leaves	0.017	0.000026	0.000068
		Onion bulb	0.019	0.000029	0.000076
Hartbeespoort					
Sept (2019)	S3	wheat grains	0.013	0.00002	0.000052
Feb (2020)	S2	soybean	0.047	0.000072	0.000188
	S3	soybean	0.122	0.000187	0.000488

3.3.3.2 Metals species in edible plants

The levels of metal species in the edible part of the vegetable samples collected around Roodeplaat and Hartbeespoort farmland sites are presented in Table 3.12. The results revealed that the levels of Aluminum (Al) ranged from 97.04 to 668.00 mg kg^{-1}, respectively. The vegetable control plant sample collected from Rietvlei site had the highest level of Al compared to other plant samples.

The levels of Cr, Mn, and Fe in vegetable samples ranged from 0.21 to 10.80 mg kg^{-1}; 3.30 to 86.00 mg kg^{-1}; and 19.64 to 734.00 mg kg^{-1}, respectively. For Cr, all vegetable samples had concentrations above the FAO/WHO, (2007) in Chiroma & Ebewele (2014) and European Commission (EC, 2006) permissible for food crops, except for soybean plants collected at site S3. In addition, Fe was observed to be above the guideline value set by FAO/WHO, (2007) and EC, (2006) for medicinal plant (Serokolo) wild ginger collected from site S4, and soybean from site S1, and a control plant from Rietvlei site.

As can be seen from Table 3.12. Ni, Cu, and Zn concentrations in edible parts of the plant's samples ranged from 0.23 to 6.20 mg kg^{-1}; 0.96 to 60.40 mg kg^{-1}; and 5.45 to 76.80 mg kg^{-1}, respectively. Soybean plant sample collected from site S4 had high level of Zn which was above the FAO/WHO, (2007) and EC, (2006) permissible guideline value set for food crops. While, for Cu only soybean from site S5 and control plant from Rietvlei were above the permissible limit guideline for food crops. Also, As, Cd, and Pb concentrations in edible part of the plant samples ranged from 0.01 to 0.20 mg kg^{-1}; 0.01 to 0.06 mg kg^{-1}; and 0.10 to 0.70 mg kg^{-1}, respectively. Lead (Pb) concentration in all plant's samples were above the FAO/WHO, (2007) and EC, (2006) permissible guideline value for food crops. The overall metal species accumulation in all collected plant samples followed the

decreasing order: Al > Fe > Mn > Zn > Cu > Cr > Ni > Pb > As > Cd, respectively. Comparing the data obtained from the current study the toxic metals in plant samples such as Zn, Cu, Ni, Cr, Fe and Mn were way much higher than the one reported by (Gebeyehu & Bayissa (2020), in tomato and cabbage crops in their study in Mojo area (central Ethiopia).

The high levels of metals such as Cr and Fe in the sampled plants, can be explained by the Cr mining activities taking place next to the farms around Brits, with the mine dumps and waste possibly contaminating the cultivated land. The elevated metals in the agricultural fields might have been also introduced via application of organic and inorganic fertilizers, and other agrochemicals such pesticides and herbicides. The accumulation of non-essential metals such as Pb, As, Cr, and Cd in all collected food crops is of concern because these metals are highly toxic even at very low concentrations and might pose human health risks via consumption of the contaminated food crops over in the long-term.

Table 3.10. Metals (mg kg^{-1}) (N = 14) in edible food crops sampled

	S2	S3	S2	S4	S1	S5	Rietvlei (plant Controls)		
Metals	**Soybean**	**Soybean**	**Soybean**	**Wild ginger**	**Soybean**	**Soybean**		**FAO/WHO, 2007 (a)**	**EU, 2006 (b)**
Al	121.38	97.04	343.00	455.00	645.00	356.5	668.00	n. a	n. a
Cr	1.28[b]	0.21	4.10[b]	3.40[b]	4.70[b]	7.3[b]	10.80[a]	20.0	1.0
Mn	18.62	3.30	43.00	76.00	54.00	50.5	86.00	500	500.0
Fe	50.05	19.64	128.00	486.00[a]	477.00[a]	260.0	734.00[a]	450.0	n. a
Ni	1.74	0.23	5.50	2.00	9.20	3.95	7.80	68.0	n. a
Cu	3.50	0.96	10.50	8.10	13.10	51.7[ab]	60.40[ab]	40.0	20.0
Zn	10.92	5.45	30.90	76.80[ab]	37.90	23.25	37.40	60.0	50.0
As	0.02	0.01	0.10[ab]	0.20[ab]	0.10[ab]	0.1	0.20[ab]	0.5	0.2
Cd	0.03	0.01	0.01	0.06	0.02	0.03	0.01	0.2	0.2
Pb	0.38[a]	0.10[a]	0.20[a]	0.70[ab]	0.20[a]	0.45[ab]	0.70[ab]	0.3	0.43

[a] indicates values above FAO/WHO threshold for metals in food crops, [b] indicates values above EU, 2006 threshold for metals in food crops, [ab]indicates values above both FAO/WHO (2007) and EC, (2006) maximum guideline values for metals in food crops

3.3.3.3 Metals in agricultural soils

The levels of metal species in soil samples collected at sampling points where vegetable samples were collected were determined to evaluate the translocation factor of metals from the soil to the edible parts of the vegetable samples. The obtained data of metals in soil samples collected from Roodeplaat and Hartbeespoort farmland sites are presented in Table 3.13. Soil samples collected from all sampling sites had presence of all metal species analyzed in this study. The mean concentration of metal species Al, Cr, Mn, Fe, Ni, Cu, Zn, As, Cd and Pb ranged from 13110 to 42135 mg kg^{-1}; 87.75 to 723.40 mg kg^{-1}; 17064.53 to 34665 mg kg^{-1}; 39.8 to 166.30 mg kg^{-1}; 17.27 to 31.45 mg kg^{-1}; 29.26 to 86.5 mg kg^{-1}; 0.75 to 9.30 mg kg^{-1}, 0.04 to 0.06 mg kg^{-1}; and 3.45 to 19.5 mg kg^{-1}, respectively. The mean levels of As, Cr, Mn, Ni, Cu and Fe were found to be higher than the guideline limit set by DEA (2010), FAO/WHO (Chiroma & Ebewele, 2014), and US Environmental Protection Agency (2011) for agricultural soils. The obtained levels of metals in soil samples clearly indicates that the cultivated soils in the study area are contaminated by metals.

The concentrations of As, Pb, Cd, Zn, Fe, and Mn obtained from the soil samples in this study were found to be lower than the ones found in cabbage and tomato samples by Gebeyehu and Bayissa (2020). In addition, levels of nickel (Ni), copper (Cu) and (Cr) in cultivated soils were found to be much higher than those reported by (Gebeyehu & Bayissa (2020). Our findings indicated that that the cultivated soils in Roodeplaat and Hartbeespoort sites are contaminated by toxic metals such as As, Cu, Cr, Ni, Mn, and Fe as their content exceeded the thresholds set for agricultural purposes.

Table 3.11. Levels of metal species (mg kg^{-1}) in agricultural soils samples collected from Roodeplaat and Hartbeespoort farmlands collected crops are grown

Metals	S1	S2	S2	S3	S4	S5	Controls	DEA (2010) **(a)**	FAO/WHO (Chiroma *et al.*, 2014) **(b)**	USEPA (Ahmad *et al.*, 2019) **(c)**
Al	30755.00±19	36090.	42135.00±5	30872	13110.00±	13085.00±	10622±1459	n. a	n. a	n. a
Cr	723.40±27.72	111.07	225±6.08[ab]	320.2	87.5±5.94[a]	87.75±2.62	250±98.99[ab]	6.5	100	n. a
Mn	821.30±23.62	734.66	459.85±6.08	407.1	982±84.85	2715±569.	625±304.06	n. a	n. a	2000
Fe	33285.00±13	22240.	22870±113.	17064	35560±274	34665±304	51335±1707	n. a	n. a	21000
Ni	166.30±6.36[a]	76.59[b]	80.9±1.84[b]	101.5	29.6±2.12	39.8±4.81	76.94±50.81	91	50	n. a
Cu	26.00±2.55[a]	17.27[a]	31.45±13.79	17.90[a]	25.1±1.13[a]	29.85±3.46	48.13±16.49	16	100	n. a
Zn	59.35±2.19	37.19	37.25±0.78	29.26	86.5±8.20	46.9±0.42	52.40±26.78	240	300	n. a
As	1.15±0.07	1.18	0.75±0.07	9.30[a]	7.65±1.06[a]	6.15±2.90[a]	6.02±3.03[a]	5.8	20	n. a
Pb	6.8±0.71	7.62	3.45±0.07	5.45	8.25±1.06	19.5±2.97	11.23±0.400	20	100	n. a
Cd	0.06±0.01	0.046	0.05±0.00	0.041	0.05±0.01	0.05±0.01	0.03±0.01	7.5	3.0	n. a

[a] ≥ DEA (2010) set threshold, [b] ≥ FAO/WHO set threshold, [c] ≥ USEPA set threshold, [ab] ≥ DEA (2010) and FAO/WHO (2007)

3.3.3.4 Metals in irrigation water

The levels of metals in the irrigation water were monitored to evaluate the potential transfer of metals from the irrigation water to the irrigated crops. The results of mean concentrations of metals species in irrigation water samples collected from Roodeplaat and Hartbeespoort irrigation canals and farm dams are presented in Table 3.14. The mean levels of metal species in water samples ranged from (Al) 0.01 to 0.88 mg L^{-1}, (Cr) 0.00 to 0.002 mg L^{-1}, (Mn) 0.010 to 1.24 mg L^{-1}, (Fe) 0.04 to 1.13 mg L^{-1}, (Ni) 0.003 to 0.006 mg L^{-1}, (Cu) 0.001 to 0.004 mg L^{-1}, (Zn) 0.025 to 0.09 mg L^{-1}, (As) 0.001 to 0.003 mg L^{-1}, and (Pb) 0.000 to 0.001 mg L^{-1}. Metal species observed from this study in the irrigation water were all within the acceptable limit set by DWAF (1996) and FAO/WHO (2007) for water meant for irrigation purpose.

The spearman correlation coefficient (r^2) between metals in irrigation water and plants samples were determined to evaluate if the metals in irrigation water are being transferred into crops via irrigation. Among all the measured metals only Lead (r = 0.874*) and Arsenic) (r = 0.809*) had a moderate positive association with metals in irrigation water and plants. Thus, indicating that Pb and As in irrigation water are being transferred into food crops via irrigation, and eventually accumulate into food crops, resulting in human health risks through consuming the crops.

Table 3.12. Levels of metal species (mg L^{-1}) in irrigation water samples collected from Roodeplaat and Hartbeespoort irrigation canals and farm dams

Sampling sites	H1	H3	H3	H4	R2	R3	FAO (1985)	DWAF (1996)
Al	0.02±0.01	0.01±0.00	0.010	0.293	0.88±0.09	0.45±0.26	5.0	5-20
Cr	0.001±0.001	0.00±0.00	0.001	0.002	0.001±0.001	0.00±0.00	n. a	0.1-1.0
Mn	0.81±0.02	0.81±0.06	1.24	0.37	0.21±0.002	0,010±0.002	0.2	0.02-10
Fe	0.07±0.001	0.04±0.03	0.12	1.13	0.13±0.005	0.05±0.02	n. a	5-20
Ni	0.004±0.00	0.003±0.00	0.004	0.006	0.005±0.001	0.005±0.002	0.2	0.2-2.0
Cu	0.001±0.00	0.001±0.00	0.001	0.001	0.01±0.001	0.004±0.001	0.2	0.2-0.5
Zn	0.09±0.03	0.03±0.01	0.012	0.025	0.06±0.04	0.01±.001	2.0	1.0-5.0
As	0.002±0.00	0.002±0.001	0.001	0.001	0.003±0.001	0.001±0.00	0.1	0.1-2.0
Pb	0.0001±0.000	0.0002±0.0002	0.000	0.000	0.001±0.0001	0.0002±0.0001	5.0	0.2-2.0

#FAO 1985 and DWAF (1996) maximum guideline values for irrigation water

3.3.3.5 Health Risk Analysis

The estimated daily intake (EDI) was determined using the mean concentrations of each metal species in the sampled food crops and the results are presented in Table 3.15. The estimated daily intake of the metals considered in this study were determined based on their mean concentration in each plant and the estimated daily consumption of the vegetables in gram. The EDI value of each metal of interest was determined by the formula used by Gebeyehu & Bayissa (2020) with slight modifications as presented in Eq. (2).

$$EDI = \frac{E_f * E_D * F_{IR} * C_M * C_f}{B_W * T_A} \quad [7]$$

Where:

E_f is exposure frequency (365 day/year);
E_D is the exposure duration (65 years), equivalent to average life time;
F_{IR} is the average food (vegetable) consumption (240 g/person/ day), which was derived from the World Health Report (Gebeyehu & Bayissa, 2020) for low fruit and vegetable intake; C_M is metal concentration (mg kg^{-1} dry weight);
C_f is concentration conversion factor for fresh vegetable weight to dry weight (which is 0.085);
B_W is reference body weight for an adult, which is 70 kg; and
T_A is the average exposure time (65yrs x 365 days) and 0.001 is unit conversion factor.

The EDI values for Al, Cr, Mn, Fe, Ni, Cu, Zn, As, Cd, and Pb were found to be ranging from 0.028 to 0.19 mg kg^{-1}; 0.00006 to 0.002 mg kg^{-1}; 0.0009 to 0.02 mg kg^{-1}; 0.005 to 0.14 mg kg^{-1}; 0.00007 to 0.003 mg kg^{-1}; 0.0003 to 0.04 mg kg^{-1}; 0.0030 to 0.02 mg kg^{-1}; 0.000003 to 0.00006 mg kg^{-1}; 0.000003 to 0.00002; and 0.0003 to 0.0002 mg kg^{-1}, respectively. The EDI values obtained for each metal in the plant samples were below the maximum tolerable daily intake of each metal species in food as indicated in Table 3.15.

The EDI for all collected plant samples across the sampling sites followed the decreasing order: Al > Fe > Mn > Cu > Zn > Cr > Ni > Pb > As > Cd, respectively. The EDI values of each metal obtained from the edible plants were lower than the data reported by Gebeyehu and Bayissa (2020) for tomato and cabbage.

Table 3.13. Estimated daily intake (EDI mg day^{-1} kg^{-1} body weight, N=14) of metals in food plants sampled

	S2	S3	S2	S4	S1	S5	
Metal	**Soybeans**	**Soybeans**	**Soybean**	**Serokolo (*Siphonochilus aethiopicus*)**	**Soybean**	**Soybean**	**MTDI**
Al	0.035	0.028	0.10	0.13	0.19	0.10	n. a
Cr	0.0003	0.00006	0.001	0.0009	0.001	0.002	0.2
Mn	0.005	0.0009	0.01	0.02	0.02	0.01	2.0-5.0
Fe	0.015	0.005	0.04	0.14	0.14	0.08	15.0
Ni	0.0005	0.00007	0.002	0.0006	0.003	0.001	0.3
Cu	0.001	0.0003	0.003	0.002	0.04	0.02	2.5-3.0
Zn	0.003	0.0030	0.009	0.02	0.01	0.007	60.0
As	0.000006	0.000003	0.00003	0.00006	0.00003	0.00003	0.13
Cd	0.00001	0.000003	0.000003	0.00002	0.000006	0.000009	0.021
Pb	0.0001	0.00003	0.00006	0.0002	0.00006	0.0001	0.21
∑ EDI	0.06	0.04	0.16	0.31	0.40	0.22	

MTDI (Maximum Tolerable Daily Intake)

3.3.3.6 Translocation factor

The translocation factor was determined to evaluate the potential transfer of metal species from soil to edible parts of the food plants, as this acts as an indirect entry route of toxic metals into the food chain. The translocation factors for all plants species collected from the cultivated lands around Roodeplaat and Hartbeespoort sites are shown in Table 3.16. From the obtained data, the translocation factor for Cd TF= 1.33 in Soybean plant collected from site S4 was > 1, indicating that Cd was rapidly being transported from the soil to the edible part of the plants. Also, Zn, TF = 1.73 in a soybean plants collected from site S5 was > 1.

From the data in Table 3.16, the transfer factors were decreasing in the following order of Cd > Cu > Zn > Pb > Ni > Mn > As > Al > Cr > Al for all collected food plants across the sampling sites, throughout sampling period. The translocation factor values observed from this current study were lower than the one reported in a similar study carried by (Gebeyehu & Bayissa (2020) in tomatoes and cabbages, except for Cd from sampling site S3 and S4, and Cu from sampling site S5 which were observed to be higher than those reported by (Gebeyehu & Bayissa, 2020).

Table 3.14. Translocation factors between vegetable crops and soils collected from Roodeplaat and Hartbeespoort farmland sites

Metal species	S2	S3	S2	S4	S1	S5
Al	0.03	0.003	0.008	0.03	0.002	0.03
Cr	0.01	0.001	0.02	0.04	0.006	0.08
Mn	0.03	0.008	0.09	0.08	0.07	0.02
Fe	0.02	0.001	0.000	0.01	0.01	0.01
Ni	0.02	0.05	0.07	0.07	0.06	0.10
Cu	0.20	0.05	0.33	0.32	0.50	**1.73**
Zn	0.29	0.19	0.83	0.09	0.64	0.27
As	0.02	0.001	0.13	0.03	0.09	0.02
Cd	0.6	0.25	0.2	**1.33**	0.36	0.67
Pb	0.05	0.02	0.06	0.89	0.03	0.02

3.4 SUMMARY

The co-existence of cyanotoxins, metals and anionic surfactants in irrigation water (canals and farm dams) and agricultural soils around Roodeplaat and Hartbeespoort Dam sites. Also, the relationship between MC levels and physicochemical parameters in irrigation water were investigated. Our field sampling revealed the presence of cyanotoxins, metals and anionic surfactants in irrigation water (canals and farm dams), and agricultural soils in all the sampled sites during the sampling period. It also emerged that pH, turbidity, EC, and TDS have a correlation with levels of MCs in the irrigation water, thus suggesting that these could be used to predict MCs levels/presence in the irrigation water from the two dams.

Findings also demonstrated the applicability of SPATT using the resin DION HP20 for the passive sampling of MCs in the irrigation water as it increased the likelihood of detecting MCs in instances where grab samples would miss or detect very low levels of the toxins. This is of significance, since grab sampling could miss some episodic HAB events, thus SPATT could be used to complement grab sampling and give early warnings in water intended for irrigation purposes. Among toxic cyanobacterial genus identified in the irrigation water from the two dams were *Microcystis,* A*nabaena* and O*scillatoria*, with *Microcystis* being the most dominant throughout the sampling sites.

The study also found that metals in irrigation water were below the DWAF (1996) recommended threshold while in agricultural soils, metals like Cr, Ni, Cu, Pb, and As were above the guideline values set for agricultural soils. The findings also showed that MCs and metals do accumulate in food crops when irrigated with contaminated water. The calculated estimated daily intake (EDI) for MCs in the collected food plants was below the WHO guideline threshold of 0.04 μg kg^{-1}. Thus, plants being irrigated by water from these two dams are still safe for human consumption.

Among the metals of concern in the food plants, Cr, Fe, Cu, Zn, As, and Pb were found above the EU and FAO/WHO threshold for these metals in food crops. However, the calculated EDI for each of the metals detected were below the maximum tolerable daily intake (MTDI), thus implying that the plants being irrigated by water from the two dams are still safe for human consumption.

The presence of anionic surfactants, metals species and cyanotoxins in irrigation water and agricultural soils in the study area as reported in the study is of concern since the risk of indirect

exposure of humans and animals via consumption of contaminated plants remains high. This is because anionic surfactants are known to promote the uptake of cyanotoxins and metals and thus increase the risk to humans. There is thus need to regularly monitor, manage and control the three pollutants investigated in this study (cyanotoxins, anionic surfactants and metals) in light of the synergic impacts of these pollutants on plants and the poor quality of sewage effluent discharge into the catchment, mining and industrial activities around the area.

CHAPTER 4: BIOACCUMULATION AND ELIMINATION (TOXICOKINETICS) OF CYANOTOXINS BY PLANTS

This chapter was prepared by Glynn K. Pindihama & Gitari W. Mugera.

4.1 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) states that special attention should be given to the presence of cyanotoxins in crop plants and their degree of accumulation, so as to take special care in avoiding their contamination in human food-stuff. 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 cyanotoxins uptake, accumulation and elimination capacities in different 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 experiments on accumulation capacity (i.e. bio-concentration factors-BCF) and elimination capacity of plants from the field and laboratory studies were determined in order to explain accumulation of cyanotoxins when plants are irrigated with water from the two dams.

Data gathered in this section is expected to contribute to further understanding of accumulation and elimination (toxicokinetics) of different MCs congeners and other cyanotoxins on *Brassica oleracea* and *Solanum tuberosum*. This part of the study is also expected to bring new information about toxicokinetics of different MC congeners and other cyanotoxins in the different plant tissue.

4.2 MATERIALS AND METHODS

4.2.1 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 3 cc, 60 mg, were purchased from Promolab Pty Ltd T/A Separations (South Africa).

4.2.2 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 20L 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 free of cyanobacterial

contamination. The soils were collected from the top 20 cm depth, air dried and passed through a 2 mm sieve before use. Upon loading the soils into the planting bags, fourteen day old cabbage seedlings were transplanted (one plant per pot) into the bags.

The potential for toxin transfer to irrigated crops was evaluated through a field experiment. Replicate cabbage and potato plants were grown with irrigation using raw dam water collected from Roodeplaat Dam and with visible cyanobacterial cells. Controls were run concurrently with the treatments (i.e. watered using cyanotoxins 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 cabbage plants was introduced where the plants were watered with 50% (v/v) field-water/deionised water. Figure 4.1 shows the layout of the plants after 27 days of planting.

Figure 4.1. Layout of the experimental set-up showing *Brassica oleraceaon* the left and *Solanum tuberosum* on the right

Plant leaves, roots, tubers, shoots were harvested at intervals and the entire plants were then destructively harvested at maturity (after 84 days for potatoes and 120 days cabbages). Samples of roots, tubers, shoots and leaves and soil were freeze-dried and subsequently toxin concentrations were measured in the plant tissues and soil using the ELISA method with verification by LC-MS/MS. The experiments were conducted in triplicates of the exposed and control plants (i.e. were watered with cyanobacteria free water).

4.2.3 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.

4.2.4 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 cyanotoxins in plants were monitored.

4.2.4.1 Estimation of chlorophyll content

To determine oxidative stress induced by cyanotoxins 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 stress 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 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 UVvis spectrophotometer (SPECTROstar Nano, BMG LABTECH, Germany). Chlorophyll a and b and total chlorophyll content were calculated using the following equations:

$$Chl\ a = 13.36\ A664.2 - 5.19\ A648.6 \quad [8]$$

$$Chl\ b = 27.43\ A648.6 - 8.12\ A664.2 \quad [9]$$

$$Total\ chlorophyll = Chl\ a + Chl\ b. \quad [10]$$

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

4.2.4.2 Data analysis

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

4.2.5 Bioaccumulation and plant biochemical responses upon exposure to cyanotoxins

4.2.5.1 Cyanotoxin accumulation capacity

For better understanding of the bioaccumulation kinetics of cyanotoxins 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 cyanotoxins). To determine

environmental relevant concentrations for the cyanotoxins characterization in raw water and in biomass were conducted with LC-MS/MS.

4.2.5.2 Quantification of cyanotoxins 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°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 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 1750 rpm. The whole process of sonication and centrifuging 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).

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 be measured using the ELISA method and a triple quadrupole LC-MS/MS system (model 8045, Shimadzu Corporation, Japan).

4.2.5.2.1 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 carried out 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 ◦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 8:

$$\text{Conc in sample } (\mu g/mL) = \left(\frac{\text{Co} * \text{Vol of extract used (L)}}{\text{Volume of sample used (L)}}\right) \quad [11]$$

Where: Co = the conc 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.

4.2.5.3 Cyanotoxin accumulation capacity data analysis

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

4.2.4.4 Estimated daily intake (EDI)

To estimate the daily intake of cyanotoxins for an average size human we using equation 9:

$$\text{EDI} = \frac{T * C}{W} \quad [12]$$

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 cabbage 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.

4.3 RESULTS AND DISCUSSION

4.3.1 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 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 leaf extracts and in potato tuber extracts and these were used to quantify the toxins in

the plant samples (Figure 4.2). The MRM chromatograms of the quantification ions for the 3 MCs at a concentration of 100 µg L^{-1} are shown in Figure 4.3.

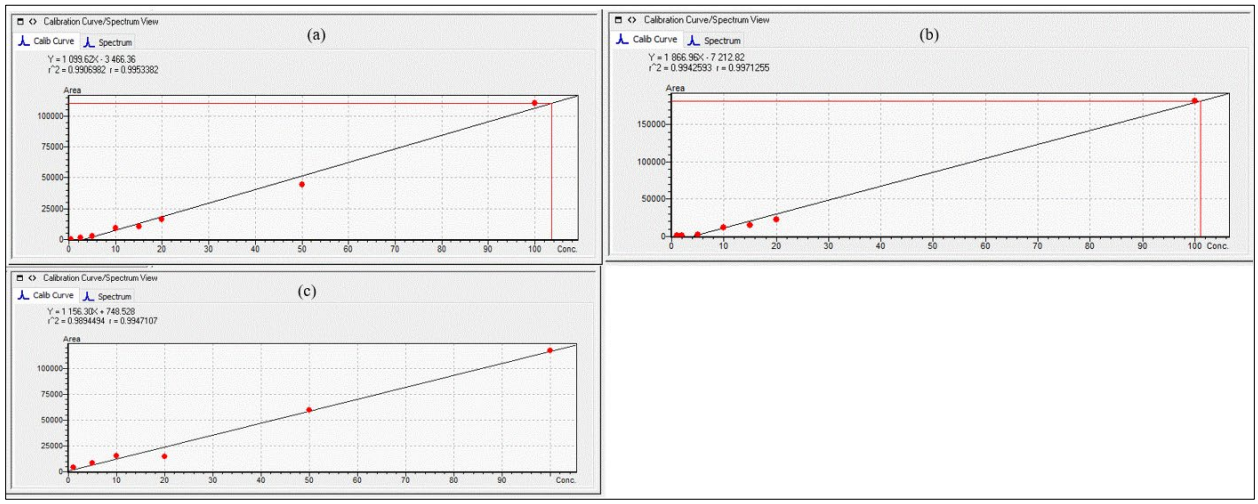

Figure 4.2. Calibration curves obtained for (a) Microcystin-RR (MC-LR); (b) MC-YR; and (c) MC-LR; in plant media.

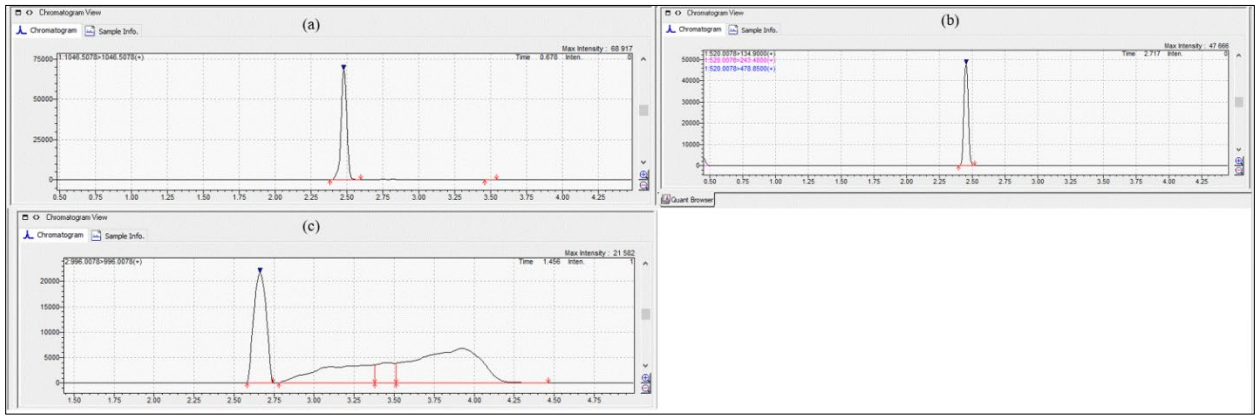

Figure 4.3. MRM chromatograms of quantification ions for the 3 MCs, (a) MC-RR; (b) MC-YR and (c) MC-LR at a concentration of 100 µg L^{-1}.

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 4.1 and 4.2 shows the cyanotoxins 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-Wallis 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 would have exceeded the total daily intake limit set by the World Health Organization.

Table 4.1. Mean (SE) of MCs accumulated in B. oleracea leaves upon 20 day exposure to the 3 treatments. Data labelled with different small letters (a-c) differed significantly at $p < 0.05$ in each row (n=6).

	Experimental	**Diluted Dam Water**	**Control**	***P*-value**	**EDI (**mg kg^{-1} of body mass day^{-1})
MCRR (µg g^{-1} DW)	0.005622 (±0.001696)[a]	0.006718 (±0.002628)[a]	0.000395 (±0.000133)[a]	0.1784 (*n.s*)	0.010
MCYR (µg g^{-1} DW)	0.004394 (±0.000263)[a]	0.002748 (±0.000494)[b]	0.0005 (±8.89E-05)[c]	< 0.0001***	0.006
MCLR (µg g^{-1} DW)	0.002073 (±0.001209)[a]	0.001709 (±0.000637)[a]	6.28E-06 (±3.67E-06)[a]	0.3371 (*n.s*)	0.003

Note: *P < 0.05, **P <0.01, ***P < 0.001, n.s = not significant.

The mean levels of MCs accumulated in *S. tuberosum* are shown in Table 4.2. Statistically significant higher levels of MC-YR and MC-LR were accumulated in the tubers ($p < .05$, 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 4.2. Mean (SE) of MCs accumulated in S. tuberosum tubers upon 20 day exposure to the 2 treatments. Data labelled with different small letters (a-c) differed significantly at $p < 0.05$ in each row (n=6).

	Experimental	**Control**	***P*-value**	**EDI** (mg kg^{-1} of body mass day^{-1})
MCRR (µg g^{-1} DW)	0.001415 (±0.000927)[a]	0.00014 (±9.15E-05)[a]	0.8361 (*n.s*)	0.002
MCYR (µg g^{-1} DW)	0.008694 (±0.000985)[a]	0.000441 (±5.85E-05)[b]	< 0.0001***	0.012
MCLR (µg g^{-1} DW)	0.135508 (±0.03338)[a]	0.014127 (±0.003535)[b]	0.0003***	0.192

Note: *P < 0.05, **P <0.01, ***P < 0.001, n.s = not significant.

Our findings did not really 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 high 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 tested here. This was contradictory to the findings of Bartos (2020) who found increasing levels of cyanotoxins 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 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.

Our findings 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 cyanotoxins via diet.

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

4.3.2 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) 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 our findings, where high MCs concentration were reported in the winter season, Mbiza (2014) reported high MCs levels in the wet season.

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.

Chlorophyll-*a* was used to estimate measure of algal biomass (Ramaraj *et al.*, 2013). South Africa like other countries across the globe, has no regulations or policies on cyanobacteria and

cyanotoxins in water intended for irrigation of food crops (Pindihama & Gitari, 2020). Algal 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.

4.3.3 Effects of MCs on *Brassica oleracea* and *Solanum tuberosum*

Previous studies have found that cyanotoxins (CYN) 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).

4.3.3.1 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 3.1 and Figure 3.1).

Table 4.3. Effect of dam water on *Brassica oleracea* seed germination

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

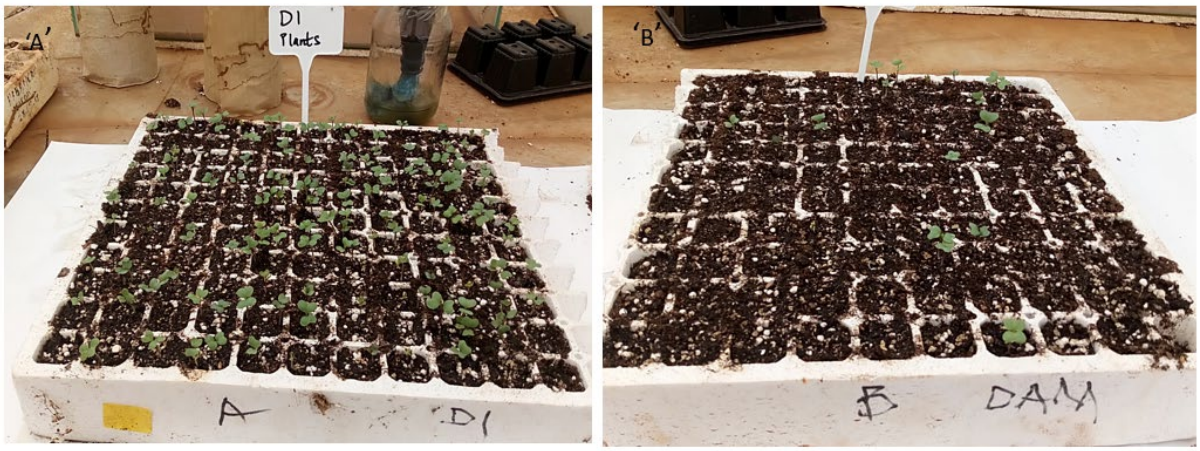

Figure 4.4. Images of germinated seeds after 8 days of watering (a) with milli-Q water (b) with dam water.

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

Purkayastha *et al.* (2010), also 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 also 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.

4.3.3.2 Effects on plant growth

Cyanotoxins 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 3.2 shows an image of the plants after 30 days of the experiment.

Figure 4.5. Image of the plants after 30 days (*Brassica oleracea* on the left and *Solanum tuberosum* on the right)

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. 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, see Appendix E).

Several studies have reported plant growth inhibition due to exposure to cyanotoxins. Growth inhibitory effects have previously been reported by several authors (McElhiney *et al.*, 2001; Crush *et al.*, 2007; Stephan Pflugmacher *et al.*, 2006). In most cases, effects on growth and leave and root development were recorded. For example, growth of potato (*Solanum tuberosum*) cultures were 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 cyanobacteria MC-extract. 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.

McElhiney *et al.* (2001), found that shown that Microcystins are inhibitors of growth and development in potato shoots and mustard seedlings under laboratory conditions. The findings suggest that exposure to Microcystins via irrigation water contaminated with toxic cyanobacteria pose a threat to the quality and yield of crop plants in the environment. The increase in growth and chlorophyll content of cultured potato shoot cultures was shown to be significantly inhibited at Microcystins-LR concentrations equivalent to 5-50 μg L^{-1} Microcystin-LR, which are representative of levels found in lake water during cyanobacterial blooms (McElhiney *et al.*, 2001).

El Khalloufi *et al.* (2011) also evaluated the effects of different concentrations of MCs on the development of symbiosis between *Medicago sativa* L. and rhizobia strains and observed a reduction in growth of both the plant and the bacterial nodules. It was also found that exposure of alfalfa seeds and seedlings to concentrations of 0, 2.22, 11.12 or 22.24 mg L^{-1} MCs affected all stages of plant development and led to reduced root length.

Overall, the inhibition effect seems to be dependent on the: (1) plant species; (2) stage of development (seedlings are generally more susceptible than adult plants); (3) time of exposure (prolonged exposures are associated with increased inhibition); (4) range of toxin concentrations applied (positive relation of toxin concentration and inhibition effects); and (5) the nature of the toxin used (e.g. purified toxin or crude extracts) (Machado *et al.*, 2017b). According to Machado *et al.* (2017b), the exposure of plants to MC-LR, either purified or contained in a crude extract, may induce histological, cytological and morphological modifications, which seem to be related to the negative impacts on the growth and development of the plants.

However, in consistent with our findings Järvenpää *et al.* (2007) did not observe any negative effects on the growth and chlorophyll 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 are able to 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.*, 2017b).

In explaining some of our 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 our study used raw dam water and natural soil conditions, it is thus not surprising that less effects on plants were reported.

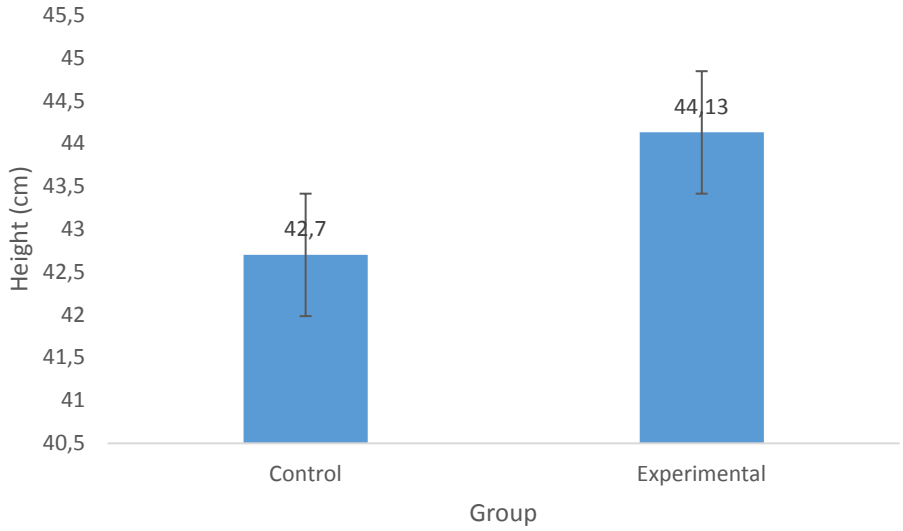

Figure 4.6. Plant height of Solanum tuberosum after 49 days

4.3.3.3 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 measured 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 3.4 and 3.5. In figure 3.4, 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. test, two-tailed P value is 0.8377, considered not significant at 95% confidence interval (CI)).

Similarly for *Brassica oleracea*, Figure 3.5 shows an increase of total chlorophyll content of the plants from the control plants to diluted dam water, with the highest total chlorophyll in raw dam water 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).

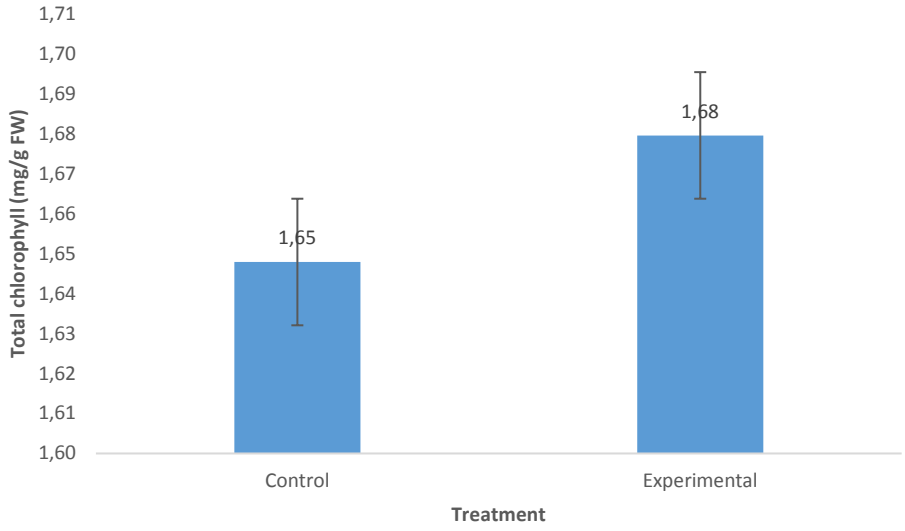

Figure 4.7. Total chlorophyll of Solanum tuberosum watered with milli-Q water and dam water

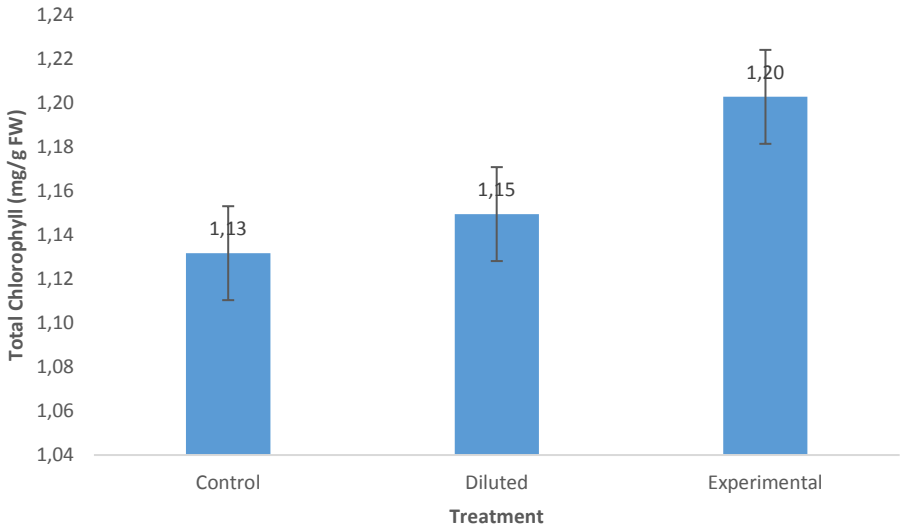

Figure 4.8. Total chlorophyll of *B. oleracea* watered with milli-Q water, diluted dam water and raw dam water

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 cultures, those exposed to toxin 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.*, 2017b). 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 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.*, 2017b). 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 our study, might not adversely affect exposed plants.

4.4 SUMMARY

The aim of this Chapter was to investigate the uptake and accumulation of MCs in edible parts of the plants *Brassica oleracea* and *Solanum tuberosum*. Pot-culture experiments with the plants were conducted, from October 2020 to January 2021. Results presented here cover the preliminary findings from the assays pending analysis of some of the samples.

Water used to irrigate the plants was collected from Roodeplaat Dam (Pretoria), next to the dam wall in 20L containers and was kept frozen till use. 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. 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. The EC for the sampled water used ranged from 296.67±13.87 to 878.67±42.44 $\mu S\ cm^{-1}$ and was in most cases higher the South African guideline and FAO limits for irrigation water.

The raw dam water did not have any effect on the germination of potato seeds, but severe effects were found on the germination of cabbage seeds, with 84.3% successful germination in the control trays and only 12.7% successful germination in the trial's trays. Such findings were in inconsistent with previous studies which have reported negative effects of cyanotoxins on seed germination and development and also highlight the possible impacts of irrigating crops with such water.

Cyanotoxins 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*.

Among variables monitored to compare the plant growth of the two groups, was the plant height of *Solanum tuberosum* after 49 days. The results showed that the experimental plants were growing better than the control plants, but the difference was not significant as shown by the results of the Student's *t* test at 95% CI. In explaining some of our 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 our study used raw dam water and natural soil conditions, it is thus not surprising that less effects on plants were reported.

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). To monitor oxidative stress possibly induced by cyanotoxins in the dam water, we 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 was found to be higher in the experimental plants compared to the control plants for both potato and cabbage plants, even though the difference was not significant (unpaired Student 't' test, and one way ANOVA at 95% CI). The findings were supported by Corbel *et al.* (2015), who 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 our study, might not adversely affect exposed plants.

Based on our findings, the levels of cyanotoxins 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 from this section demonstrated that the two plants can bio-accumulate 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. Our findings, together with findings in other studies discussed here, demonstrates terrestrial food crops can accumulate cyanotoxins to levels that can pose human-health risks when exposed to naturally relevant levels of cyanotoxins.

CHAPTER 5: ACCUMULATION OF CYANOTOXINS AND TOXIC METALS IN THE PRESENCE OF LAS ON *BRASSICA OLERACEA* AND *SOLANUM TUBEROSUM*

This chapter was prepared by Glynn K. Pindihama & Gitari W. Mugera.

5.1 INTRODUCTION

The bulk of water allocated to agriculture in SA goes to commercial farming, with small-scale farming mostly relying on hand watering and rain-fed agriculture (Turton, 2016). For crop production to be profitable and sustainable, irrigation water has to be of acceptable quality and irrigating with poor 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).

Other water quality problems facing irrigated agriculture in SA 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). In addition to inorganic pollutants, the emerging organic pollutants of concern in SA 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 (Meyer *et al.*, 2014).

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.*, 2017a). In effect, a study by Wang *et al.* (2012) demonstrated that the uptake of MC-LR in the presence of the anionic surfactant linear alkylbenzene sulfonate (LAS) increased compared to when plants are exposed to MC-LR alone. 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 bio-accumulate (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. Such findings underline the potential importance of multiple stressors and the need for further research to understand the joint effects of cyanotoxin mixtures in addition to other contaminants under South African conditions.

To investigate if cyanotoxins and metal species uptake and accumulation in different parts of the plants is affected by the presence of LAS, 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 experiments on accumulation capacity of plants from the field and laboratory assays were determined in order to explain accumulation of cyanotoxins and metal species when plants are irrigated with water from hypertrophic reservoirs which are also likely to be contaminated with LAS and other anionic surfactants.

Data gathered in this section is expected to contribute to further understanding of uptake and accumulation of MCs in the presence of other pollutants such as LAS and metal species. This is of importance, since terrestrial food plants can be exposed to numerous anthropogenic pollutants and other stressors such as linear alkylbenzene sulfonate (LAS) and toxic metal species, which may enhance cyanotoxins accumulation from the surrounding environment after irrigation.

This section of the study thus aimed at studying the impact of multiple stressors by examining the accumulation of cyanotoxins in the presence of linear alkylbenzene sulfonate (LAS) and metal pollutants.

5.2 MATERIALS AND METHODS

5.2.1 Sampling

A field survey was conducted from the 23rd of March to the 26th of March, 2021 to identify and collect field water suitable for the experiments. Water samples were collected from canals and farm dams from the two sites namely Roodeplaat and Hartbeespoort Dam sites. TDS, EC, pH and turbidity of the water were monitored in-situ and anionic surfactants, nutrients (dissolved phosphates and nitrates), chlorophyll-*a* and MCs were measured ex-situ.

5.2.2 Pot-culture Experimental set-up

The cabbage seeds were purchased from NTK Agricultural Products & Services (S.A) 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 200 mm plant pots filled with non-contaminated soil. The cabbage 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). The background levels of metal elements in the soil used are presented in *Table 1*. With regards to the three main nutrients, P, K, Total N and Organic matter, the soil had 25.86 (mg kg^{-1}); 184 (mg/kg); 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 approx. 15 kg of the soil was placed into 350 mm plastic pots for the experiments.

All the potato seeds were first washed with distilled water before being planted into 20L plant pots 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 depth, air dried and passed through a 2 mm sieve before use. Upon loading the soils into the planting bags, fourteen day old cabbage seedlings were transplanted (one plant per pot) into the bags.

The potential for cyanotoxins and metals transfer to irrigated crops was evaluated through a pot-culture experiment. Replicate cabbage and potato plants were grown with irrigation using raw dam water collected from Roodeplaat Dam which had visible cyanobacterial cells. Controls were run concurrently with the treatments (i.e. watered using cyanotoxins free water). The plants were watered daily with 500 mL of their respective water (Figure 5.1 shows the layout of the plants after 30 days of planting).

Table 5.1. Background level of Cations in the Soil

Element	Background level
B mg/kg	<280
V mg/kg	9.14±12.91
Cr mg/kg	56.01±33.40
Mn mg/kg	1709.85±160.28
Co mg/kg	60.40±18.85
Ni mg/kg	27.67±9.63
Cu mg/kg	126.28±48.07
Zn mg/kg	46.97±1.75
As mg/kg	0.79±0.26
Se mg/kg	0.09±0.02
Sr mg/kg	9.83±2.08
Mo mg/kg	<3
Cd mg/kg	0.06±0.01
Sn mg/kg	0.09±0.01
Sb mg/kg	<1
Ba mg/kg	72.48±2.53
Hg mg/kg	0.01±0.01
Pb mg/kg	11.53±1.69
Al g/kg	14.06±1.80
Fe g/kg	33.29±31.73
Ca g/kg	1.88±0.20
K g/kg	0.43±0.13
Mg g/kg	0.99±0.04
Na g/kg	0.29±0.01
P g/kg	0.17±0.00
Si g/kg	0.56±0.09

Figure 5.1. Layout of the experimental set-up showing the 4 treatments *of Brassica oleracea*.

To investigate the effects of different concentrations of LAS on cyanotoxins and metal species (Mn, Al and Sr) accumulation in *Brassica oleracea* and *Solanum tuberosum*, a series of concentrations of LAS (as determined by field study) (0.13 and 3.4 mg L^{-1}) were introduced to the dam water with a known fixed concentration of Microcystins (±15 µg L^{-1}) and Mn (0.257 mg L^{-1}), Al (0.6 mg L^{-1}) and Sr (0.16 mg L^{-1}) for 14 days and this was then used to irrigate the plants daily for a period of 30 days, with each pot receiving 500 mL of water daily.

In order to maintain approximately constant concentrations of the toxicants, the medium and toxicant concentrations were refreshed every day. At days 5 and 14, the accumulations of

cyanotoxins and metal species (MCs) in *Brassica oleracea* and *Solanum tuberosum* were measured (Table 1).

Table 5.2. Experimental design and parameters monitored in each treatment

Treatments	Parameters monitored
Control: no toxicants Treatment 1 (**T1**)	On day 5: cyanotoxins + Mn, Al & Sr in *Brassica oleracea* & *Solanum tuberosum* On day 14: cyanotoxins + Mn, Al & Sr in *Brassica oleracea* & *Solanum tuberosum*
Fixed cyanotoxin & metal exposure (fixed conc of cyanotoxins + fixed Mn, Al & Sr exposure) (as determined by field study) (**T2**)	On day 5: cyanotoxins + Mn, Al & Sr in *Brassica oleracea* & *Solanum tuberosum* On day 14: cyanotoxins + Mn, Al & Sr in *Brassica oleracea* & *Solanum tuberosum*
Single LAS exposure: 0.13 (**T3i**) and 3.4 mg L^{-1} (**T3ii**)	On day 5: cyanotoxins + Mn, Al & Sr in *Brassica oleracea* & *Solanum tuberosum* On day 14: cyanotoxins + Mn, Al & Sr in *Brassica oleracea* & *Solanum tuberosum*
Joint exposure: 0.13 (**T4i**) and 3.4 mg L^{-1} LAS (**T4ii**) and fixed conc of cyanotoxins + fixed Mn, Al & Sr exposure. (as determined by field study)	On day 5: cyanotoxins + Mn, Al & Sr in *Brassica oleracea* & *Solanum tuberosum* On day 14: cyanotoxins + Mn, Al & Sr in *Brassica oleracea* & *Solanum tuberosum*

On day 5, *Brassica oleracea* and *Solanum tuberosum* (20 plants each species) were taken out to determine the uptake of cyanotoxins and toxic metals (Al, Mn and Sr). At the end of experiment (day 14), the Fresh Weight and accumulation of cyanotoxins and metals (Mn, Al & Sr) were measured (Table 2.1).

5.2.3 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.

TDS, pH, 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 a nylon syringe filters (0.22 µm, 25 mm) prior to analysis to remove suspended solids.

5.2.4 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 cyanotoxins in plants were monitored.

5.2.4.1 Estimation of chlorophyll content

To determine oxidative stress induced by cyanotoxins 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 stress 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 were 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:

$$\text{Chl } a = 13.36\ A664.2 - 5.19\ A648.6 \quad [13]$$

$$\text{Chl } b = 27.43\ A648.6 - 8.12\ A664.2 \quad [14]$$

$$\text{Total chlorophyll} = \text{Chl } a + \text{Chl } b. \quad [15]$$

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

5.2.5 Metal species extraction and determination

The concentrations of Mn, Sr and Al in the plant parts was determined by microwave digestion method. 0.5 g of dried tissues were weighed into closed Teflon vessels and digested with 3 mL of concentrated HCl and 1 mL of concentrated HNO_3 at 450 W for 4 min. Then 3 mL of H_2O_2 was added to the mixture after cooling and once again irradiated at 450 W for 4 min. The solution was then diluted to 25 mL with distilled water after filtering and Mn, Sr and Al analysis was done using ICP-MS. All analyses were carried out in triplicates.

5.2.6 Data analysis

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

5.2.7 Cyanotoxin uptake and accumulation

For better understanding of the uptake and accumulation of cyanotoxins in the presence of LAS in various plant organs, accumulation experiments with the two plant species using water collected from the Roodeplaat Dam were conducted. The levels of cyanotoxins in the various plant tissues were determined at intervals as stated earlier in section 5.2.2 (using ELISA and LC-MS/MS).

5.2.7.1 Quantification of cyanotoxins 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 and quantification was as described in section 4.2.5.2 of the previous Chapter. The chromatographic conditions applied were similar to those described in section 4.2.5.2.1 and the EDI estimation was calculated as applied in section 4.2.4.4.

5.2.7.2 Cyanotoxin accumulation data analysis

One way analysis of variance was used to detect significant differences in bioaccumulation as a function of different cyanotoxins, metals and LAS treatments. All analyses were carried out at 5% significance level.

5.3 RESULTS AND DISCUSSION

5.3.1 Physicochemical parameters of the soil and field water

Table 3.1 shows that the pH for the water was slightly alkaline (pH 6.62±0.71 to 9.53±0.29). With regards to pH the water used for irrigation in the two sites is within the permissible limit according to the SA and FAO guidelines set at 6.5-8.4. The EC for the sampled water used ranged from 371±13.87 to 713±42.44 µS cm^{-1} and was in most cases higher than the South African guideline and FAO limits for irrigation water set at ≤400 µS cm^{-1} and 700 µS cm^{-1} respectively.

Table 5.3. Physicochemical parameters of the irrigation water monitored in-situ

Sampling point	pH	TDS (mg L^{-1})	EC(µs cm^{-1})	Turbidity (ntu)
Roodeplaat canal	7.82	266	438	4.50
Roodeplaat ARC farm dam	9.53	223	371	143.2
Roodeplaat Dep of Agric farm dam	9.02	228	380	8.33
Hartbeespoort weir	7.55	357	599	0.79
Hartbeespoort canal	8.14	429	713	22.35
Hartbeespoort farm dam 1	6.62	365	607	0.99
Hartbeespoort farm dam 2	9.34	289	483	50.32

Chlorophyll-*a* was used to estimate measure of algal biomass (Ramaraj *et al.*, 2013). South Africa like other countries across the globe, has no regulations or policies on cyanobacteria and cyanotoxins in water intended for irrigation of food crops (Pindihama & Gitari, 2020). Algal biomass as chlorophyll-*a* was relatively high in the sampled water, ranging from 10.74±0.728 µg L^{-1} to 1290.11±13.817 µg L^{-1}, with the highest readings reported in one farm dam at the Hartbeespoort site and ARC farm dam.

Table 5.4. Physicochemical parameters of the irrigation water monitored ex-situ

Sampling point	Nitrates (mg L^{-1})	Phosphates (mg L^{-1})	Chlorophyll-a (µg L^{-1})	MCs (µg L^{-1})	Anionic Surfactants (mg L^{-1})
Roodeplaat canal	2.7±0.141	0.75±0.021	49.97±0.933	0.31±0.184	0.19±0.177
Roodeplaat ARC farm dam	1.85±0.071	0.17±0.000	440.24±328.147	13.03±3.599	1.64±0.163
Roodeplaat Dep of Agric farm dam	7.58±1.520	0.45±0.000	373.68±0.309	0.21±0.007	0.15±0.078
Hartbeespoort weir	4.00±1.697	0.88±0.000	10.74±0.728	0.14±0.028	0.26±0.028
Hartbeespoort canal	9.35±0.354	0.58±0.014	423.51±25.321	0.16±0.035	0.27±0.085
Hartbeespoort farm dam 1	2.25±0.212	0.93±0.021	15.88±1.301	0.14±0.049	0.16±0.007
Hartbeespoort farm dam 2	2.45±0.354	0.25±0.007	1290.11±13.817	0.50±0.021	3.49±0.000

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. 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.

Based on these findings (Tables 3.1 and 3.2) water to be used for the experiments was collected from the ARC farm dam (Roodeplaat site). The water is alkaline, with a pH of 9.02, has high EC and TDS levels (228 mg L^{-1} and 380 µs cm^{-1} respectively), high cyanobacterial biomass (Chlorophyll-a 440.24±328.147 µg L^{-1}) and contained a significant load of anionic surfactants (1.64±0.163 mg L^{-1}). Of importance, the water had very high levels of MCs (13.03±3.599 µg L^{-1}), making it ideal for the intended experiments. The water was then collected from ARC farm dam in 20L containers and kept frozen till use.

5.3.2 Metal elements in irrigation water

The levels of metal elements in the irrigation water from the two dams are shown in Table 3.3 and Table 3.4. The results show that all the elements (essential and non-essential) are in trace levels

and none of them exceeds the DWA (1996) and the FAO (1985) guidelines for these elements in irrigation water. The only concern here is the pH of the water (as discussed in section 3.2 of this report) which is alkaline and makes some of these metal elements to be available in the soils after repeated irrigation over the years, resulting in these elements accumulating in the soils and being taken up by plants. When accumulated to some extent in soils, metal elements such as Cu, Cd, Pb, Zn, Ni, Hg, and Cr can be toxic to plants, animals and humans due to their characteristic of bioaccumulation and persistence.

Table 5.5. Metal species in irrigation water from Roodeplaat Dam

Parameter	June, 2019	September, 2019	South African guidelines	FAO guidelines
Al (mg/ L^{-1})	0.376 (±0.817)	0.624 (±0.726)	5-20	5.0
As (mg L^{-1})	0.002 (±0.001)	0.001 (±0.000)	0.1-2.0	0.1
B (mg L^{-1})	0.059 (±0.008)	0.068 (±0.005)	0.5-6.0	0.7
Ba (mg L^{-1})	0.049 (±0.015)	0.152 (±0.056)	-	-
Cu (mg L^{-1})	0.006 (±0.003)	0.006 (±0.007)	0.2-0.5	0.2
Mn (mg L^{-1})	0.257 (±0.179)	0.158 (±0.109)	0.02-10	0.2
Ni (mg L^{-1})	0.006 (±0.003)	0.008 (±0.003)	0.2-2.0	0.2
Pb (mg L^{-1})	0.004 (±0.004)	0.001 (±0.001)	0.2-2.0	5.0
Sr (mg L^{-1})	0.144 (±0.116)	0.118 (±0.020)	-	-
Zn (mg L^{-1})	0.083 (±0.080)	0.090 (±0.033)	1.0-5.0	2.0

N.B: The 1996 South African and 1985 FAO 1985 guidelines do not have a value for Barium and Strontium.

Table 5.6. Metal species in irrigation water from Hartbeespoort Dam

Parameter	June,2019	September, 2019	South African guidelines	FAO guidelines
Al (mg L^{-1})	0.216 (±0.477)	0.411 (±0.393)	5-20	5.0
As (mg L^{-1})	0.001 (±0.001)	0.001 (±0.000)	0.1-2.0	0.1
B (mg L^{-1})	0.046 (±0.007)	0.048 (±0.005)	0.5-6.0	0.7
Ba (mg L^{-1})	0.036 (±0.014)	0.136 (±0.037)	-	-
Cu (mg L^{-1})	0.008 (±0.003)	0.004 (±0.005)	0.2-0.5	0.2
Mn (mg L^{-1})	0.124 (±0.042)	0.161 (±0.081)	0.02-10	0.2
Ni (mg L^{-1})	0.005 (±0.002)	0.005 (±0.002)	0.2-2.0	0.2
Pb (mg L^{-1})	0.021 (±0.029)	0.001 (±0.000)	0.2-2.0	5.0
Sr (mg L^{-1})	0.149 (±0.082)	0.165 (±0.021)	-	-
Zn (mg L^{-1})	0.049 (±0.018)	0.085 (±0.027)	1.0-5.0	2.0

N.B: The 1996 South African and 1985 FAO 1985 guidelines do not have a value for Barium and Strontium.

Based on our findings from deliverable 2 and also presented here in Tables 3.3 and 3.4, Al, Sr and Mn were found to detectable in the irrigation water compared to other elements in the two sampling events and thus were selected for the assays.

5.3.3 Soil properties

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). The background levels of metal elements in the soil used are presented in Table 5.6. With regards to the three main nutrients, P, K, Total N and Organic matter, the soil had 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 approx. 10 kg of the soil was placed into 350 mm plastic pots for the experiments.

Table 5.7. The background level of metal elements in the soil

Element	Background level
B mg kg^{-1}	<280
V mg kg^{-1}	9.137±12.906
Cr mg kg^{-1}	56.014±
Mn mg kg^{-1}	1709.849±160.279
Co mg kg^{-1}	60.404±18.852
Ni mg kg^{-1}	27.666±9.631
Cu mg kg^{-1}	126.278±48.071
Zn mg kg^{-1}	46.968±1.745
As mg kg^{-1}	0.792±0.256
Se mg kg^{-1}	0.093±0.018
Sr mg kg^{-1}	9.826±2.081
Mo mg kg^{-1}	<3
Cd mg kg^{-1}	0.055±0.014
Sn mg kg^{-1}	0.092±0.007
Sb mg kg^{-1}	<1
Ba mg kg^{-1}	72.481±2.527
Hg mg kg^{-1}	0.011±0.007
Pb mg kg^{-1}	11.534±1.694
Al g kg^{-1}	14.060±1.799
Fe g kg^{-1}	33.286±31.726
Ca g kg^{-1}	1.877±0.196
K g kg^{-1}	0.425±0.130
Mg g kg^{-1}	0.985±0.044
Na g kg^{-1}	0.291±0.012
P g kg^{-1}	0.168±0.000
Si g kg^{-1}	0.558±0.094

5.3.4 Uptake and accumulation of Mn, Sr and Al in presence of anionic surfactants

The levels of Mn, Sr and Al taken up by *B. oleracea* after 5 days of exposure to four treatments is presented in Figure 5.2 (a, b, c respectively). A Kruskal-Wallis Test (Nonparametric ANOVA) showed no significant differences (P> .05) in the levels of Mn, Sr and Al taken up by the plants after days of exposure (P values of 0.6530; 0.1973 and 0.8514 for Mn, Sr and Al respectively, (Appendix E-G).

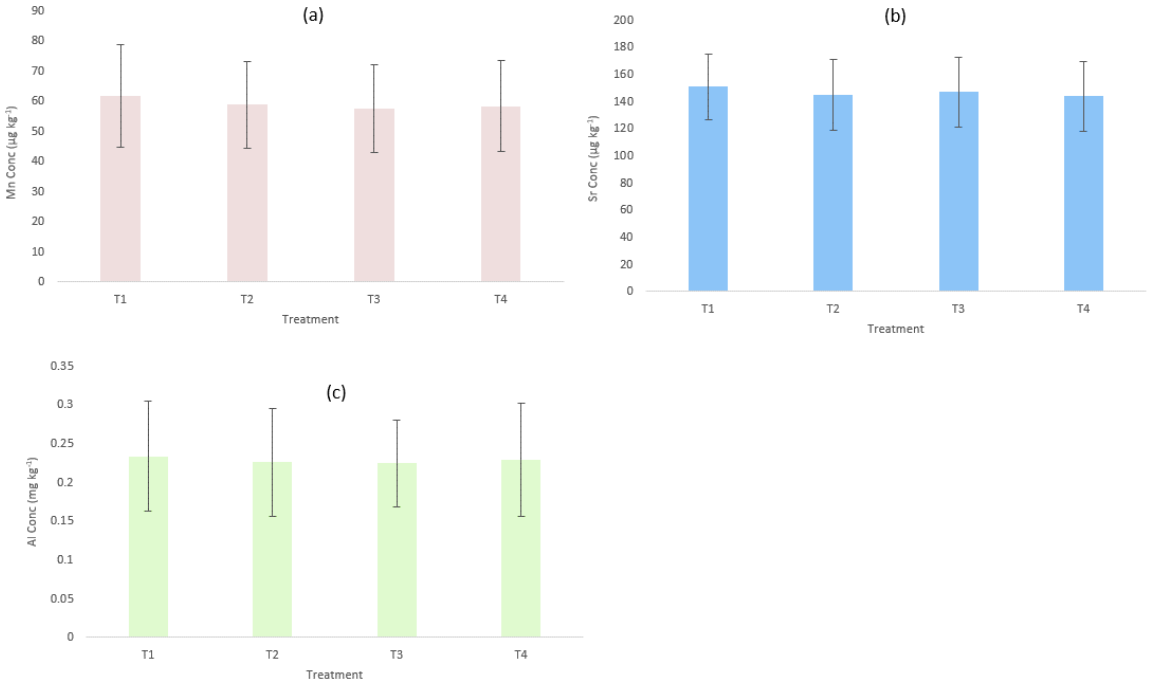

Figure 5.2. Accumulation of metals in *B. oleracea* after 5 days (a) Mn (µg kg^{-1}) (b) Sr (µg kg^{-1}) (c) Al (mg kg^{-1}).

Figure 5.3 shows the levels of Mn, Sr and Al accumulated by *B. oleracea* after 20 days of exposure to the 4 treatments. As can be seen in Figure 5.3 and also by the significance tests (Appendix H to J), there no significance differences in the accumulation of the metals in the plants (P> .05) except for Al uptake which showed a slightly significant difference in the uptake of between treatment 2 (T2) (raw dam water) and treatment 4 (T4) (raw dam water spiked with LAS and the metals), with a significantly higher uptake observed in treatment 2 compared to treatment 4. The significantly higher accumulation of Al in T2 compared to T4 was contrary to what we had hypothesised, since we expected the increased presence of LAS in T4 to enhance uptake of the metals in T4 compared to other treatments.

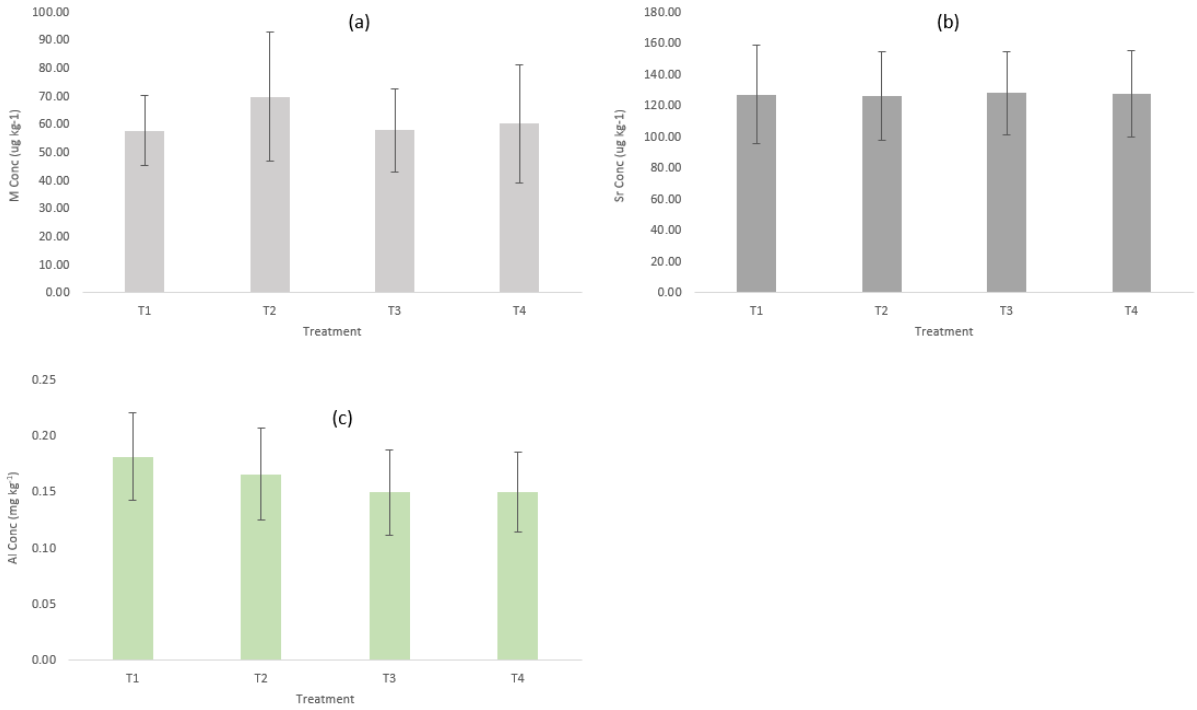

Figure 5.3. Accumulation of metals in *B. oleracea* after 20 day exposure (a) Mn (µg kg^{-1}) (b) Sr (µg kg^{-1}) (c) Al (mg kg^{-1}).

Figure 5.4 (a, b, c) shows the levels of Mn, Sr and Al accumulated by *S. tuberosum* after exposure the treatments for 20 days. Higher uptake of Mn and Sr were observed in T2 and T3 whereas for Al, higher uptake was observed in T1. Regardless of the noticeable differences in the accumulation of the 3 metals exposed to the 4 treatments in Figure 5.4, no statistical significant differences were found among the 4 treatments for all the 3 metals (P> .05, Kruskal-Wallis Test, Appendices K-M). No significant differences were also reported within the same treatments with different levels of LAS (T3i vs T3ii and T4i vs T4ii) for all the 3 metals and for both plants (P> .05, Mann-Whitney Test and Student 't' test, Appendices N-Y). This showed that, at the levels applied, LAS did not influence uptake of the 3 metals in the two plants tested.

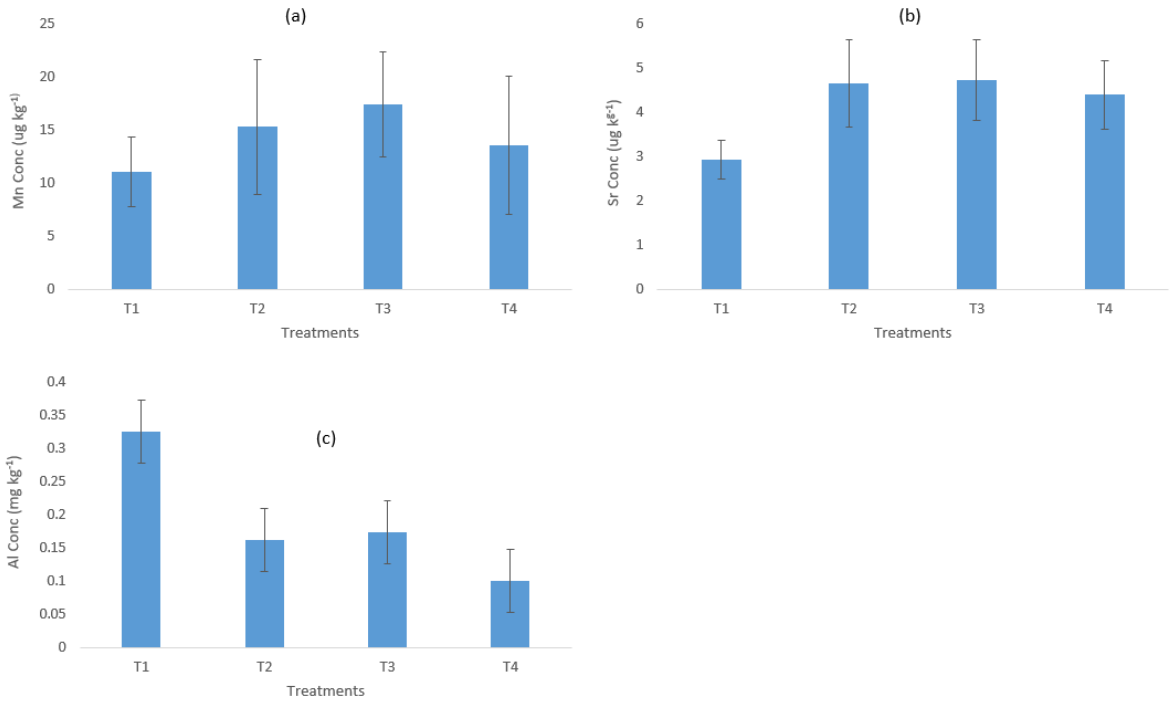

Figure 5.4. Accumulation of metals in *S. tuberosum* after 20 day exposure (a) Mn (µg kg^{-1}) (b) Sr (µg kg^{-1}) (c) Al (mg kg^{-1}).

5.3.5 Uptake and accumulation of other major & trace cations in presence of anionic surfactants

With regards to other major and trace cations which were not intentionally spiked in the treatments, Figure 5.5 and Figure 5.6 shows a higher uptake of the major cations K, Mg, Ca, P and Fe by *S. tuberosum* and also evidently high accumulation of minor cations B, V, Cr, Mn, Co, Ni, Cu, Zn and Ba. Except for B which showed higher uptake in T4 (Figure 5.6), the other trace and major elements showed high accumulation in T1 compared to other treatments, even though no statistically significant differences were reported for all the monitored cations accumulated in *S. tuberosum* thus confirming that the presence of LAS not having an impact on the uptake of major and trace cations.

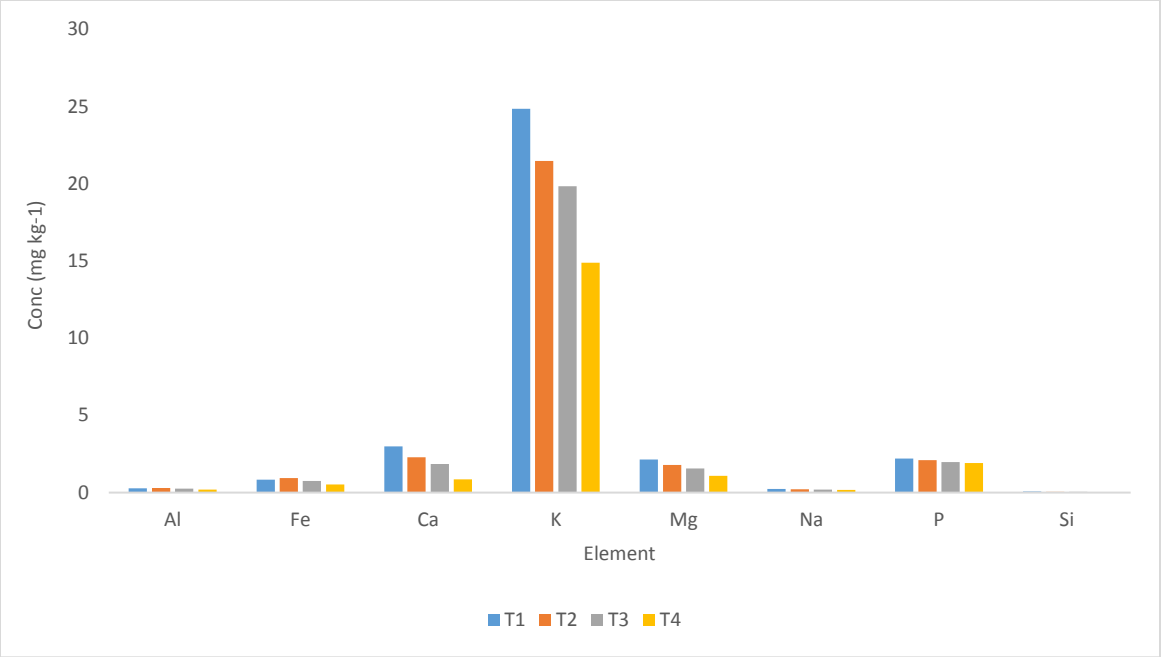

Figure 5.5. Accumulation of major cations (mg kg^{-1}) in *S. tuberosum* after 20 day exposure (mg kg^{-1}).

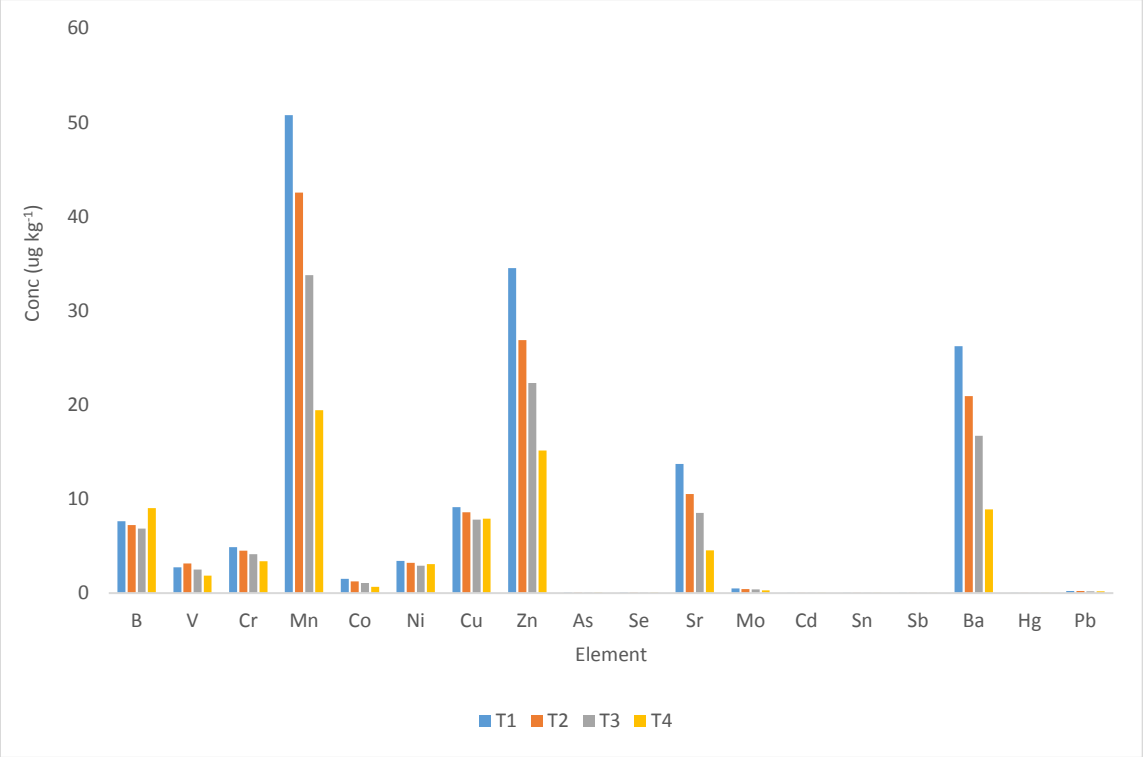

Figure 5.6. Accumulation of minor cations (µg kg^{-1}) in *S. tuberosum* after 20 day exposure (µg kg^{-1}).

Figures 5.7 and 5.8 shows the levels of major and minor cations accumulated by *B. oleracea* over the 20 day exposure period. There was no evident pattern of uptake when comparing the uptake of each cation in *B. oleracea* among the 4 treatments. This is despite the introduction of LAS in treatments 3 and 4 which was expected to enhance uptake of these elements.

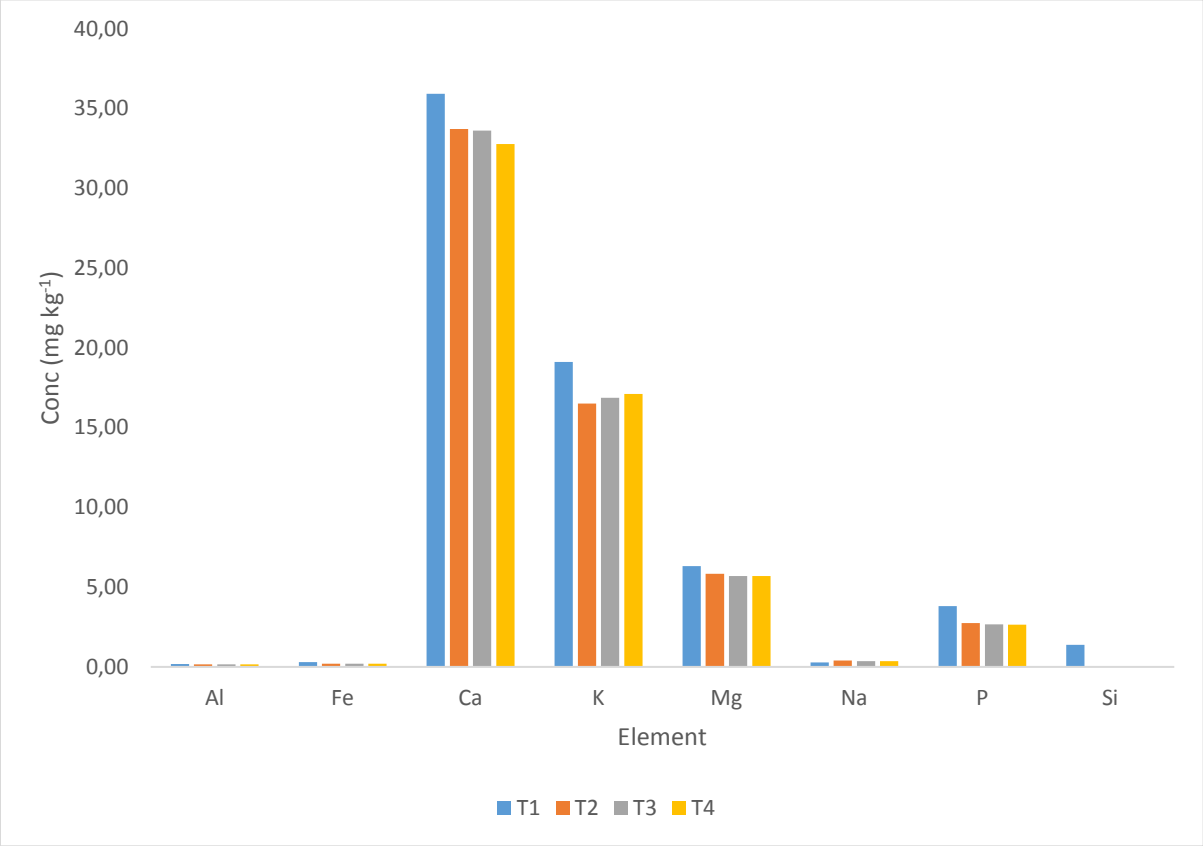

Figure 5.7. Accumulation of major cations (mg kg^{-1}) in *B. oleracea* after 20 day exposure (mg kg^{-1}).

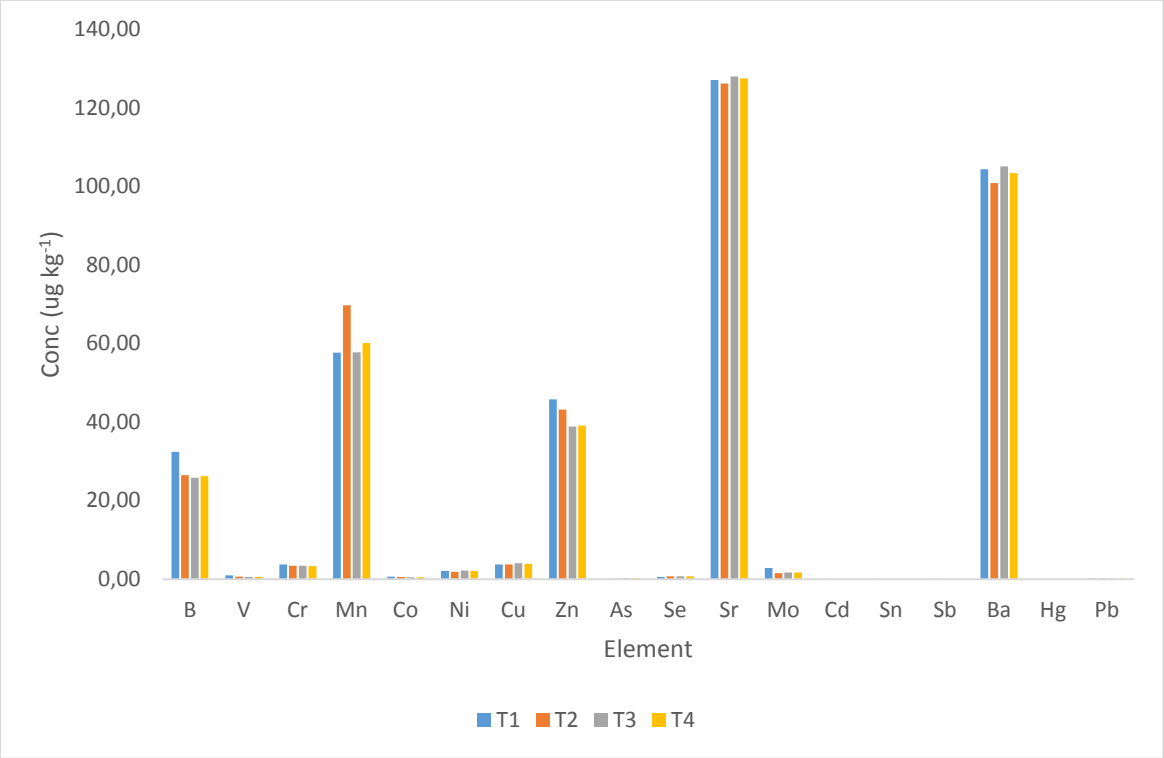

Figure 5.8. Accumulation of minor cations (mg kg^{-1}) in *B. oleracea* after 20 day exposure (µg kg^{-1}).

5.3.6 MCs accumulation

When exposed to the 4 different treatments for a period of 20 days, Table 5.8 shows the mean (± SE) concentrations of MCs accumulated in the tubers of *S. tuberosum tubers*. 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 toxins accumulated were reported for MC-LR and MC-YR whereas MC-RR did show any statistically significant differences (ANOVA/Kruskal-Wallis Test, at 95% CI). 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 high 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 which exceeded the recommended TDI.

Table 5.8. Mean (±SE) MCs accumulated in S. tuberosum tubers upon 20 day exposure to the 4 treatments. Data labelled with different small letters (a-d) differed significantly (ANOVA/ Kruskal-Wallis Test) at $p < 0.05$ in each row (n=6).

	Treatment 1	**Treatment 2**	**Treatment 3**	**Treatment 4**	***P*-value**	**EDI** (mg kg^{-1} of body mass day^{-1})
MCRR (µg g^{-1} DW)	0.000253 (±0.0001552)^{a}	0.004851 (±0.0009748)^{a}	0.000535 (±0.0001112)^{a}	0.00382 (±0.001209)^{a}	0.0793 (n.s)	0.007
MCYR (µg g^{-1} DW)	0.000554 (±7.95E-05)^{a}	0.006404 (±0.001069)^{a,b}	0.001167 (±0.0003442)^{b}	0.005309 (±0.0007399)^{a,c}	0.0007***	0.009
MCLR (µg g^{-1} DW)	0.016041 (±0.008997)^{a}	0.200387 (±0.05003)^{b}	0.022709 (±0.009017)^{a,c}	0.150868 (±0.0458)^{a,b,d}	0.0013 **	0.284

Note: *P < 0.05, **P <0.01, ***P < 0.001, n.s = not significant.

With regards to the accumulation of the toxins in *B. oleracea* leaves, Tables 5.9 and 5.10 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. Statistically significant differences in treatments were found among the treatments for MC-YR and MC-LR after 5 days of exposure, with significantly higher accumulations found in *T2* followed by *T4* compared to the other treatments (ANOVA/Kruskal-Wallis Test at *P*= .05).

Findings in Table 5.10 shows that statistically significant differences were found among the treatments for all the 3 congeners of MCs (ANOVA/Kruskal-Wallis Test at p= .05). Similar to be patterns observed for *S. tuberosum* tubers and for *B. oleracea* leaves after 5-day exposures, higher levels of MCs were accumulated in *T2*, followed by *T4* compared to the other treatments. The findings imply that the presence of LAS in T3 did not have any impact on MCs uptake from the soil (without exposure to MCs in irrigation water) and that presence of LAS in raw dam water did not enhance the uptake and accumulation of MCs by the plants.

Table 5.9. Mean (±SE) MCs accumulated in B. oleracea leaves upon 5 day exposure to the 4 treatments. Data labelled with different small letters (a-d) differed significantly (ANOVA/ Kruskal-Wallis Test) at $p < 0.05$ in each row (n=6).

	Treatment 1	**Treatment 2**	**Treatment 3**	**Treatment 4**	***P*-value**	**EDI** (mg kg^{-1} of body mass day^{-1})
MCRR (µg g^{-1} DW)	0.000583 (±0.0005829)[a]	0.011646 (±0.006789)[a]	0.000884 (±0.000884)[a]	0.012962 (±0.008763)[a]	0.5427 (*n.s*)	0.018
MCYR (µg g^{-1} DW)	0.00059 (±5.80E-05)[a]	0.005786 (±0.0002905)[b]	0.000603 (±3.10E-05)[a]	0.005473 (±0.0003488)[b]	< 0.0001 ***	0.008
MCLR (µg g^{-1} DW)	1.14E-05 (±6.82E-06)[a]	0.003271 (±0.000625)[b]	2.66E-05 (±6.20E-06)[a]	0.001076 (±0.000435)[a]	< 0.0001***	0.005

Note: *P < 0.05, **P <0.01, ***P < 0.001, n.s = not significant.

Table 5.10. Mean (±SE) MCs accumulated in B. oleracea leaves upon 20 day exposure to the 4 treatments. Data labelled with different small letters (a-d) differed significantly (ANOVA/ Kruskal-Wallis Test) at $p < 0.05$ in each row (n=6).

	Treatment 1	**Treatment 2**	**Treatment 3**	**Treatment 4**	***P*-value**	**EDI** (mg kg^{-1} of body mass day^{-1})
MCRR (µg g^{-1} DW)	0.000155 (±0.000155)[a]	0.083923 (±0.02975)[b]	0.000683 (±0.000683)[a]	0.034973 (±0.01751)[a,b]	0.0143*	0.119
MCYR (µg g^{-1} DW)	0.00063 (±5.82E-05)[a]	0.006938 (±0.000273)[b]	0.000521 (±1.41E-05)[a]	0.005134 (±0.000344)[c]	< 0.0001***	0.010
MCLR (µg g^{-1} DW)	2.17E-05 (±8.64E-06)[a]	0.005741 (±0.000651)[b]	1.41E-05 (±8.17E-06)[a]	0.002531 (±0.000969)[c]	< 0.0001***	0.008

Note: *P < 0.05, **P <0.01, ***P < 0.001, n.s = not significant.

Much of the work on the combined ecotoxicological 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's permeability, activity of enzymes and structure of tissue in organisms (Wang *et al.*, 2011). Unlike our findings where the presence of LAS did not impact of the accumulation of MC-LR in plants, Wang *et al.* (2011) reported higher accumulation rates when lettuce seedling where exposed to a combination of MC-LR and LAS compared to MC-LR in 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. Unlike our findings, Wang *et al.* (2012), found enhanced uptake of MC-LR in duckweed even at the lowest concentrations of 3 μg mL^{-1}, which were similar to the highest level of 3.4 μg mL^{-1} we applied here.

According to Mao *et al.* (2015) at low concentrations, surfactants build up at liquid to liquid or at 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 aqueous phase gain more mobility and thus 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).

The interaction and combination of LAS and other contaminants like microcystins 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 and other contaminants such metals which were detected in the water used. Consistent to our findings, Zhang *et al.* (2008) did not find increased uptake of Cd uptake by soybean 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, plant species, concentrations tested and the duration of exposure. In this study factors such as faster biodegradation of LAS by microbes, a reduction in the exchangeable metals available in the media and low concentrations of

LAS tested could all have affected LAS, metals and other contaminants activity and toxicity to the plants.

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 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 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.

5.3.7 The synergic effects of MCs, metals (Sr, Mn, Al) and anionic surfactants on plants

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 measured 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 both plants, after 20 days of exposure to 4 treatments.

The total chlorophyll of the plants after 20 days of exposure to the treatments is shown in Figure 5.9 (a & b). In Figure 5.9 (a), the total chlorophyll of *B. oleracea* was found to be comparable in all the 4 treatments significant (P> .05, One-way Analysis of Variance (ANOVA) at 95% confidence interval (CI), Appendix Z). Similarly, for *S. tuberosum* Figure 5.9 (b), no significant differences in the total chlorophyll content among the treatments (P> .05, One way ANOVA, 95% CI, Appendix ZA). These findings indicated lack of oxidative stress nor noticeable effects on both plants as a result of exposure to cyanotoxin and metal contaminated water in the presence of LAS, this is regardless the fact that anionic surfactants are known to induce contaminants uptake as observed by Wang *et al.* (2012).

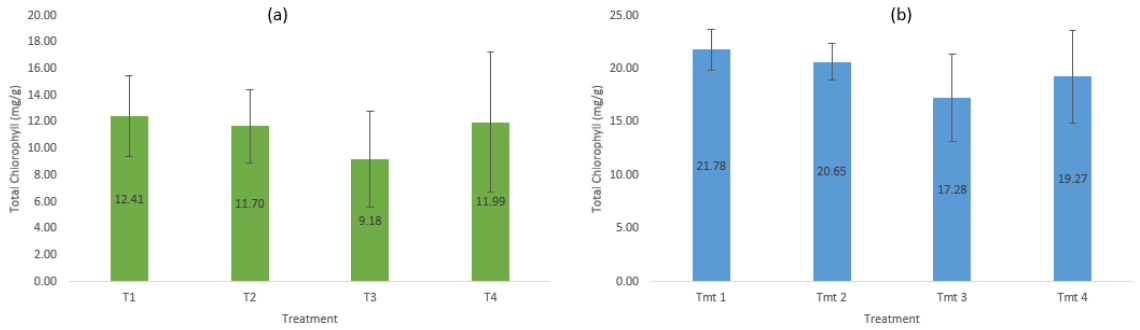

Figure 5.9. Total Chlorophyll (a) *B. oleracea* after 20 day exposure (b) *S. tuberosum* after 20 day exposure.

Figure 5.10 (a & b) shows the total chlorophyll *S. tuberosum* exposed to different levels of LAS after 20 days (a) in DI water (T3i & T3ii) (b) in spiked dam water (T4i & T4ii). Exposure to increased levels of LAS as observed in sections 5.3.4 and 5.3.5 did not result in the increased uptake of the contaminants and as a result did not induce any possible oxidative stress, hence no differences in the total chlorophyll levels (P> .05, unpaired t test).

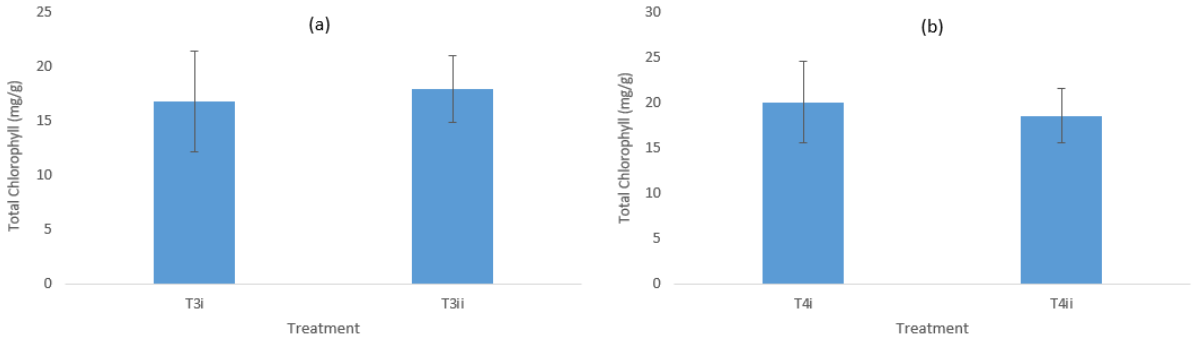

Figure 5.10. Comparison of total chlorophyll levels of *S. tuberosum* exposed to different levels of LAS after 20 days (a) in DI water (b) in spiked dam water

With regards to total chlorophyll in *B. oleracea*, Figure 5.11 shows a comparison of total chlorophyll levels of the plants when exposed to different levels of LAS after 20 days (a) in spiked DI water (*T3i* & *T3ii*) (b) in spiked dam water (*T4i* & *T4ii*). Increased levels of LAS in DI water did not seem to affect the uptake of MCs, and other ions in the surrounding medium, hence very little/ no effect on the plant health and total chlorophyll levels (P> .05). Inasmuch as a statistical significant difference (*P*< .05) was observed for spiked dam water (*T4*) (Figure 5.11b), higher

chlorophyll levels were expected in T4i which was exposed to less LAS compared to T4ii which was exposed to higher LAS levels. Higher total chlorophyll levels in *T4ii* thus assumes other external factors at play, such as pests attacking the plants.

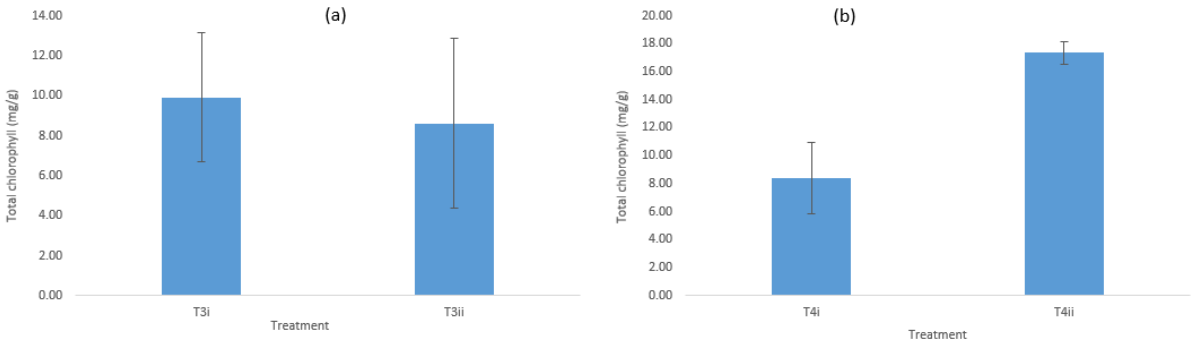

Figure 5.11. Comparison of total chlorophyll levels of *B. oleracea* exposed to different levels of LAS after 20 days (a) in DI water (b) in spiked dam water

As discussed in section 2.5.1, LAS alters the permeability of membranes and thus enhances the uptake and accumulation of other toxicants (Wang *et al.*, 2012) both in water and soil. Despite previous studies have reported increased uptake of metals in presence of LAS (Pierattini *et al.*, 2018) and the increased metal levels inhibiting chlorophyll production and inducing oxidative stress (Sulaiman & Hamzah, 2018), our findings indicated that the presence of LAS, metals and MCs did not induce synergic effects on the plants. This was demonstrated by the comparable levels of total chlorophyll and metals accumulated in both plant species upon exposure to contaminated water containing different levels of LAS.

5.4 SUMMARY

The current chapter detailed work done on the fourth aim of the project which aimed to investigate the uptake and the accumulation of MCs and metal species in different parts of the plants *Brassica oleracea* and *Solanum tuberosum* in the presence of the anionic surfactant LAS. The findings indicated that the dam water which was used to irrigate the plants was alkaline, with a pH of 9.02, had high EC and TDS levels (228 mg L^{-1} and 380 µs cm^{-1} respectively), high cyanobacterial biomass (Chlorophyll-a 440.24±328.147 µg L^{-1}) and contained a significant load of anionic surfactants (1.64±0.163 mg L^{-1}). Of importance, the water had very high levels of MCs (13.03±3.599 µg L^{-1}). Based on the findings, the presence of anionic surfactants did not induce

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 different 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 here. 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 high 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 which 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 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 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.

CHAPTER 6: DEVELOPMENT & APPLICATION OF A CROSSLINKED CHITOSAN-BASED SOLID PHASE ADSORPTION TOXIN TRACKING TECHNOLOGY (SPATT) ADSORBENT

This chapter was prepared by Glynn K. Pindihama, Gitari W. Mugera & Rabelani Mudzielwana

6.1 INTRODUCTION

Harmful cyanobacteria blooms (CyanoHABs) are comprised of naturally occurring photosynthetic prokaryotes found in a wide variety of aquatic environments and capable of producing toxic secondary metabolites (cyanotoxins). CyanoHABs can thrive under a wide range of environmental conditions and are especially prolific and competitive under high nutrient conditions often associated with eutrophic waters. Due to the toxicological effects from microcystins (MCs), exposure to the toxins presents a health hazard and numerous MC poisonings have been documented in pets, livestock, wildlife, and humans (Preece *et al.*, 2017).

Microcystins (MCs) are the most prevalent and abundant cyanobacterial toxins in the environment, which can be frequently found in water reservoirs used for drinking water supply (Buratti *et al.*, 2017; Huisman *et al.*, 2018). Consumption of MC-contaminated drinking water has been recognised as an important exposure route, with potentially chronic, repeated exposure patterns (Jaša *et al.*, 2019). Considering chronic toxicity of MCs, namely their tumor-promoting and hepatocarcinogenic activity, the World Health Organization (WHO) established a provisional guidance value of 1 mg L^{-1} for MC-LR concentration in drinking water, and even stricter limits (0.1-0.3 mg L^{-1}) have been implemented by some regulatory agencies (Buratti *et al.*, 2017). Long-term compliance with such limits requires a multi-barrier approach, which includes a monitoring system for cyanobacteria and cyanotoxins, in order to optimize and facilitate their effective removal during drinking water treatment operation (He *et al.*, 2016; Sklenar *et al.*, 2016). Therefore, there is a continuous effort to develop rapid, simple, sensitive, and cost-effective methods which would allow either real-time or time-integrative monitoring of cyanobacteria and MCs.

Sampling and monitoring of cyanotoxins, especially in large water bodies and rivers, can be problematic because cyanotoxins levels can vary rapidly with changing environmental and hydrological conditions. Current sampling practices (e.g. grab samples) provide only a snapshot

of cyanotoxins present at one point in time and may miss areas or times of highest risk. These issues are particularly relevant when sampling rivers where toxic benthic mats may sporadically release toxins to the water and flows transport toxins rapidly (Wood *et al.*, 2011). Passive in situ methodology known as solid phase adsorption toxin tracking technology (SPATT) has been shown to be a simple and sensitive means of warning of toxic micro-algal bloom development and associated shellfish contamination in the marine environment (MacKenzie *et al.*, 2004; Piletska *et al.*, 2008; Pizarro *et al.*, 2008). SPATT involves suspending in the water body small bags containing adsorption substrates which accumulate toxins. 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 (Fux *et al.*, 2009; MacKenzie, 2010), but a range of technical problems remain to be solved for use of SPATT with highly water soluble toxins such as cylindrospermopsin and saxitoxins.

Many different sorbents have been used for passive sampling all over the world, from HP-20 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 microalgal toxins using different bulk polymeric sorbents (Zendong *et al.*, 2014), most of these sorbents are synthetic and relatively costly to buy. This study aims to synthesize and assess the physicochemical properties of modified/crosslinked chitosan sorbents for their potential/ability to adsorb a range of MCs and to determine the potential applicability of SPATT for tracking MCs in irrigation water.

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 not been studied, much especially for passive sampling in SPATT format. Based on literature, we believe that the insertion of multi-walled carbon nanotubes into the network has a synergistic effect, of improving the mechanical and adsorptive characteristics of the hydrogel. In this way, the

innovation of this work is 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. The aim of this work was to develop chitosan-based sorbents for MCs sampling in a SPATT bag format.

6.2 MATERIALS AND METHODS

6.2.1 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 polystyrenic matrices (HP20 and SP700) were purchased from Rochelle Chemicals (Johannesburg, S.A). De-ionized water obtained from a MilliQ water purification system (Merck-Millipore Ltd., Germany) to 18 mΩ quality was used to prepare all the solutions.

6.2.2 Preparation of glutaraldehyde crosslinked chitosan hydrogel

Chitosan (1 g) was dissolved 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 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.

6.2.3 Preparation of chitosan/MWCNT composite

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 the gel. After, the hydrogel 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.

6.2.4 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 using ca.20 mg samples. The surface morphology of the samples was characterized by FEI Nova Nano SEM 230.

6.2.5 Adsorption and desorption studies

6.2.5.1 Adsorption experiment

A 5 μg L^{-1} MC-LR solution was used for the adsorption experiment. The toxin solution (2 mL) was placed in 15 mL glass centrifuge tubes with 0.01 g and 0.02 g of the ChMWCNT composite and the mixture was shaken at 145 rpm for 30 min at room temperature. The adsorption solutions were centrifuged at 700 × g for 10 min and the supernatants were collected for analysis using the ELISA method. Adsorption amounts were equal to the initial content minus the residual toxins in the supernatants.

6.2.5.2 Desorption experiment

The residual contents of the centrifuge tubes used in the adsorption experiment were dried and 2 mL of 80% methanol solution was added to release the toxin. The mixtures were shaken at were shaken at 145 rpm for 30 min at room temperature. The desorption solutions were centrifuged at 700 × g for 10 min and the supernatant collected for ELISA analysis.

6.2.6 Adsorption and desorption in SPATT bag format

6.2.6.1 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 an appropriate mass (0.1 g for ChGLA hydrogel and ChMWCNT composite and 1.5 g for DIAION HP20) and then the fourth side was sewn to form a finished SPATT bag of approximately 55 × 55 mm dimension (see Figure 6.1). The SPATT bags were activated by soaking in 100% MeOH for 48 hours, then rinsed thoroughly in in de-ionized water (Milli-Q) for removal of any MeOH residues and kept in Milli-Q water at 4-6°C prior to use.

Figure 6.1. SPATT samplers packed with the 3 different materials.

6.2.6.2 Adsorption experiment with spiked Milli-Q water

A 5 µg L^{-1} MC-LR solution 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 0.1 g of the ChGLA hydrogel and ChMWCNT composite and 1.5 g of the resin HP20 were introduced to each bottle and the solutions were shaken at 145 rpm for 30 min at room temperature. The SPATT bags were removed after the 30 mins, rinsed in Milli-Q water and kept at 4-6°C prior to extraction.

6.2.6.3 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 0.1 g of the ChGLA hydrogel and ChMWCNT composite and 1.5 g of the resin HP20 were introduced to each bottle and the solutions were shaken at 145 rpm for 30 min at room temperature (Figure 6.2). The SPATT bags were removed after the 30 mins, rinsed in Milli-Q water and kept at 4-6°C prior to extraction.

Figure 6.2. Laboratory Adsorption experiment with field water.

6.2.6.4 SPATT Extraction

The SPATT bags used for the adsorption studies were oven dried at 40°C, then cut open and the material was placed in 15 mL centrifuge tubes. The toxins were extracted using 80% MeOH through shaking at 145 rpm for 30 mins and then centrifuging at 700 g for 10 mins. The supernatant was collected and dried at 50°C under a stream of nitrogen. The dried material was then reconstituted with 1 mL of the ELISA buffer solution which was supplied with the ELISA kits used (ELISA test kits, EUROFINS (Kit Lot No: 19I1120: PN 520011)).

6.2.7 Determination of MCs levels in the residues and extracted material

A EUROFINS Microcystin Plate Kit was used for the MCs assay with the ELISA technique. It is based on a direct competitive ELISA for quantitative detection of MCs and nodularins according to the manufacturer's instructions and on the polyclonal antibodies. In detail, 100 µL of negative control, calibrator, and sample was added to their respective wells before being incubated for 90 min at room temperature. After that, to each well, 100 µL of microcystins-enzyme conjugate was added and mixed. After another incubation of 30 minutes, a wash step with deionized water was repeated four times. Then, 100 µL of substrate was added to each well before another incubation of 30 min at room temperature. Finally, 100 µL of stop solution was added to each well. The obtained color and its related absorbance, where toxin concentration is inversely proportional to color development, were read at 450 nm within 15 min. MCs values were calculated using a standard curve (0.15-5.0 µg L^{-1}).

To determine the microcystin concentrations adsorbed to the SPATT resin, the following formula was used:

$$\text{ug/g-resin} = \frac{(ug/L{-}extract\ x\ 0.001\ L\ extract{-}vol\ )}{0.1\ g{-}resin} \quad [16]$$

Where: the extract volume is 0.001 L and there are 0.1 g of resin per sampler for ChGLA and ChMWCNT; and 1.5 g for HP20 resin.

6.3 RESULTS AND DISCUSSION

6.3.1 Characterization of the synthesized material

6.3.1.1 Fourier Transform Infrared (FTIR) analysis

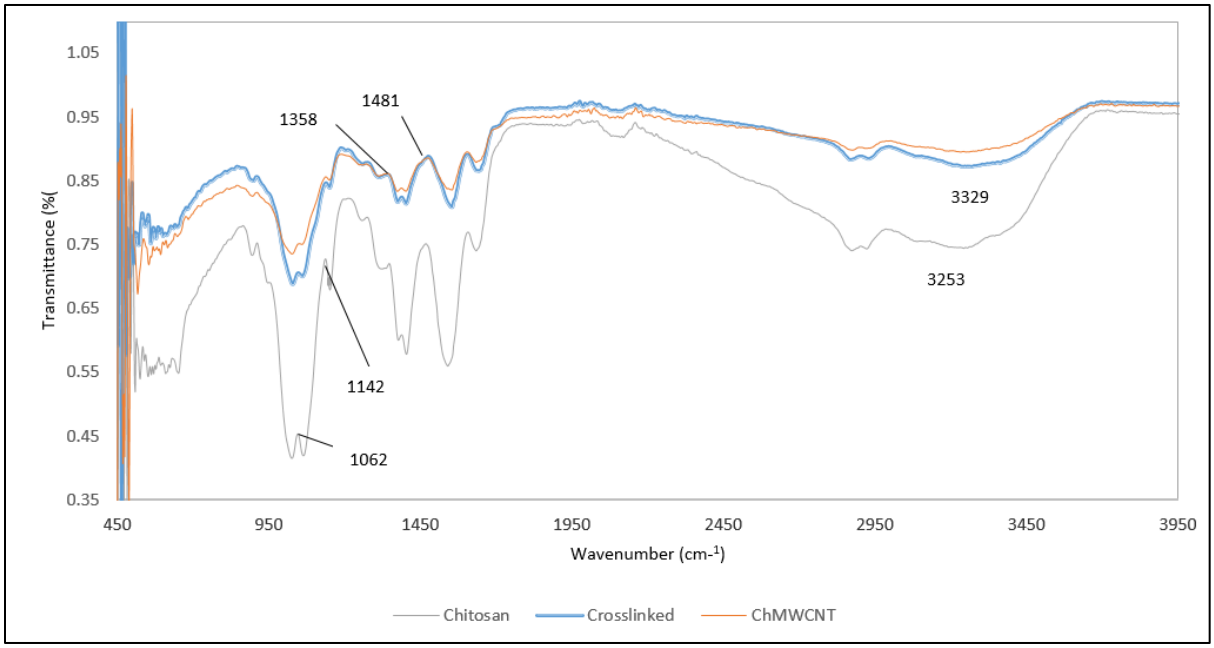

Figure 6.3. FTIR spectra of Ch, ChGLA and ChMWCNT

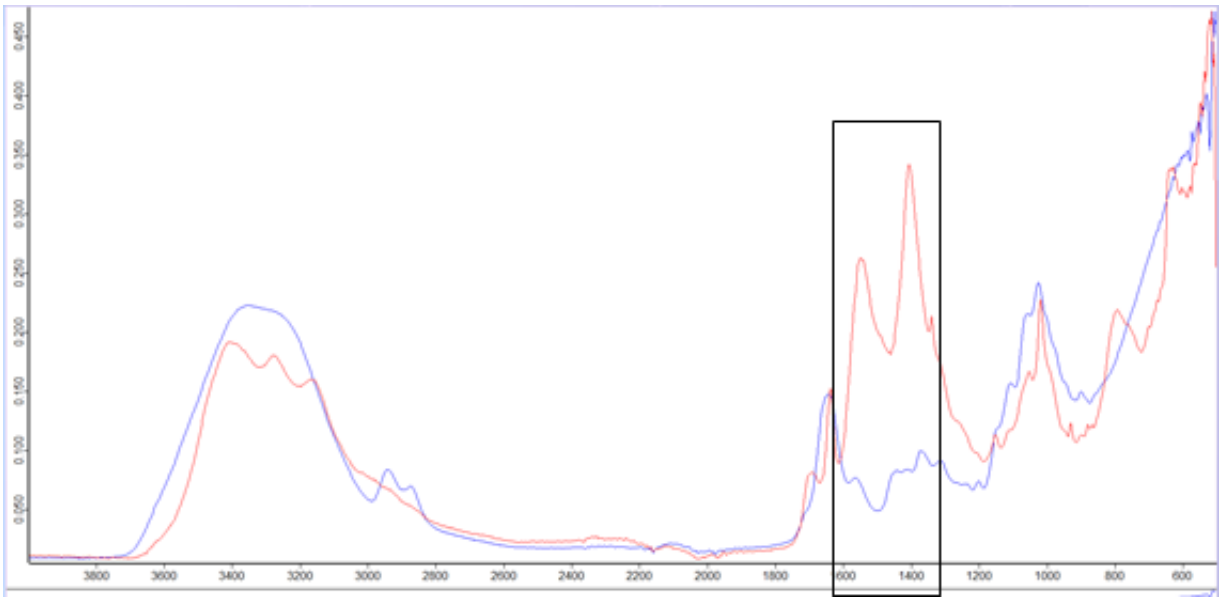

Figure 6.4. FTIR spectra of chitosan (CH) and glutaraldehyde crosslinked chitosan (CHGLA) showing formation of a new bond

The FTIR spectra of chitosan (Ch), chitosan-GLA (ChGLA) and chitosan-Multi-Walled Carbon Nanotubes (ChMWCNTs) are shown in Figure 6.3. The spectrum of chitosan displays a number of absorption peaks, an indication of different types of functional groups presents in chitosan sorbents. The broad and strong band ranging from 3200 to 3600 cm^{-1} corresponds to the presence of -OH and -NH_2 groups, which is consistent with the peak at 1062 and 1142 cm^{-1} assigned to alcoholic C-O and C-N stretching vibration. The 1358 cm^{-1} band is characteristic of glutaraldehyde and C=N bond, which confirms the crosslinking between chitosan and glutaraldehyde (clearly seen in Figure 6.4). The addition of multi-walled Carbon Nanotubes (ChMWCNT) resulted in a decrease of the 1481 cm^{-1} band, due to the increase in the amount of C. These results confirm that in the ChGLA hydrogel and ChMWCNT composite, glutaraldehyde was successfully crosslinked with the chitosan chains. Also, in the ChMWCNT, it was confirmed the insertion of activated carbon into the hydrogel structure.

6.3.1.2 Scanning Electron Microscopy (SEM) analysis

Figure 6.5 shows the surface morphology of raw chitosan at different magnifications. The SEM images clearly shows a flat, smooth and dense surface. This can be compared to the surface morphologies of gluteraldehyde crosslinked freeze-dried chitosan (ChGLA) in Figure 6.6 (A) and

that of gluteraldehyde crosslinked chitosan modified with multiwalled carbon nanotubes (ChCWCNT) in Figure 6.7 (A). Compared to the smooth surface of raw chitosan, ChGLA displays a rough, porous surface as also found by Li *et al.* (2013). Li *et al.* (2013) described the microspheres of chitosan crosslinked with gluteraldehyde as spherical and having a smooth outer surface. Differences in structures of gluteraldehyde crosslinked chitosan can be due to degree of crosslinking and levels of deacetylation in the synthesis of chitosan itself which obviously affects its morphology and size (Li *et al.*, 2013).

Upon adsorption of MCs, Figure 6.6 (B) shows a clear contrast in the surface morphology of the ChGLA as it becomes hollower and less compact.

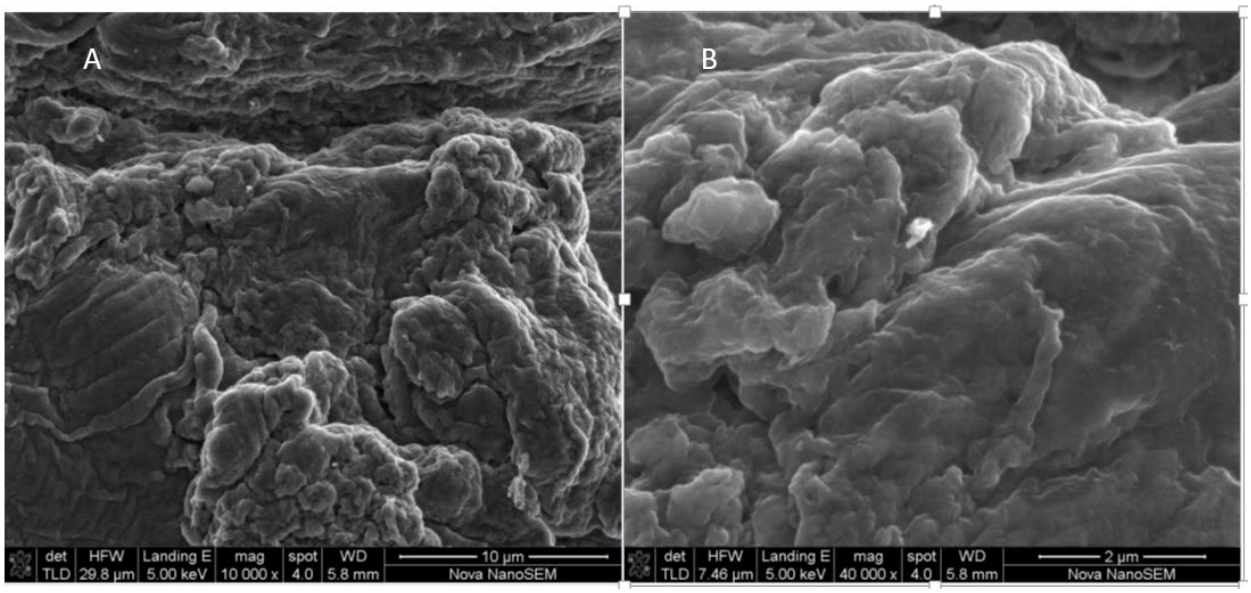

Figure 6.5. SEM images for Chitosan A) x10 000 magnification B) x40 000 magnification

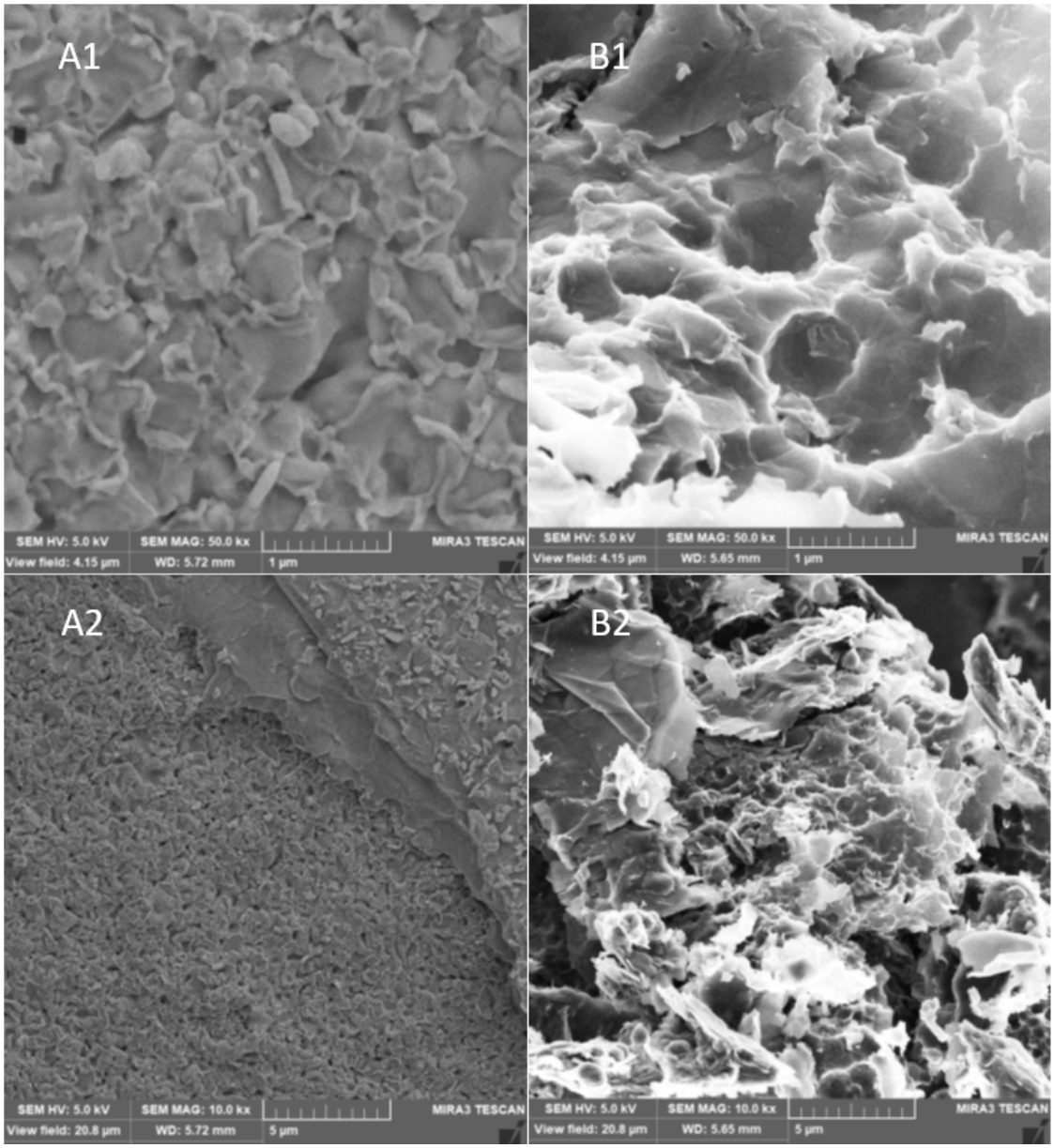

Figure 6.6. SEM images for ChGLA A) before adsorption B) after adsorption

Figure 6.7 shows the morphology of ChMWCNT at different magnifications (a) raw material before adsorption and (b) after adsorbing MCs. Compared to the smooth and flat surface of chitosan and the rough, porous surface of ChGLA, the introduction of multiwalled CNTs seems to make the crosslinked chitosan structure more porous and granular. 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 6.7 (A vs B) with the structure becoming deformed and irregular upon adsorption.

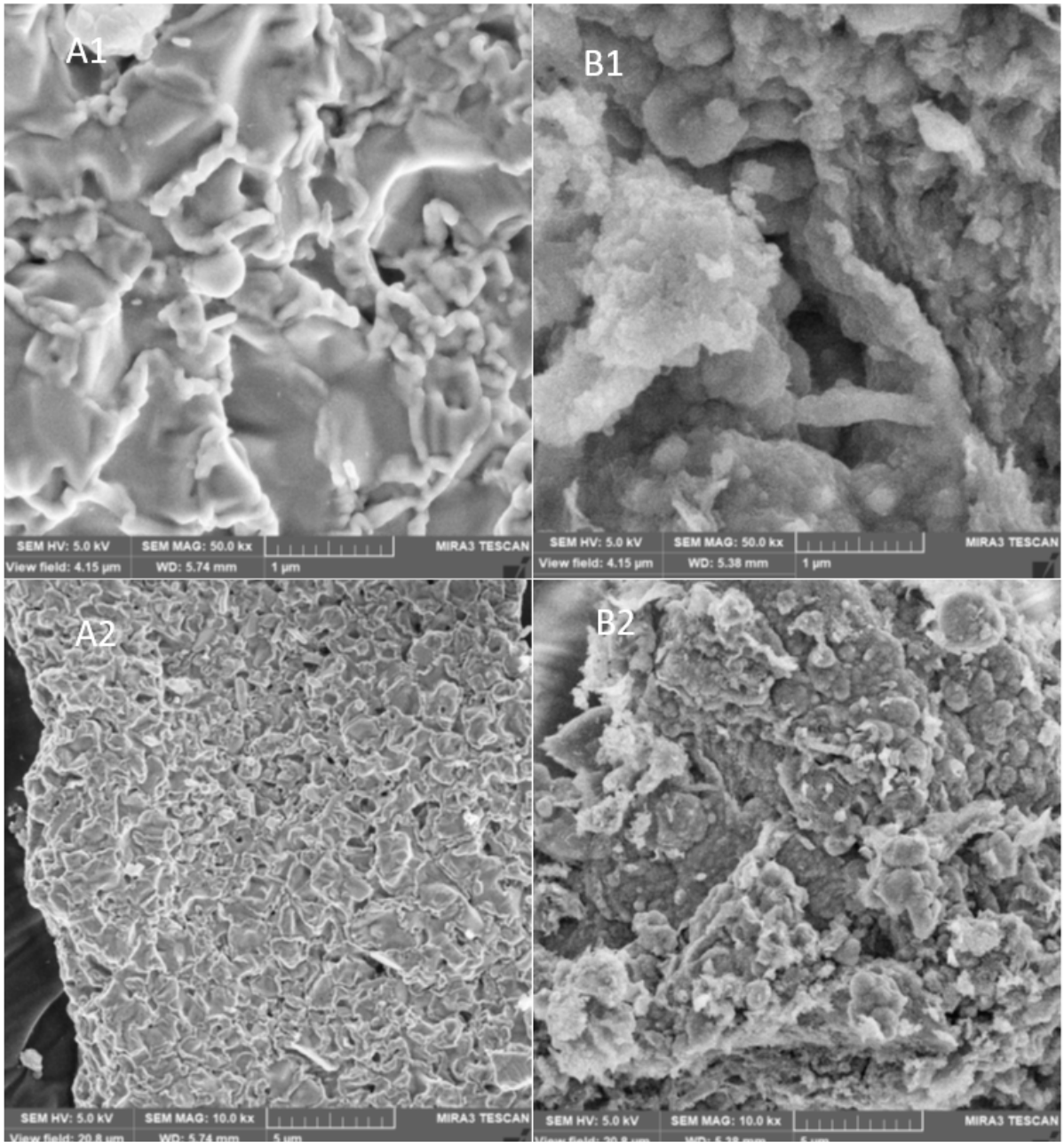

Figure 6.7. SEM images for ChMWCNT A) before adsorption B) after adsorption

6.3.1.3 Brunauer-Emmett-Teller (BET) analysis

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 volume (Zhao *et al.*, 2013). Table 6.1 shows the surface area and pore size of the raw chitosan, synthesized ChGLA, ChMWCNT and the commercial DION HP20 resin, which was evaluated using the Brunauer-Emmett-Teller (BET) method. Findings indicate that the 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 materials, raw chitosan had the lowest, followed by ChGLA, ChMWCNT. HP20 displayed far more superior surface area and pore volume compared to any of the synthesized material, but the ChMWCNT had much greater pore sizes compared to 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 HP20, its higher pore sizes makes it ideal of the adsorption of MCs, as Li *et al.* (2011), reiterated 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 HP20, ChGLA, ChMWCNT and Chitosan

	HP20	ChGLA	ChMWCNT	Chitosan
Surface area (m^2/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 (cm^3/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

6.3.2 Physicochemical parameters of the raw dam water

The raw dam water used for the adsorption studies was collected from Roodeplaat Dam (Pretoria), next to the dam wall in 20L containers and frozen till use. The water was collected in July, 2021. Table 2.1 shows the physicochemical properties of the raw dam water. The Microcystins (MCs) levels of the water used were 5.041 μg L^{-1}. The pH for the water was slightly alkaline (pH 8.84) and the EC was 346 μS cm^{-1} and the turbidity was 34.05 ntu.

Table 6.2. Physicochemical parameters of the raw dam water

pH	EC (us cm^{-1})	TDS (mg L^{-1})	Turbidity (NTU)	MC-LR (ug L^{-1})
8.84	346	234	34.05	5.041

6.3.3 Adsorption and desorption characteristics of the sorbents for microcystins

6.3.3.1 Adsorption characteristics without SPATT bags

Upon exposing the 0.01 g and 0.02 g of the synthesized ChMWCNT composite, to 5 ug L^{-1} of MC-LR (2 mL) for 30 minutes with agitation of 145 rpm, Figure 6.8 shows the adsorption efficiency of the synthesized material (ChMWCNT). Based on the findings in Figure 6.8, there was a 96.1% reduction of MC-LR in solution when 0.01 g of the composite was used, and a 95.6% adsorption/removals of MC-LR when 0.02 g of the composite was used. This can be compared to only 11.8% loss which was noted in the control solutions which were not exposed to any adsorbent. These finding indicate that, the ChMWCNT composite is a good adsorbent for the toxin and that there is no difference in the % removal if 0.01 g or 0.02 g of the material is used.

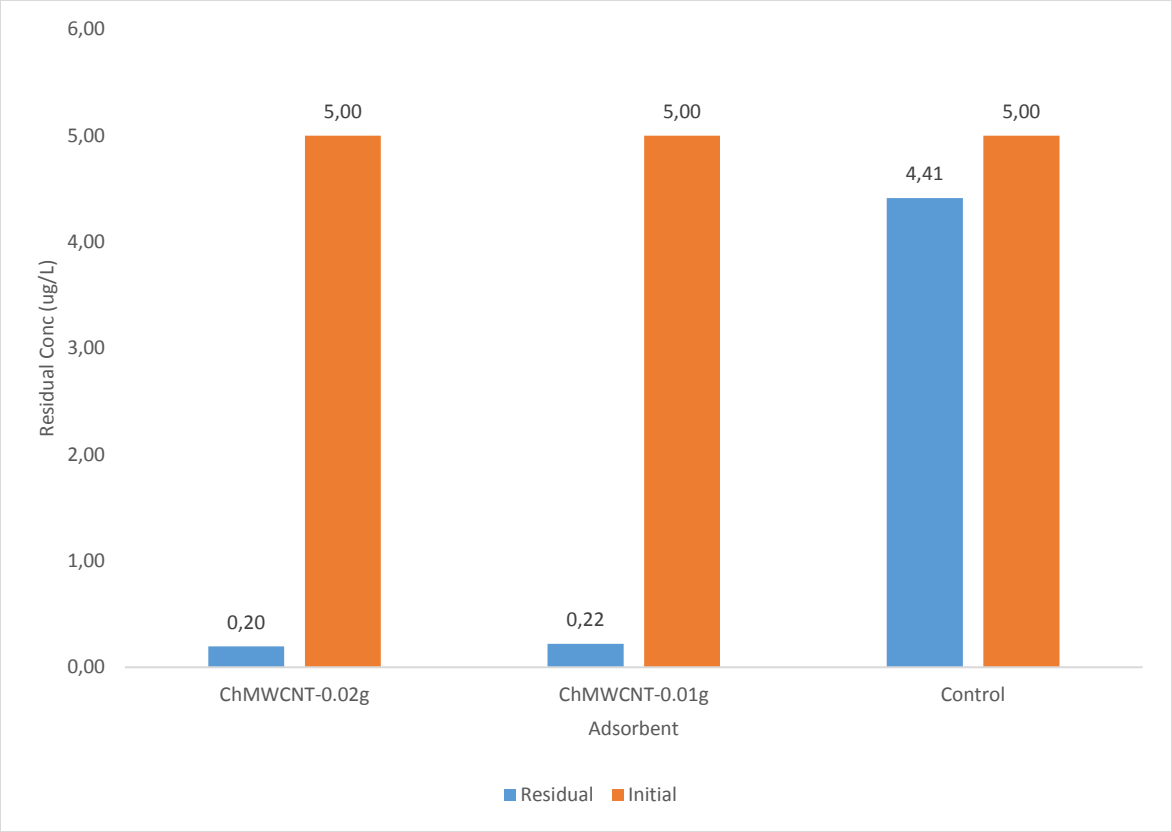

Figure 6.8. Adsorption efficiency of ChMWCNT (0.01 g and 0.02) after exposure to an initial conc of 5 µg L^{-1} (2 mL) for 30 mins, shaking at 145 rpm

6.3.3.2 Desorption characteristics without SPATT bags

Having adsorbed MC-LR, the next important stage was to see if the adsorbent ChMWCNT could desorb the toxin, for the adsorbent to be used for passive sampling purposes. After subjecting the ChMWCNT exposed to 5 ug L^{-1} of MC-LR and having observed the adsorption efficiency (section 6.3.3.1), the recovered residue upon centrifugation, was exposed to 5 mL of 80% MeOH for 30 mins and agitated at 145 rpm and 0.0461 ± 0.021 µg g^{-1} was recovered from the composite (ChMWCNT).

6.3.3.3 Adsorption and desorption characteristics in SPATT bags using spiked water

After establishing the synthesized sorbents adsorption and desorption capacities as free material, the next step was to pack the material in SPATT bags which were constructed and packed as described in section 6.2.7.1. The SPATT bags were activated by soaking them in 100% MeOH for 24 hours and thereafter thoroughly rinsed with deionized water prior to use. Upon exposure to

spiked deionized water with an initial concentration of 5 ug L^{-1} of MC-LR, the levels of MC-LR were reduced to 0.032; 0.022 and 0.016 in ChMWCNT (0.1 g), ChGLA (0.1 g) and HP20 (1.5 g) respectively, within 30 mins (Figure 6.8). This accounted for 99.4%, 99.6% and 99.7% removal/adsorption in the three respective adsorbents (ChMWCNT (0.1 g), ChGLA (0.1 g) and HP20 (1.5 g), Table 6.3).

Of importance to note was the relatively comparable adsorption capacities of the two synthesized sorbents (ChGLA and ChMWCNT) at a very low dosage of 0.1 g, compared to 1.5 g dosage applied for the commonly used resin (HP20) applied commonly for the passive sampling of MCs. The synthesized resins have very low density compared to the HP20 resin; thus equal dosages could not be applied in the SPATT bags.

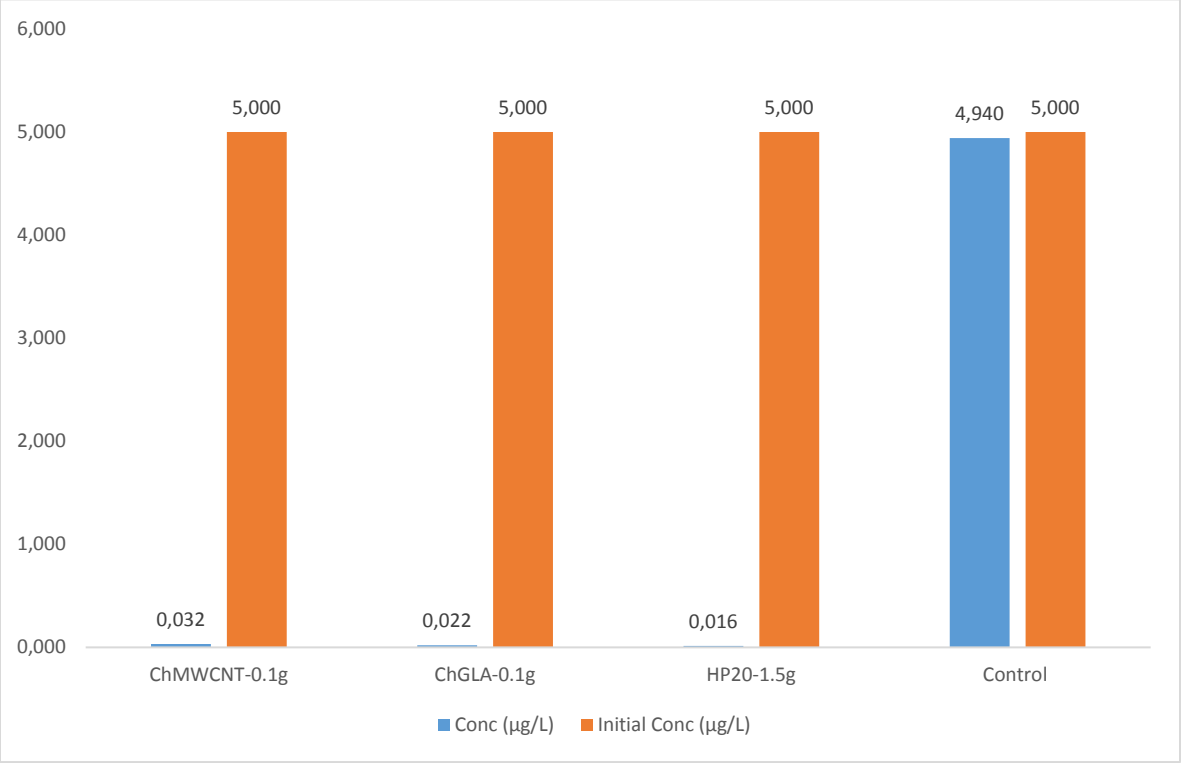

Figure 6.9. Adsorption efficiency of ChGLA and ChMWCNT (0.1 g) after exposure to an initial conc of 5 µg L^{-1} (20 mL) for 30 mins, shaking at 145 rpm

Table 6.3. Adsorption efficiency of ChGLA and ChMWCNT (0.1 g) after exposure to an initial conc of 5 µg L^{-1} (20 mL) for 30 mins, shaking at 145 rpm

Composite/hydrogel	Adsorption (%)
ChGLACNT- 0.1 g	99.4%
ChGLA- 0.1 g	99.6%
HP20- 1.5 g	99.7%
Control	1.2%

Upon desorption of the adsorbed toxins, results of SPATT sampling are normally expressed as µg g^{-1}- resin or µg g^{-1}- resin/day. To better compare the adsorption efficiencies of the 3 material, the adsorption efficiencies were converted to µg g^{-1}-resin (see equation 12). The finding in Table 2.3 shows that, inasmuch as the 3 materials demonstrated above 99% adsorption efficiencies within 30 mins, the newly synthesized sorbents had much higher desorption efficiencies when 80% of MeOH is used as the desorption solution. The ChGLA hydrogel showed 6.8 times desorption efficiency and the ChMWCNT composite had 2.7 times desorption efficiency better than the most commonly used HP20 resin. When comparing the two newly synthesized hydrogels, the ChGLA showed a desorption efficiency that was at least 2.6 times better than that of ChMWCNT, proving that, among the 3 materials tested here, the glutaraldehyde crosslinked chitosan (ChGLA) is the most ideal material to be used for MCs passive sampling in SPATT bag format.

Table 6.4. Composite/hydrogel Adsorption capacities in SPATT bags

	ChGLA	ChMWCNT	HP20
MCs adsorbed to SPATT resin (µg g^{-1} of resin)	0.0459 ± 0.0063	0.0180 ± 0.0026	0.006759± 0.0044

6.3.3.4 Adsorption characteristics in SPATT bags using field water

The three adsorbents were also tested for their adsorption and desorption efficiency when exposed to field water as described in section 2.2.7.3. The physicochemical properties of the filed water are described in section 2.3.2. Based on the findings in Table 2.4, the MCs levels in the desorbed extracts showed that the HP20 resin had higher levels of MCs compared to the two sorbents (ChGLA and ChMWCNT). However, the adsorbents' desorption efficiency, which takes the amount of the material used in the adsorption process showed that the ChGLA had a better desorption efficiency compared to the other two materials. When applied in field water in SPATT

bag format, the ChGLA hydrogel desorbed MCs 1.2 times better than ChMWCNT composite and 11.4 times better than the HP20 resin.

In as much as the ChGLA hydrogel performed better than the ChMWCNT composite in field water, the differences were relatively comparable in field water than in spiked deionized water. However, the ChGLA desorption efficiency when using 80% MeOH as the desorption solution was much higher in field water (11.4 times higher) than in spiked deionized water (6.8 times). These finding once again confirmed that, under the conditions applied, the ChGLA hydrogel is a much better sorbent to be used for the passive sampling of MCs in SPATT bag format compared to ChMWCNT composite and the HP20 resin.

Table 6.5. Composite/hydrogel Adsorption capacities in SPATT bags with field water

	Field water (ug L^{-1})	**SPATT extract (µg L^{-1})**	**SPATT (µg g^{-1}-resin)**
ChGLA 0.1 g	5.041 ± 0.144	15.519± 2.461	0.155± 0.025
ChMWCNT 0.1 g	5.041 ± 0.144	12.665± 4.207	0.127± 0.042
HP20 1.5 g	5.041 ± 0.144	20.487± 2.036	0.014± 0.001

Since no optimization of the equilibration time and other conditions were conducted in these trials, the equilibration time and conditions for the adsorption and desorption experiments were derived from literature. Zhao *et al.* (2013) conducted detailed experiments looking into the adsorption and desorption mechanisms of the intra- and extra-cellular toxins MC-LR and [Dha^7] MC-LR produced by *Microcystis aeruginosa* on aromatic resins HP20 and SP700 and found that the adsorption equilibration times for microcystins by the HP20 and SP700 resins were 30 min and 15 min, respectively. Hence equilibration times of 30 mins for both adsorption and desorption were adopted in this trial.

Of the adsorbents used to date, the aromatic resin DIAON HP20 has proven particularly efficient in monitoring a wide range of marine and fresh water bio-toxins, including microcystins (Zhao *et al.*, 2013), hence the synthesized chitosan-based sorbents were compared to the aromatic resin DIAON HP20. In our laboratory trials with both spiked deionized water and field water, we found

that ChGLA had much higher recoveries compared to ChMWCNT and HP20, demonstrating that ChGLA had weaker affinity for MCs compared to the other two, thus making it the ideal candidate for passive sampling of MCs in SPATT format. Kudela (2011), conducted trails using Pinto Lake water and seawater and found saturation values of up to 18,4 $\mu g\ g^{-1}$ resin for HP20, thus proving that the HP20 used in the current study could not have been saturated during the trials.

Kudela (2011) applied sequential extraction and found excellent extraction efficiencies with up to 100% recoveries using 50% MeOH extractions. Kudela (2011) also found differences in the adsorption of different MC congeners, thus future work will need to look into adsorption efficiencies and recoveries of the different congeners on the synthesized adsorbents and if sequential extractions using different MeOH solutions will have an impact on the recoveries.

In our laboratory trials with field water we found adsorption capacities of 0.155± 0.025; 0.127± 0.042; and 0.014± 0.001 for ChGLA, ChMWCNT and HP20 respectively, with ChGLA showing much better adsorption capacities and recoveries. These figures are very much comparable to the 0.0783-0.2455 ug/g-resin reported by Davis and Hansen (2013) in Black Lake – West Site (UK), when they used the HP20 resin to monitor the lake for MCs. Based on our findings, the adsorption of MCs by the ChMWCNT composite and the ChGLA hydrogel will need further investigation and possible application in the field in a SPATT bag format. We had hypothesized thatbiopolymer chitosan could be crosslinked and/or modified to be used for this purpose since it has a wide presence of hydroxyl groups (-OH) and primary amine ($-NH_2$) (adsorption sites), making it an efficient adsorbent (Alves *et al.*, 2019). Because of the stability difficulty and the limitations on using chitosan powder for an adsorption operation in aqueous medium, an alternative was chemical modification of this polymer, to intensify its potential and increase its applicability (Gonçalves *et al.*, 2017). As applied by Alves *et al.* (2019) crosslinking chitosan and then insertion of multiwalled CNTs, then followed by freeze-drying were expected to increase its surface area and give it better stability in aqueous solutions.

6.4 SUMMARY

The current work describes the successful crosslinking and addition of multi-walled CNTs onto the chitosan structure crosslinked with gluteraldehyde as was confirmed by the FITR results. The SEM images confirmed the successful crosslinking of chitosan by gluteraldehyde and also 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 compared to the HP20 resin, suggested better capacities for the ChMWCNT to adsorb MCs.

Both the gluteraldehyde crosslinked chitosan hydrogel (ChGLA) and the chitosan-multi-walled CNT (ChMWCNT) composite were applied for the adsorption and desorption of MCs. The findings indicate that both adsorbents are good sorbents for MC-LR and their adsorption capacities are much better compared to the commonly used aromatic resin HP20. The desorption efficiencies of the two synthesized chitosan sorbents were also much better compared to that of the HP20 resin, making them ideal candidates for application in the SPATT bag format for the passive sampling of MCs.

SPATT using the synthesized chitosan-based adsorbents has the potential to be integrated into current cyanobacterial monitoring programmes and would be a very useful and economical tool for early warning and monitoring of toxic cyanobacterial events in water intended for irrigation.

CHAPTER 7: FIELD APPLICATION OF A CROSSLINKED CHITOSAN-BASED SOLID PHASE ADSORPTION TOXIN TRACKING TECHNOLOGY (SPATT) ADSORBENT

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7.1 INTRODUCTION

Harmful cyanobacteria blooms (CyanoHABs) are comprised of naturally occurring photosynthetic prokaryotes found in a wide variety of aquatic environments and capable of producing toxic secondary metabolites (cyanotoxins). CyanoHABs can thrive under a wide range of environmental conditions and are especially prolific and competitive under high nutrient conditions often associated with eutrophic waters. Due to the toxicological effects from microcystins (MCs), exposure to the toxins presents a health hazard and numerous MC poisonings have been documented in pets, livestock, wildlife, and humans (Falconer, 2005).

Microcystins (MCs) are the most prevalent and abundant cyanobacterial toxins in the environment, which can be frequently found in water reservoirs used for drinking water supply (Buratti *et al.*, 2017; Huisman *et al.*, 2018). Consumption of MC-contaminated drinking water has been recognised as an important exposure route, with potentially chronic, repeated exposure patterns (Jaša *et al.*, 2019). Considering chronic toxicity of MCs, namely their tumor-promoting and hepato-carcinogenic activity, the World Health Organization (WHO) established a provisional guidance value of 1 mg L^{-1} for MC-LR concentration in drinking water, and even stricter limits (0.1-0.3 mg L^{-1}) have been implemented by some regulatory agencies (Buratti *et al.*, 2017). Long-term compliance with such limits requires a multi-barrier approach, which includes a monitoring system for cyanobacteria and cyanotoxins, in order to optimize and facilitate their effective removal during drinking water treatment operation (He *et al.*, 2016; Sklenar *et al.*, 2016). Therefore, there is a continuous effort to develop rapid, simple, sensitive, and cost-effective methods which would allow either real-time or time-integrative monitoring of cyanobacteria and MCs.

Sampling and monitoring of cyanotoxins, especially in large water bodies and rivers, can be problematic because cyanotoxins levels can vary rapidly with changing environmental and hydrological conditions. Current sampling practices (e.g. grab samples) provide only a snapshot

of cyanotoxins present at one point in time and may miss areas or times of highest risk. These issues are particularly relevant when sampling rivers where toxic benthic mats may sporadically release toxins to the water and flows transport toxins rapidly (Wood *et al.*, 2011). Passive in situ methodology known as solid phase adsorption toxin tracking technology (SPATT) has been shown to be a simple and sensitive means of warning of toxic micro-algal bloom development and associated shellfish contamination in the marine environment ((MacKenzie *et al.*, 2004; Piletska *et al.*, 2008; Pizarro *et al.*, 2008). SPATT involves suspending in the water body small bags containing adsorption substrates which accumulate toxins. 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 (Fux *et al.*, 2009; MacKenzie, 2010) but a range of technical problems remain to be solved for use of SPATT with highly water soluble toxins such as cylindrospermopsin and saxitoxins.

Many different sorbents have been used for passive sampling all over the world, from HP-20 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 microalgal toxins using different bulk polymeric sorbents (Zendong *et al.*, 2014) most of these sorbents are synthetic and relatively costly to buy. This study aims to synthesize and assess the physicochemical properties of modified/crosslinked chitosan sorbents for their potential/ability to adsorb a range of MCs and to determine the potential applicability of SPATT for tracking MCs in irrigation water.

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 not been studied, much especially for passive sampling in SPATT format. Based on literature, we believe that the insertion of multi-walled carbon nanotubes into the network has a synergistic effect, of improving the mechanical and adsorptive characteristics of the hydrogel. In this way, the

innovation of this work is 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. The aim of this work was to develop chitosan based hydrogel/composite for MCs sampling in a SPATT bag format.

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 polystyrenic matrices (HP20 and SP700) were purchased from Rochelle Chemicals (Johannesburg, S.A). De-ionized water obtained from a MilliQ water purification system (Merck-Millipore Ltd., Germany) to 18 mΩ 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 nanotubes 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 an electric sewing machine and form an open bag of 55 mm width. The SPATT bags were filled with 3 g (dry weight) of DION HP20 and 0.2 of ChGLA and ChMWCNT per each bag, then sewn on the fourth side forming a 55 x 55 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 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 DION HP20 (b) synthesized ChGLA (c) synthesized ChMWCNT.

7.2.5 Laboratory exposures

To gain an insight on the approximate number of days the samplers take to be saturated, eighteen, one litre amber bottles (nine for ChGLA and nine for HP20) were filled with raw unfiltered Roodeplaat Dam water which contained visible cyanobacteria cells and known to be contaminated with the cyanotoxin microcystin (MC). SPATT bags containing ChGLA and HP20 were suspended in each bottle and three samplers (SPATT bags) were removed from each treatment after 24 hrs, 48 hrs and 72 hrs and the residual concentration of MCs monitored at each of those three intervals. The bottles containing the samplers were continuously agitated at 100 rpm using reciprocal shaker for the duration of the experiment and light illumination was kept at a minimum during the experiment (see Figure 7.2).

Figure 7.2. Laboratory exposure experiment set-up.

Individual samplers were removed daily, rinsed in distilled water and refrigerated in sealed Ziplock bags. Water samples (5 mL) were also taken daily and analysed the same day or frozen for later microcystins analysis using the EUROFINS (USA) high ELISA kits.

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 Hartbeespoort site. 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.

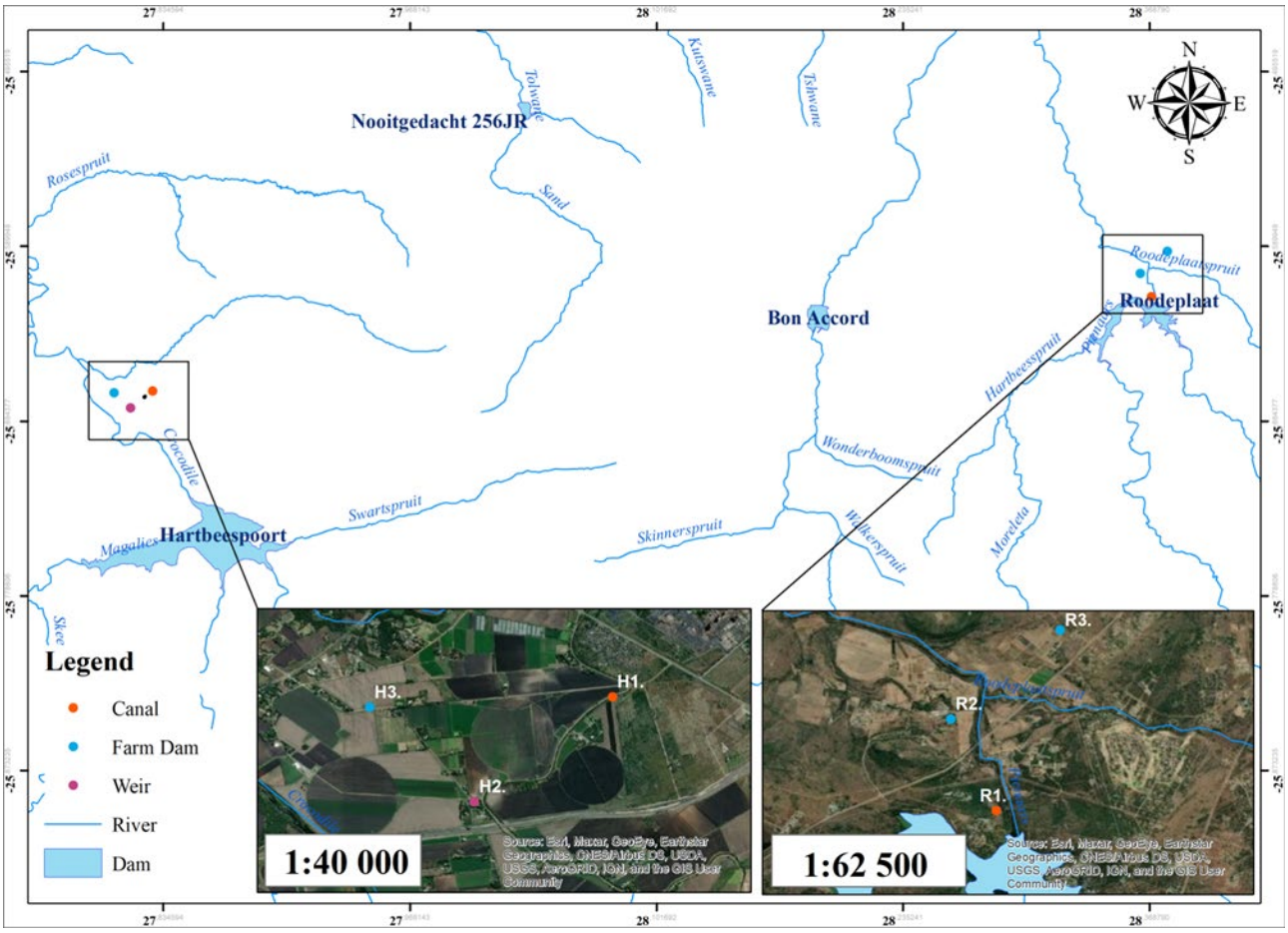

Figure 7.3. Location of the sampling points & sites for the SPATT samplers' deployment.

Six SPATT samplers (two containing each of the sorbent, ChGLA, ChMWCNT and DION HP20) were deployed for 2 days in the field during the period 10 January 2022 to 13 January 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 7.4. SPATT samplers' configuration for field deployment

7.2.7 Toxin extraction and analysis from the SPATT samplers

For both laboratory and field SPATT samplers, the 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 decanted into a 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 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 extraction being 5 mL each for each sample and the resulting supernatants pooled 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 microcystins 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 the following formula:

$$\text{Total dissolved MCs (} \mu g\ g^{-1} \text{ resin)} = \frac{(MCs\ conc\ \ \mu g/L - \text{extract})\ x\ (0.002L\ extract - VOL)}{(3\ g\ resin)} \quad [17]$$

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 DIAON HP20 resin used in the SPATT bags (0.2 g for ChGLA and ChMWCNT).

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 cyanobacterial biomass in water samples according to Lawton *et al.* (1999).

7.3 RESULTS AND DISCUSSION

7.3.1 Laboratory exposure test

The SPATT samplers loaded with 0.2 g of ChGLA and DION HP20 were exposed to field to try and estimate the time they take to be saturated when exposed to field water with approximately 5 ug L^{-1} of MCs. Only two of the three sorbents were tested due to the availability of the ELISA kits and previous performance of the adsorbents in the previous experiments (refer to deliverable 7 page 16). Findings of the laboratory trial in Figure 7.5 show that both adsorbents were saturated within 24 hours when exposed to field water with an initial concentration of 5-9 ug L^{-1} of MCs. Of importance to note was the fact that the resin HP20 retained the MCs throughout the 72 hour period of exposure, whereas the ChGLA hydrogel seemed to leach the adsorbed MCs as indicated by the decline in the recovered MCs as the time of exposure was increased (from 24 hrs through to 72 hrs).

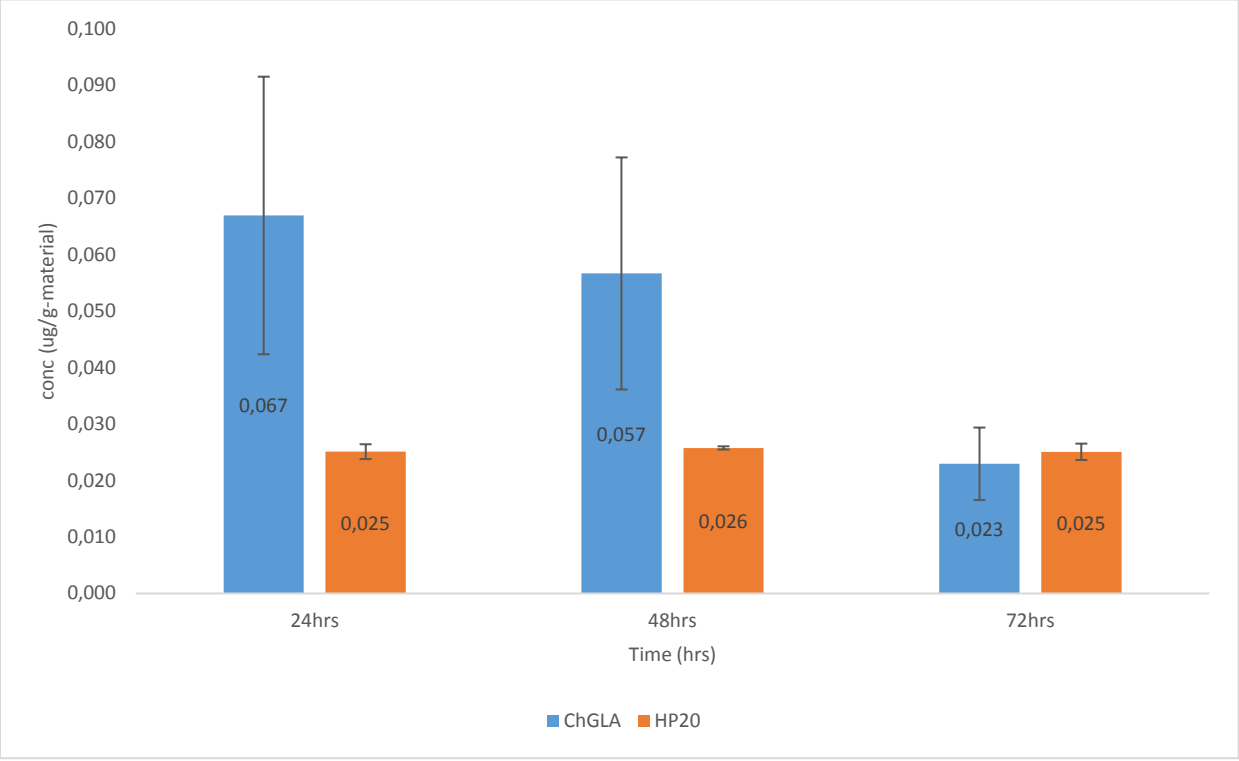

Figure 7.5. MCs adsorbed by the sorbents in SPATT bags (µg g^{-1}-material) over time when exposed to field water during laboratory trails.

With regards to the residual MCs levels in the water upon exposure to the samplers, Figure 7.6 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. Whereas, bottles in which the field water was exposed to the ChGLA loaded samplers, the MCs levels in the bottles was gradually going down from an initial concentration of ± 5 µg L^{-1} to ± 2 µg L^{-1} after 72 hours of exposure. Our previous experiments had shown us that ChGLA adsorbed MCs better than HP20, thus 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 HP20 and only 0.2 g for ChGLA, since ChGLA is less dense than HP20, hence the SPATT bags could not take up more of the adsorbent.

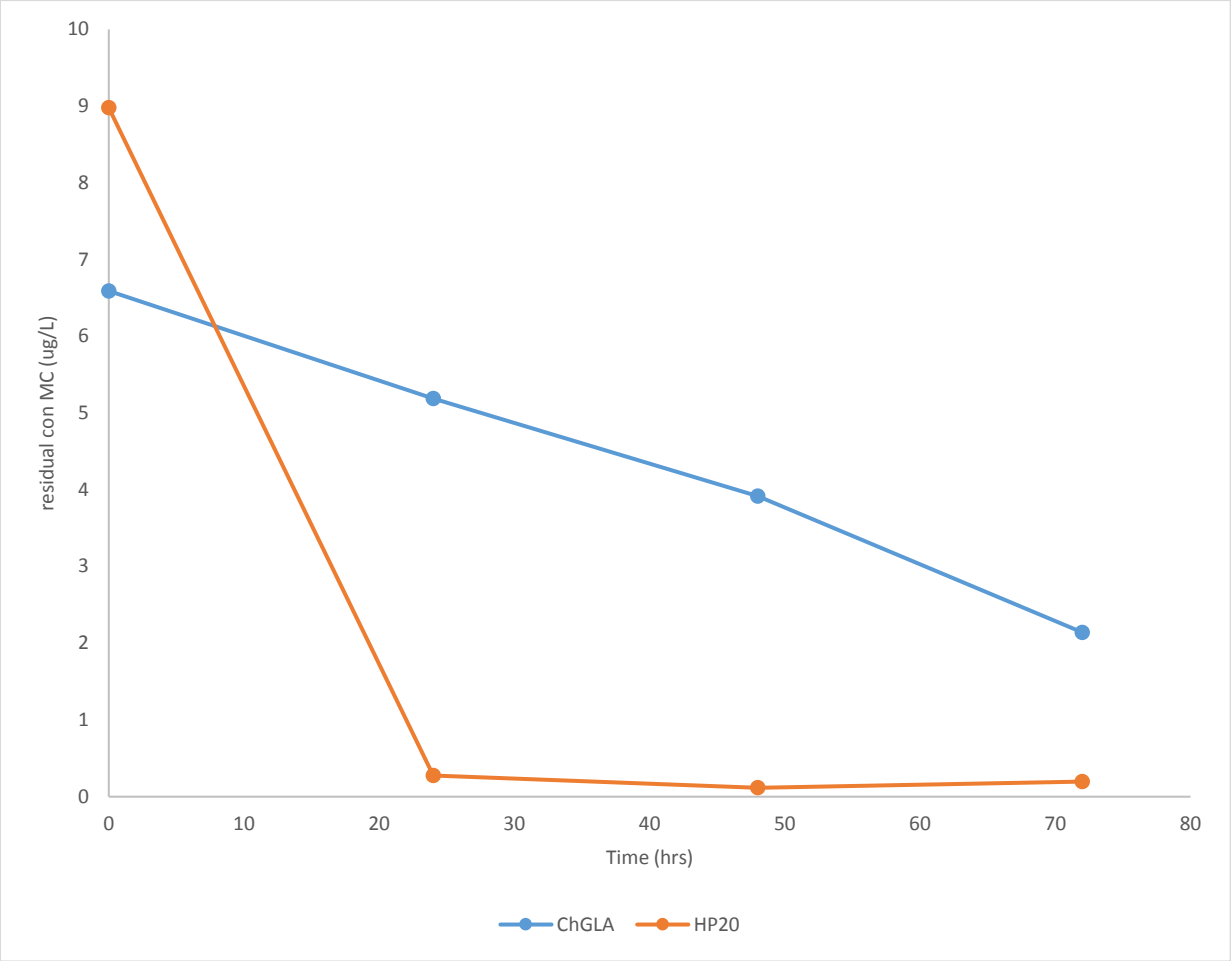

Figure 7.6. Residual MCs levels in the bottles with SPATT samplers with HP20 and ChGLA over time

7.3.2 Field Monitoring

7.3.2.1 Levels of MCs detected by the SPATT samplers

Despite the laboratory study having shown that the samplers were getting saturated after 24 hours, the samplers with the three sorbents (ChGLA, ChMWCNT and 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 prior to the field visit. Findings of the field monitoring confirmed that the field water in both sites had traces of MCs (below 1 ug L^{-1}) in five out of the six sites based on the grab samples (Table 7.1).

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. The samplers were detecting higher MCs levels in the Roodeplaat site compared to the Hartbeespoort site (Figure 7.7). Contrary to our preliminary laboratory findings in the previous report, the ChMWCNT adsorbent was adsorbing MCs better than the other two adsorbents in water from the Hartbeespoort site, whereas the adsorption (µg g^{-1}-adsorbent) of all the three adsorbents was comparable for all the samplers deployed at the three points at the Roodeplaat site (Figure 7.7).

Table 7.1. Levels of MCs monitored by different samplers/methods

Site	Sampling point	Water	ChGLA		ChMWCNT		HP20	
		(grab µg L^{-1})	mg L^{-1}-extract	mg g^{-1}-adsorbent	mg L^{-1}-extract	mg g^{-1}-adsorbent	mg L^{-1}-extract	mg g^{-1}-adsorbent
Hartbeespoort	H1	0.597	0.459	0.009	2.732	0.055	0.823	0.011
	H2	0.173	0.149	0.003	13.208	0.264	12.207	0.163
	H3	0.178	7.019	0.140	0.291	0.006	1.691	0.023
Roodeplaat	R1	0.116	5.811	0.116	5.290	0.106	9.557	0.127
	R2	0.493	9.061	0.181	7.360	0.147	11.783	0.157
	R3	3.316	12.191	0.244	12.767	0.255	12.509	0.167

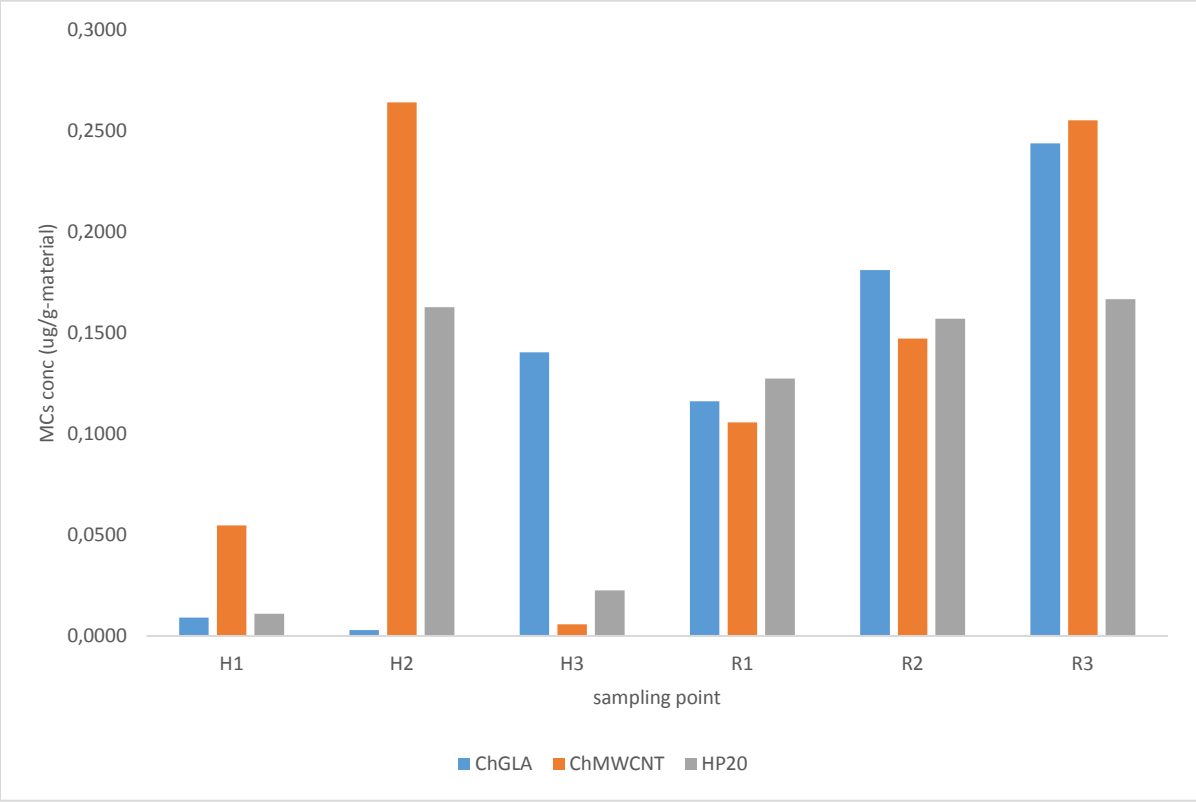

Figure 7.7. MC levels adsorbed by the three samplers in µg g^{-1}-adsorbent over the 2 days.

A comparison of the detection abilities of the three samplers and grab samples collected upon deployment and upon retrieval of the samplers is shown in Figure 7.8. Generally, the three samplers demonstrated a similar trend in detecting MCs to grab samples, particularly in the three Roodeplaat sampling points. The few exceptions were point H2 where the ChMWCNT and HP20 detected quite high levels of MCs compared to the ChGLA sampler and the grab samples and in sampling point H3 where the ChGLA detected high levels of MCs compared to the other two samplers and the grab samples. Some of the reasons for the patterns are better explained in Table 2.3 which looks at the physicochemical parameters and the levels of MCs detected by the different samplers and sampling method.

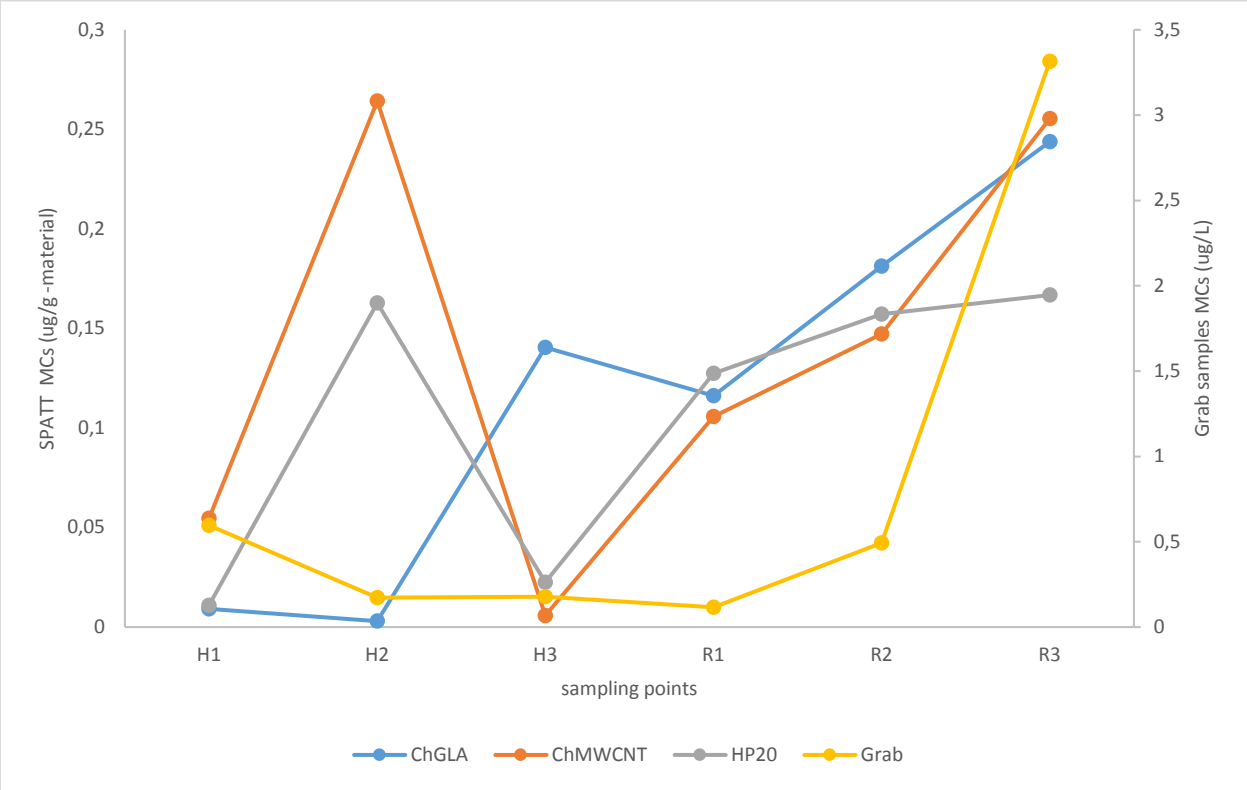

Figure 7.8. Comparison of the levels of MCs adsorbed by the samplers vs grab samples.

7.3.2.2 MCs SPATT sampler detection and physicochemical parameters

Among the physicochemical parameters monitored in-situ and ex-situ during the sampling period were Electrical Conductivity (EC) (us/cm); TDS (ppm); Salinity (psu); pH; DO (mg L^{-1}); Temp $^{\circ}C$; 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 Hartbeespoort site was exceeding the guideline

recommended for irrigation water, the pH of the water for both sites was slightly alkaline but within the DWAF (1996) limits. The phosphate levels for all the sampling points were within the FAO guideline thresholds but the nitrate levels were above for sampling point H2 and all the Roodeplaat sampling points.

The concentration of chlorophyll-*a* present in the water is usually directly related to the number of algae living in the water. Generally, concentration levels of chlorophyll-a above 10 µg L^{-1} results in eutrophication which ultimately increases the likelihood, and rapid growth of cyanobacterial bloom in the aquatic ecosystem (Kansas Department of Health and Environment, 2011). With regards to the chlorophyll-*a*, samplings H3 and R2 had chlorophyll-*a* levels above the 10 ug L^{-1} threshold, implying 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 high levels of cyanobacteria are present in a waterbody, 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. Two of the six sampled sites (H2 and 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. Physicochemical properties of the irrigation water monitored

Site	EC (us/cm)	TDS (ppm)	Salinity (psu)	pH	DO (mg L^{-1})	Temp ^{o}C	Turbidity (ntu)	Phosphates (mg L^{-1})	Nitrates (mg L^{-1})	Chl *a* (µg L^{-1})
H1	434.8	213.6	0.26	7.88	10.27	36.5	71.25	0.68	3	2.888
H2	414.1	203.4	0.25	7.5	5.44	26	9.28	1	8	1.185
H3	592.4	290.8	0.339	8.04	9.65	30.1	29.45	1.05	4	23.844
R1	325.1	159.8	0.205	6.94	2.35	20.2	11.83	1.08	7	5.998
R2	330.1	162.3	0.21	8.28	7.34	29	35.28	1.2	5	35.322
R3	296.5	145.8	0.194	8.88	13.49	29.6	36.14	0.73	6	7.331
Guidelines	≤400*	260*		6.5-8.4*				0-2**	< 5	

*Department of Water Affairs (DWA, 1996), **Food and Agriculture Organization (FAO) guidelines of 1985

Besides the anticipated strong positive correlations between EC and TDS, EC and salinity and salinity and TDS, important positive correlations were recorded for water pH vs MC levels in grab samples, pH vs MC levels (µg g^{-1}) detected by the ChGLA samplers, DO vs MCs in grab samples, nitrate levels vs MC levels in HP20 and ChMWCNT samplers, chlorophyll-*a* vs MC levels in ChGLA, MC levels in grab vs ChGLA and ChMWCNT samplers and MC levels in HP20 vs ChMWCNT samplers (Table 7.3). The findings of the study in Table 7.3 also show strong negative correlations for TDS vs MCs in MCs in ChMWCNT; MCs in HP20 vs TDS; Salinity vs MCs in ChMWCNT; salinity vs MCs in HP20; temperature vs HP20 and turbidity vs MCs in HP20. Based on these findings, it was deduced that, factors strongly positively correlated with MC levels based on grab sampling were: pH and DO. Factors/parameters strongly positively correlated with MCs levels detected in ChGLA are: pH and chlorophyll-*a*. MCs detection in ChMWCNT was strongly positively correlated with nitrates and MCs detection in the resin DION HP20 was also strongly positively correlated with nitrates. MC detection by HP20 and ChMWCNT was strongly negatively correlated with: TDS and salinity and MC detection in HP20 was further strongly negatively correlated to temperature and turbidity.

Importantly, there was a strong positive correlation between MC adsorption by HP20 and MC adsorption by ChMWCNT (also supported in Figure 7.8). In addition most parameters/factors significantly correlating to the MC detection by the HP20 samplers were similarly affecting the ChMWCNT samplers. The levels of MCs detected through grab sampling was also strongly positively correlated with MC detection by ChGLA and ChMWCNT.

In this study chlorophyll-*a* was strongly positively correlated to MC levels detected by ChGLA and not any other samplers and 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 microcystin concentration detected by SPATT samplers using the resin DION HP20. Kudela (2011) also found algae biomass as chlorophyll concentrations as best single predictor for MC loads in Pinto Lake (U.S.A) in both grab and SPATT samplers using DION HP20.

Table 7.3. Pearson correlation coefficients between physicochemical parameters monitored and MCs in SPATT samplers used

	Conductivity (us/cm)	*TDS (ppm)*	*Salinity (psu)*	*pH*	*DO (mg L^{1})*	*Temp °C*	*Turbidity (ntu)*	*Phosphates (mg L^{-1})*	*Nitrates (mgL^{-1})*	*Chl a (µgL^{-1})*	*Grab (µg L^{-1})*	*ChGLA (µg g^{-1})*	*ChMWCNT (µg g^{-1})*	*HP20 (µg g^{-1})*
Conductivity (us cm^{-1})	1													
TDS (ppm)	1	1												
Salinity (psu)	1.000**	1.000**	1											
pH	-0.098	-0.097	-0.077	1										
DO (mg L^{-1})	0.122	0.123	0.139	0.903**	1									
Temp °C	0.351*	0.351*	0.359*	0.579**	0.760**	1								
Turbidity (ntu)	0.115	0.116	0.119	0.439*	0.633**	0.904**	1							
Phosphates	0.055	0.055	0.053	-0.360*	-0.664**	-0.602**	-0.638**	1						
Nitrates	-0.460*	-0.460*	-0.465*	-0.370*	-0.544**	-0.815**	-0.865**	0.316*	1					
Chl a	0.149	0.150	0.161	0.355*	0.071	0.080	0.023	0.647**	-0.351*	1				
Grab	-0.477*	-0.477*	-0.463*	0.769**	0.739**	0.218	0.224	-0.592**	0.022	-0.160	1			
ChGLA	-0.354*	-0.354*	-0.339*	0.634**	0.399*	-0.142	-0.089	0.141	-0.023	0.522**	0.641**	1		
ChMWCNT	-0.643**	-0.644**	-0.64**	0.235	0.043	-0.282	-0.395*	-0.112	0.740**	-0.322*	0.512**	0.1401	1	
HP20	-0.763**	-0.763**	-0.76**	0.107	-0.227	-0.582**	-0.599**	0.299	0.804**	0.007	0.353*	0.3734	0.872**	1

** Strong correlation between parameters; * Moderate correlation between parameters

N.B: If the coefficient value lies between ± 0.50 and ± 1, then it is said to be a strong correlation. Moderate degree: If the value lies between ± 0.30 and ± 0.49, then it is said to be a medium correlation. Low degree: When the value lies below 0.29, then it is said to be a small correlation.

We also found that nitrate levels in the water were strongly positively correlated to the MC levels in HP20 and ChMWCNT but not in grab samples. Kudela (2011) also found total dissolved nitrogen (TDN) to be significantly correlated to toxin concentrations in both grab and SPATT samplers, but Howard *et al.* (2017) 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. These strong correlations between toxin levels and nitrates and chlorophyll-*a*, gives an indication that managing nutrient loads might be the ideal primary strategy to curb harmful cyanobacterial blooms and toxin levels in the catchment.

With regards to our two newly synthesized sorbents, preliminary field findings seem to suggest that:

- Factors correlating to MC detection by HP20 also correlate to ChMWCNT
- Physicochemical parameters such as conductivity, salinity and TDS have an impact on the performance of ChMWCNT in the field water
- pH has a strong positive correlation with MC detection by ChGLA

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. In contrary to our findings, 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.

7.4 SUMMARY

This Chapter describes the successful field deployment of SPATT samplers using the two newly synthesized chitosan sorbents namely, gluteraldehyde 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 55 * 55 mm SPATT bags, whereas 3 g of the commercially available DION HP20 resin was used for both laboratory and field trials. The laboratory trial with field water having ± 5 μg L^{-1} of MCs showed that the commercial HP20 resin

was getting saturated after 24 hours of exposure and the ChGLA and ChMWCNT sorbents were getting saturated after 24 hours and thereafter leaching out some of the adsorbed MCs.

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 sampling period. The findings indicated that MCs were detectable in all the two sites and in all the six sampling points in all the three types of samples 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 were grab samples detected traces of the toxin.

The findings showed a good correlation of the toxins detected by the 3 samplers compared to the grab samples, but strong positive correlation were recorded for grab samples vs the ChGLA samplers, whereas the ChMWCNT samplers showed a strong positive correlation with the HP20 samplers. Among physicochemical parameters monitored, there was a strong positive correlation between chlorophyll-*a* and MCs in grab samples and ChGLA. There was also a strong positive correlation between dissolved nitrate levels and toxin levels detected by HP20 and ChMWCNT.

Importantly, factors correlating to MC detection by HP20 also correlated to ChMWCNT and the physicochemical parameters such as conductivity, salinity and TDS had a correlation with the MC levels detected by ChMWCNT and HP20 in the field water. In addition, pH had a strong positive correlation with MC detection by ChGLA

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 a very 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.

- Use a different method to detect and quantify the toxins, since ELISA does not differentiate the different congeners of MCs and is affected by a host of other external factors. This will give valuable information on the effectiveness of the synthesized material in adsorbing and desorption of the different congeners of MCs commonly found in the catchment under study.
- Optimize the adsorption and desorption conditions for the sorbents and find the optimum mass to pack in SPAT bags for field applications.

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

This chapter was prepared by Glynn K. Pindihama & Gitari W. Mugera.

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 the bioavailability, toxicokinetics and effects of a range of cyanobacterial metabolites on terrestrial food plants. Chitosan-based sorbents were also developed and evaluated for their sorption and desorption of MCs and applied in passive samplers to monitor the bioavailability of cyanotoxins 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) Occurrence of cyanobacteria, cyanotoxins, toxic metals and anionic surfactants in irrigation water and agricultural soils in the crocodile West & Marico Water Management Area, potential risk of transfer of cyanotoxins into food plants human and health risks associated with consuming contaminated plants, 3) Bioaccumulation and elimination (toxicokinetics) of cyanotoxins by plants, 4) Accumulation of cyanotoxins and toxic metals in the presence of las on *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 part of the study gave 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 major 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 on productivity and the possible human and animal health impacts. In addition, the literature review also highlighted the urgent need for guidelines and policies on cyanotoxins in irrigation water, food plants and water used for livestock.

8.2.2 Occurrence of cyanobacteria, cyanotoxins, toxic metals and anionic surfactants in irrigation water and agricultural

In the second part of the study, the co-existence of cyanotoxins, toxic metals and anionic surfactants in irrigation water and agricultural soils and the health risks associated with consuming cyanotoxins contaminated plants in the Crocodile (West) Marico Water Management Area, was studied. This part also evaluate the applicability of a passive sampling technology (SPATT) to monitor and detect cyanotoxins using the DIAON HP20 resin as an adsorbent in the study area. Our field sampling revealed the presence of cyanotoxins, metals and anionic surfactants in irrigation water (canals and farm dams), and agricultural soils in all the sampled sites during the sampling period. It also emerged that pH, turbidity, EC, and TDS have a correlation with levels of MCs in the irrigation water, thus suggesting that these could be used to predict MCs levels/presence in the irrigation water from the two dams. Findings also demonstrated the applicability of SPATT using the resin DION HP20 for the passive sampling of MCs in the irrigation water as it increased the likelihood of detecting MCs in instances where grab samples would miss or detect very low levels of the toxins. This is of significance, since grab sampling could miss some episodic HAB events, thus SPATT could be used to complement grab sampling and give early warnings in water intended for irrigation purposes. Among toxic cyanobacterial genus identified in the irrigation water from the two dams were *Microcystis, Anabaena* and *Oscillatoria*, with *Microcystis* being the most dominant throughout the sampling sites.

The study also found that metals in irrigation water were below the DWAF (1996) recommended threshold while in agricultural soils, metals like Cr, Ni, Cu, Pb, and As were above the guideline

values set for agricultural soils. The findings also showed that MCs and metals do accumulate in food crops when irrigated with contaminated water. The calculated estimated daily intake (EDI) for MCs in the collected food plants was below the WHO guideline threshold of 0.04 μg kg^{-1}. Thus, plants being irrigated by water from these two dams are still safe for human consumption. Among the metals of concern in the food plants, Cr, Fe, Cu, Zn, As, and Pb were found above the EU and FAO/WHO threshold for these metals in food crops. However, the calculated EDI for each of the metals detected were below the maximum tolerable daily intake (MTDI), thus implying that the plants being irrigated by water from the two dams are still safe for human consumption.

The presence of anionic surfactants, metals species and cyanotoxins in irrigation water and agricultural soils in the study area as reported in the study is of concern since the risk of indirect exposure of humans and animals via consumption of contaminated plants remains high. This is because anionic surfactants are known to promote the uptake of cyanotoxins and metals and thus increase the risk to humans.

8.2.3 Bioaccumulation and elimination (toxicokinetics) of cyanotoxins by plants

In the third part, the accumulation and elimination capacities of MCs in different parts of the plants *Brassica oleracea* and *Solanum tuberosum* was studied. Water used to irrigate the plants 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.

Findings indicated that the raw dam water did not have any effect on the germination of potato seeds, but severe effects were found on the germination of cabbage seeds, with 84.3% successful germination in the control trays and only 12.7% successful germination in the trial's trays. Such findings were inconsistent with previous studies which have reported negative effects of cyanotoxins on seed germination and development and also highlight the possible impacts of irrigating crops with such water. Data on the toxin transfer to irrigated crops; cyanotoxin kinetics in plants exposed to cyanobacterial blooms and cyanotoxin elimination capacities of the plants is still outstanding and will be included in the final report.

8.2.4 Accumulation of cyanotoxins and toxic metals in the presence of LAS on *Brassica oleracea* and *Solanum tuberosum*

The fourth part investigated the uptake and the accumulation of MCs and metal species in different parts of the plants *Brassica oleracea* and *Solanum tuberosum* in the presence of the anionic surfactant LAS. The findings indicated that the dam water which was used to irrigate the plants was alkaline, with a pH of 9.02, had high EC and TDS levels (228 mg L^{-1} and 380 µs cm^{-1} respectively), high cyanobacterial biomass (Chlorophyll-a 440.24±328.147 µg L^{-1}) and contained a significant load of anionic surfactants (1.64±0.163 mg L^{-1}). Of importance, the water had very high levels of MCs (13.03±3.599 µg L^{-1}). Based on the findings, the presence of anionic surfactants did not induce 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 different levels of LAS. Data on uptake and accumulation of MCs in presence of anionic surfactants is still missing in this draft report and will be included in the final report.

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

The fifth part looked at synthesising a crosslinked chitosan sorbent that can be used in SPATT format to passively sample cyanotoxins. 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 also 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 compared to the HP20 resin, suggested better capacities for the ChMWCNT to adsorb MCs.

Both the glutaraldehyde crosslinked chitosan hydrogel (ChGLA) and the chitosan-multi-walled CNT (ChMWCNT) composite were applied for the adsorption and desorption of MCs. The

findings indicate that both adsorbents are good sorbents for MC-LR and their adsorption capacities are much better compared to the commonly used aromatic resin HP20. The desorption efficiencies of the two synthesized chitosan sorbents were also much better compared to that of the HP20 resin, making them ideal candidates for application in the SPATT bag format for the passive sampling of MCs. Since chitosan can easily be made by deacetylation of chitin which occurs in abundance as a by-product in the food industry from crab and shrimp shells, this could be a cheaper and readily available alternative to the commercial resins currently applied in SPATT samplers.

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 HP20) were deployed for 48 hours. The findings indicated that MCs were detectable in all the two sites and in all the six sampling points in all the three types of samplers and grab samples. The three samplers were easily detecting MCs even in areas were grab samples detected traces of the toxin.

The findings showed a good correlation of the toxins detected by the 3 samplers compared to the grab samples, but strong positive correlation were recorded for grab samples vs the ChGLA samplers, whereas the ChMWCNT samplers showed a strong positive correlation with the HP20 samplers. Among physicochemical parameters monitored, there was a strong positive correlation between chlorophyll-*a* and MCs in grab samples and ChGLA. There was also a strong positive correlation between dissolved nitrate levels and toxin levels detected by HP20 and ChMWCNT.

Importantly, factors correlating to MC detection by HP20 also correlated to ChMWCNT and the physicochemical parameters such as conductivity, salinity and TDS had a correlation with the MC levels detected by ChMWCNT and HP20 in the field water. In addition, pH had a strong positive correlation with MC detection by ChGLA. Based on these findings, it was concluded that SPATT using the synthesized chitosan-based adsorbents has the potential to be integrated into current cyanobacterial monitoring programmes and would be a very 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 catchments such as the Crocodile (West) and Marico catchment.

Both the field surveys and pot-culture experiments showed a low risk of transfer of contaminants from irrigation water to the plants for both microcystins and metal contaminants even in the presence of anionic surfactants. The study also found that metals like Cr, Ni, Cu, Pb, and As were above the guideline values set for agricultural soils. Among the metals of concern in the food plants were Cr, Fe, Cu, Zn, As, and Pb which found to be found above the EU and FAO/WHO threshold for these metals in food crops. However, the calculated EDI for each of the metals detected were below the maximum tolerable daily intake (MTDI), thus implying that the plants being irrigated by water from the two dams are still safe for human consumption.

Based on our findings, the levels of cyanotoxins 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 from this section demonstrated that the two plants can bio-accumulate 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. Our findings, together with findings in other studies discussed here, demonstrates terrestrial food crops can accumulate cyanotoxins to levels that can pose human-health risks when exposed to naturally relevant levels of cyanotoxins.

The findings of the study showed that the presence of anionic surfactants did not induce 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 different 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 here. 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 high 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 which 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 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 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 for possible use as an early warning sign for the presence of MCs in irrigation water in eutrophicated catchments in South Africa.

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 inability to sample for a full calendar year due to Covid-19 related lockdowns and restrictions
- The failure to factor in the dilution factor due to precipitation in the levels of pollutants monitored in the field.
- 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 also 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 did not look into 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 policy-makers with local evidence-based data to guide the process of policy formulation with regards to 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 prioritizes research addressing issues specific to cyanotoxins in irrigation water and development of local guidelines/regulations for cyanotoxins in agricultural water.

Findings of the field survey indicated the presence of anionic surfactants, metals contaminants and cyanotoxins in irrigation water and agricultural soils in the study area and the risk of indirect exposure to humans and animals via consumption of contaminated plants. It is thus recommended that these pollutants be regularly monitored and managed in light of the synergic impacts of these pollutant on plants and eventually in human. This considering the prevailing poor quality of sewage effluent being discharge into the catchment and the impact of the mining and industrial activities taking place in and around the area.

The pot culture experiments showed that the existing levels of anionic surfactants, metal contaminants and microcystins being found in hyper-eutrophicated reservoirs such 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. Our 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. Inasmuch 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 in the near future.

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 carried 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 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 and using a different method to detect and quantify the toxins are recommended, since the ELISA method does not differentiate the different congeners of MCs and is affected by a host of other external factors. The use of techniques such as LCMS will give valuable information on the effectiveness of the synthesized material in adsorbing and desorption of the different congeners of MCs and other cyanotoxins commonly 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 SPAT 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.

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APPENDIX A. CAPACITY BUILDING

SALPHINA NTOMBIKAYISE SATHEKGE

MSc in Environmental Sciences, 2022. Assessment of co-occurrence of cyanotoxins, toxic metals and anionic surfactants in irrigation water, agricultural soils, and Food crops. ***MSc Thesis***. University of Venda. Submitted for Examination, February 2022.

Abstract: Globally, the occurrence of cyanobacterial blooms in freshwater ecosystems has become a concern. Cyanobacteria produces secondary metabolites, known as cyanotoxins that cause acute and chronic poisoning in animals and humans. History of mining, industrial activities, and poor maintenance of wastewater treatment infrastructure are the main causes of the hyper-eutrophic conditions affecting most dams in South Africa. The co-occurrence of multiple stressors in agricultural waters and soils potentially pose a human and animal risk if contaminated water and plants are ingested.

The study investigated the co-existence of cyanotoxins, anionic surfactants and metal species in irrigation water, agricultural soils and food crops and determine the health risks associated with consuming cyanotoxins contaminated plants in the Crocodile (West) Marico Water Management Area, which covers parts of Gauteng and Northwest Provinces. Lastly the study assessed the applicability of passive sampling technology in monitoring of cyanotoxins using DIAON HP20 resins as an adsorbent. Water, food crops and soil samples were collected from Roodeplaat and Hartbeespoort dam sites in irrigation canals and cropping fields in June 2019, September 2019, February 2020, and March 2021. Seven sites were selected for sampling of water for cyanotoxins, anionic surfactants and toxic metals, while 4 farmland sites were selected for agricultural soils and food crops in Roodeplaat and Hartbeespoort sites. Physicochemical parameters of the irrigation water (pH, temperature, EC, TDS, DO), chlorophyll-a and dissolved nutrients were also monitored using Spectrophotometer and Spectro-Quant® Merck Pharo 100 with the photometric test kits from Merck, respectively. The levels of Microcystins (MCs), anionic surfactants, and metals were detected and quantified using the ELISA method, anionic surfactant portable photometer and inductively coupled plasma mass spectrometry (ICP-MS), respectively. The results are presented for each chapters below.

The results for chapter 1 revealed the co-existence of cyanotoxins, metal species and anionic surfactants in the irrigation water, and agricultural soils, across sampling sites, throughout sampling period. The microcystins in irrigation water ranged from 0.00 to 15.57 µg/L. Total anionic surfactants in irrigation water and agricultural soil ranged from 0.01 to 3.49 mg/L and 1.81 to 5.46 mg/kg, respectively. Among all the physicochemical parameters only pH ($p = 0.624$), TDS ($p = -0.466$), EC ($p = -0.445$), and turbidity ($p = 0.521$) correlated with MCs. Moreover, total anionic surfactant showed to have positive moderate relationship with levels of MCs in irrigation water ($p = 0.342$). Metal species in irrigation water were decreased in the following order: Al > Mn > Fe > B > Zn > Ni > Cu > Pb > Cr > As and were all below the maximum DWAF acceptable limit, implying that the water was safe for irrigation use. Metal species in other soil sampling sites such as 16534.61 – 33285 mg/kg (Fe), 111.25 – 723.4 mg/kg (Cr),4.44 – 23.93 mg/kg (Pb), 0.80 – 9.70 mg/kg (As), 22.11 – 33.95 mg/kg (Cu), and 33.70 – 85.885 mg/kg (Ni) were above the maximum limit set by DEA, USEPA, and FAO/WHO for agricultural use. Thus, soils from Roodeplaat and Hartbeespoort farmland sites are contaminated by the mentioned metals.

Findings from the second chapter of results revealed the bioaccumulation of microcystins and metals in food crops. The estimated daily intake (EDI) for MCs in all food crops for both adults and children were below 0.04 µg/kg DW acceptable value set by World Health Organisation, implying that the crops were safe for human consumption by adult and children population. Metal species levels accumulated in plants samples collected from different sampling sites, showed that 0.21 to 10.80 mg/kg (Cr), 19.64 to 734.00 mg/kg (Fe), 5.45 to 76.80 mg/kg (Zn), 0.01 to 0.20 mg/kg (As), 0.96 to 60.40 mg/kg (Cu), and 0.10 to 0.70 mg/kg (Pb) were above the EU and FAO/WHO guideline standards. Spearman correlation between metals in plants and water showed that only Pb ($p = 0.874$) and As ($p = 0.809$) in irrigation water had a positive moderate association with metals in plants collected from the sampling sites. The estimated daily intake (EDI) of metals via consumption of the crops were found to be below the maximum tolerable daily intake (MTDI) proposed for each metal. The translocation factors (TF) showed that only Cu and Cd were rapidly transported to the plant's edible parts from the soil. Moreover, target hazard quotient (THQ) for each metal were below 1, indicating that consuming the food crops will not cause carcinogenic effect to the adult population, while hazard index (HI) for other sites was found to be >1 for crop plants, thus plants from these sites pose a health hazards to adult population. In addition, the target

cancer risk (TCR) value for Cr and Ni in crops from other sampling sites were above the maximum threshold implying that there is a potential cancer risk to adult population over a long-term.

In addition, findings from the third chapter showed that SPATT was applicable in monitoring and detecting MCs across all sampling sites and sampling months. The MCs levels in grab and SPATT bags ranged from 0.14 to 13.03 µg/L and 0.99 to 2.28 ng/g resin throughout the sampling sites and months, respectively. Thus, showing the persistence of MCs in canals and farm dams of Roodeplaat and Hartbeespoort. A spearman correlation revealed that pH ($p = 0.776$), Turbidity ($p = 0.699$) and DO ($p = 0.829$) had a significant positive association with total toxins in grab samples, while total dissolved MCs in SPATT samples showed negative moderate relationship with TDS ($p = -0.615$) and EC ($p = -0.602$). Total toxin concentrations in SPATT bags and Grab samples did not show any correlation this is because SPATT bags detect and collect microcystins within water column overtime, unlike point (Grab sampling), hence, there is no relationship between the two-sampling method. Overall results showed that SPATT bags with DIAON HP20 resin as an adsorbent proved to be applicable in monitoring and detecting microcystins in the irrigation water of Roodeplaat and Hartbeespoort sites.

Key words: Cyanobacteria, cyanotoxins, toxic metals, anionic surfactants, Irrigation water, agricultural soils, food crops, solid phase adsorption toxin tracking (SPATT)

SHIRLEY MUKANGAYA

Honors in Environmental Sciences, 2022. Application of Solid Phase Adsorption Toxin Tracking Technology (SPATT) for Monitoring Microcystins in irrigation water around Levubu of Limpopo Province. ***Honors Mini-dissertation***. University of Venda. Submitted for Examination, February 2022.

Abstract: South Africa is a water scarce country and among its Provinces, Limpopo is one of the water scarce Province. Incidents of harmful algal blooms (HABs) have been on the rise in all provinces of S.A due to inadequate treatment of wastewater and poor catchment management. Incidents of HABs pose a risk to agriculture since some species of cyanobacteria produce secondary metabolites (cyanotoxins) which are harmful to humans upon consumption. The major route of exposure remains contaminated drinking water, but there have been reports of indirect

exposure through consumption of crops irrigated with cyanobacteria infested water. The aim of the study was to use Solid Phase Adsorption Toxin Tracking Technology (SPATT) using the resin DIAION HP20 for passive sampling of the microcystins (a cyanotoxin) in farm dams around Levubu and to determine the physicochemical parameters that correlate to toxin loads in the farm dams. To achieve this, three farm dams being fed by three different rivers were selected around the Levubu area in Limpopo Province for sampling. Physiochemical parameters such as pH, temperature, EC, TDS, turbidity was monitored for a period of 5 months. Nutrients (phosphates and nitrates), cyanobacterial biomass (as chlorophyll-*a*) and microcystins (using grab sampling and SPATT samplers) were also monitored over the 5 months. Findings indicated that most of the physicochemical parameters were within S. A's guidelines for irrigation water, but also with the optimum range for cyanobacterial growth. Nutrients (dissolved N and P) were in very low levels and in most cases below the detection limit and could be attributed to the low chlorophyll-*a* (cyanobacterial biomass) reported. Both grab and SPATT samplers detected MCs in all the farm dams throughout the sampling period and there was a similar pattern in their detection of MCs, even though there was no correlation between the MC levels detected by these two methods. None of the physicochemical parameters monitored correlated with cyanobacterial biomass and MC levels both in grab and SPATT samples.

Keywords: Microcystins, physicochemical parameters, SPATT, irrigation water

Pindihama Glynn Kuziva (PhD in Environmental Sciences expected to submit, 2022). Bioaccumulation and toxicokinetics of Cyanotoxins on terrestrial food plants and the development of a novel sorbent for monitoring cyanotoxins in irrigation water. ***PhD Thesis***. University of Venda (Pending submission)

Abstract: South Africa (SA) is known as a water-scarce country and thus management of this key resource is of paramount importance. As is the case in other parts of the world, eutrophication of freshwater resources is a serious problem in South Africa. 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. Among the impacts of eutrophication is the increased growth and dominance of cyanobacteria which produce metabolites posing a threat to aquatic ecosystems, animals and human health (Matthews *et al.*, 2010). Recently, a number of studies have

reported the toxic effects of MCs on terrestrial plants including plants used for food. Ever since, the use of surface water contaminated with cyanobacteria and cyanotoxins for agricultural purposes has been receiving growing attention (Lee *et al.*, 2017a).

The study looked at the bioavailability, toxicokinetics and effects of a range of cyanobacterial metabolites on terrestrial food plants and also developed and evaluated the use of crosslinked chitosan sorbent in Solid Phase Adsorption Tracking Technology (SPATT) to monitor the bioavailability of cyanotoxins in water intended for irrigation.

The first part of the study looked at the accumulation and elimination capacities of MCs in different parts of the plants *Brassica oleracea* and *Solanum tuberosum*. To investigate cyanotoxins uptake, accumulation and elimination capacities in different parts of the plants pot-culture experiments with cabbage (*B. oleracea*) and cultivated potato (*S. tuberosum*) were conducted.

Water used to irrigate the plants had 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 than the South African guideline and FAO limits for irrigation water. Findings indicated that the raw dam water did not have any effect on the germination of potato seeds, but severe effects were found on the germination of cabbage seeds, with 84.3% successful germination in the control trays and only 12.7% successful germination in the trial's trays. Such findings were in inconsistent with previous studies which have reported negative effects of cyanotoxins on seed germination and development and also highlight the possible impacts of irrigating crops with such water. Data on the toxin transfer to irrigated crops; cyanotoxin kinetics in plants exposed to cyanobacterial blooms and cyanotoxin elimination capacities of the plants is still outstanding and will be included in the final report.

The second part of the study investigated the uptake and the accumulation of MCs and metal species in different parts of the plants *Brassica oleracea* and *Solanum tuberosum* in the presence of the anionic surfactant LAS. To investigate if cyanotoxins and metal species uptake and accumulation in different parts of the plants is affected by the presence of LAS, pot-culture experiments with the two plant species were conducted. The findings indicated that the dam water which was used to irrigate the plants was alkaline, with a pH of 9.02, had high EC and TDS levels

(228 mg L^{-1} and 380 µs cm^{-1} respectively), high cyanobacterial biomass (Chlorophyll-a 440.24±328.147 µg L^{-1}) and contained a significant load of anionic surfactants (1.64±0.163 mg L^{-1}). Of importance, the water had very high levels of MCs (13.03±3.599 µg L^{-1}). Based on the findings, the presence of anionic surfactants did not induce 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 different levels of LAS. Data on uptake and accumulation of MCs in presence of anionic surfactants is still missing in this draft report and will be included in the final report.

The third part of the study looked at synthesising a crosslinked chitosan sorbent that could be used in SPATT format to passively sample cyanotoxins. For the synthesis of the in-house adsorbent for potential use as an alternative to the commercial resins, chitosan was crosslinked with glutaraldehyde to form a chitosan-glutaraldehyde crosslinked copolymer, which was then further modified with the addition of multi-walled carbon nanotubes (CNTs). The synthesised crosslinked chitosan (ChGLA) and the ChMWCNTs sorbents were then characterized by BET, SEM-EDX and FTIR-ATR. The findings indicated the 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 also 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 compared to the HP20 resin, suggested better capacities for the ChMWCNT to adsorb MCs.

Both the glutaraldehyde crosslinked chitosan hydrogel (ChGLA) and the chitosan-multi-walled CNT (ChMWCNT) composite were applied for the adsorption and desorption of MCs. The findings indicate that both adsorbents are good sorbents for MC-LR and their adsorption capacities are much better compared to the commonly used aromatic resin HP20. The desorption efficiencies of the two synthesized chitosan sorbents were also much better compared to that of the HP20 resin, making them ideal candidates for application in the SPATT bag format for the passive sampling

of MCs. Since chitosan can easily be made by deacetylation of chitin which occurs in abundance as a by-product in the food industry from crab and shrimp shells, this could be a cheaper and readily available alternative to the commercial resins currently applied in SPATT samplers.

The last part evaluated the developed chitosan-based sorbents, application as passive samplers in SPATT format in the study area. To investigate the applicability of a chitosan-based biopolymer as a sorbent to be used for the adsorption of MCs in SPATT bag format in the field, a glutaraldehyde crosslinked chitosan hydrogel (ChGLA) and a multi-walled carbon nanotube composite of the ChGLA (ChMWCNT) was synthesised. The two newly synthesized materials were packed in a SPATT bag format (in nylon cloth, 55 mm * 55 mm) and applied in a laboratory trial to determine the time required for the adsorbent to be saturated then later deployed in the field. The ChGLA and ChMWCNT sorbents were deployed in the field together with the commonly used commercial DION HP20 resin. Grab samples were also collected upon deployment and retrieval of the SPATT samplers and physicochemical parameters such as pH, EC, DO, salinity, chlorophyll-a, temperature, nutrients, turbidity and TDS were also monitored.

The findings showed a good correlation of the toxins detected by the 3 samplers compared to the grab samples, but strong positive correlation were recorded for grab samples vs the ChGLA samplers, whereas the ChMWCNT samplers showed a strong positive correlation with the HP20 samplers. Among physicochemical parameters monitored, there was a strong positive correlation between chlorophyll-*a* and MCs in grab samples and ChGLA. There was also a strong positive correlation between dissolved nitrate levels and toxin levels detected by HP20 and ChMWCNT.

Importantly, factors correlating to MC detection by HP20 also correlated to ChMWCNT and the physicochemical parameters such as conductivity, salinity and TDS had a correlation with the MC levels detected by ChMWCNT and HP20 in the field water. In addition, pH had a strong positive correlation with MC detection by ChGLA. Based on these findings, it was concluded that SPATT using the synthesized chitosan-based adsorbents has the potential to be integrated into current cyanobacterial monitoring programmes and would be a very useful and economical tool for early warning and monitoring of toxic cyanobacterial events in water intended for irrigation.

The pot-culture experiments showed a low risk of transfer of contaminants from irrigation water to the plants for both microcystins and metal contaminants even in the presence of anionic

surfactants. 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 for possible use as an early warning sign for the presence of MCs in irrigation water in eutrophicated catchments in South Africa.

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.

Key words: cyanotoxins, metals, Linear alkylbenzene sulphonates (LAS), Irrigation water, food crops, solid phase adsorption toxin tracking (SPATT), chitosan

APPENDIX B: TECHNOLOGY TRANSFER AND PUBLICATIONS

PUBLICATIONS IN PEER-REVIEWED JOURNALS

Pindihama, G.K. & Gitari, W.M. (**2020**). Cyanobacterial Toxins: An Emerging Threat in South African Irrigation Water. *Water and Environment Journal*. 0. P. 1-11. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/wej.12473

Pindihama, G.K. & Gitari, W.M. (**2022**). The Effect of Linear Alkylbenzene Sulphonates on the Bioaccumulation of Al, Sr & Mn by *Brassica Oleracea* & *Solanum Tuberosum.* Submitted to *Water, Air, & Soil Pollution*.

PATENTS

Chitosan-multiwalled carbon nanotubes (ChMWCNT) composite for passive sampling of cyanotoxins in SPATT bag format. Status: *Pending*

CONFERENCE PAPERS

G.K. Pindihama & W.M. Gitari. **(2021)**. Bioaccumulation and Elimination of Cyanotoxins by *Solanum tuberosum* and *Brassica oleracea*. Presented at the virtual conference of AQUA≈360: Water for All – Emerging Issues and Innovations 31st August 2021 – 2nd September 2021, University of Exeter, United Kingdom.

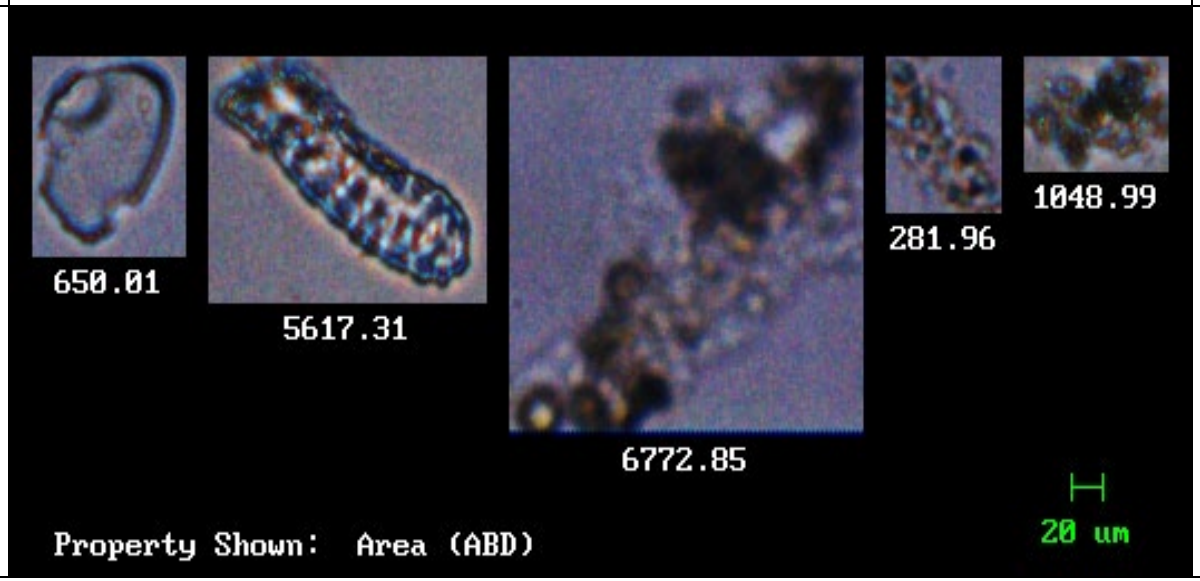
S.N. Sathekge, G.K. Pindihama & W.M. Gitari. **(2021)**. An Assessment of the co-occurrence of cyanotoxins, metals, and Anionic surfactants in irrigation water and agricultural soils. Poster, presented at the virtual conference of AQUA≈360: Water for All – Emerging Issues and Innovations 31st August 2021 – 2nd September 2021, University of Exeter, United Kingdom.

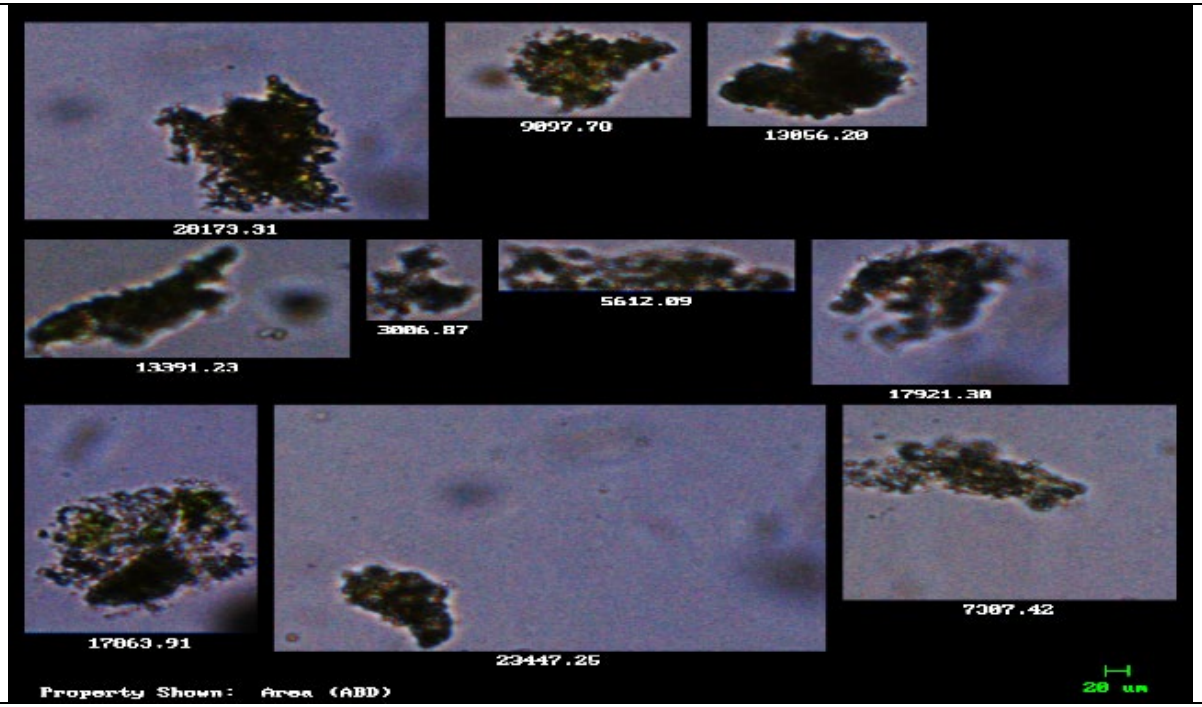
G.K. Pindihama, W.M. Gitari & S. Mukangaya. **(2022)**. Application of Solid Phase Adsorption Toxin Tracking Technology (SPATT) for Monitoring Microcystins in farm dams. Abstract submitted for the WISA 2022 Biennial Conference. To be held at the Sandton Convention Centre, Johannesburg.

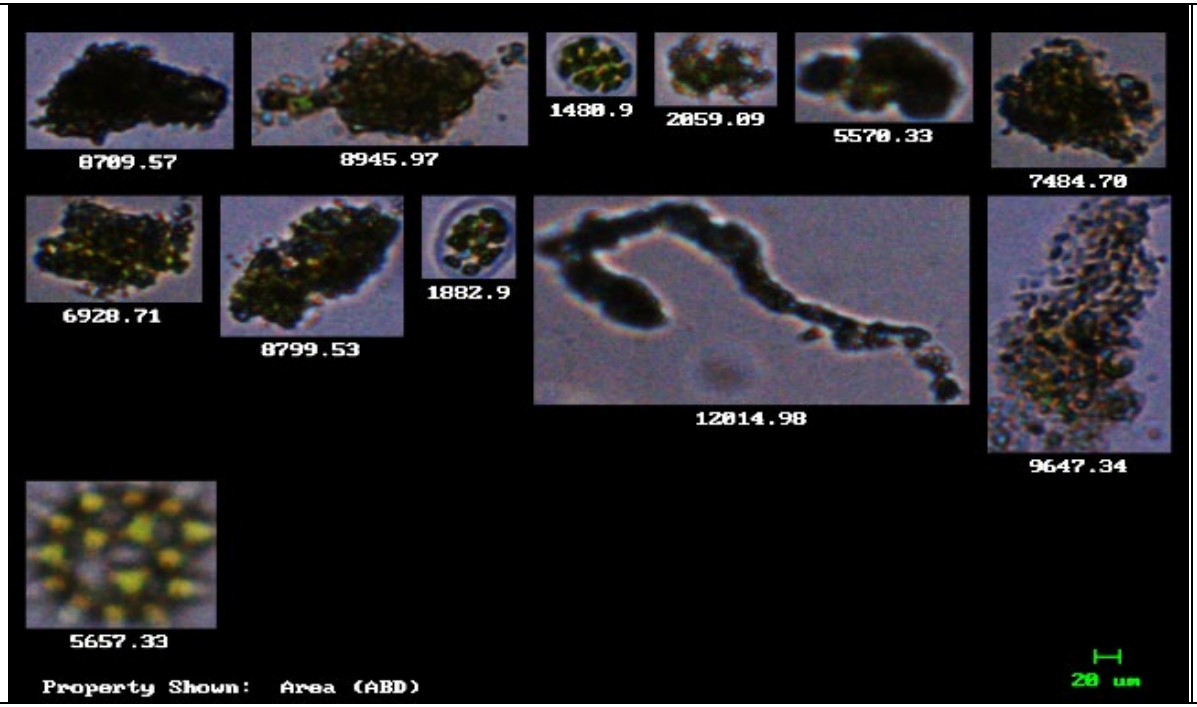
POPULAR ARTICLE

G.K. Pindihama, W.M. Gitari & S.N. Sathekge (**2021**). Could farmers be bearing the cost of poor catchment management? (Popular article submitted to the Water Wheel magazine (Nov/Dec 2021 edition).

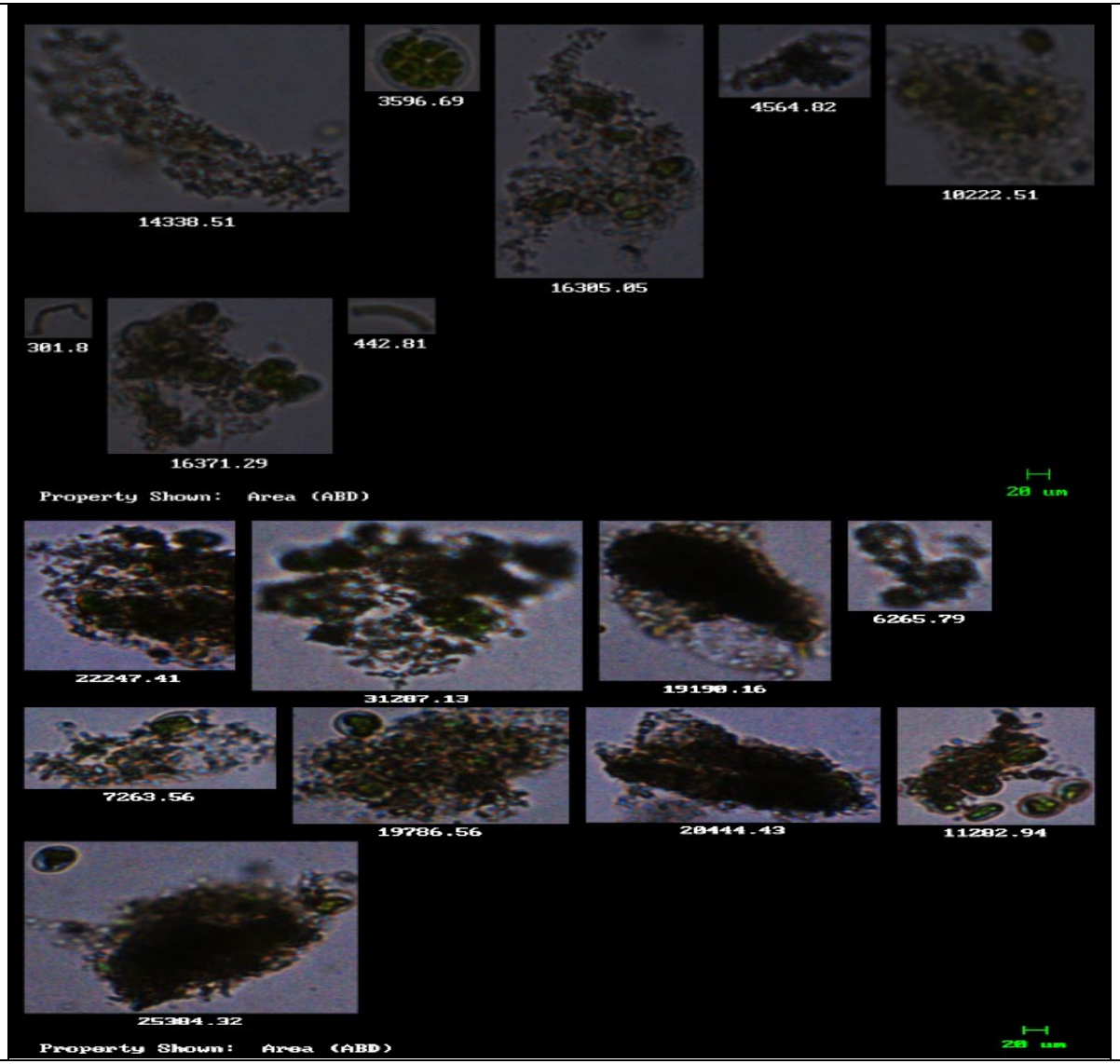
APPENDIX C. THE DIFFERENT COMPOSITION OF HARMFUL CYANOBACTERIA IN IRRIGATION CANALS AND FARM DAMS OF HARTBEESPOORT SITES.

Sampling sites	Images of harmful cyanobacteria	Species	References
H1		(6772.85; 281.96; 1048.99) *Microcystis*; (5617.31) *Dinoflagelate*; (650.01) *Anabaena*	Huynh & Serediak, 2006 Tshifura, 2018

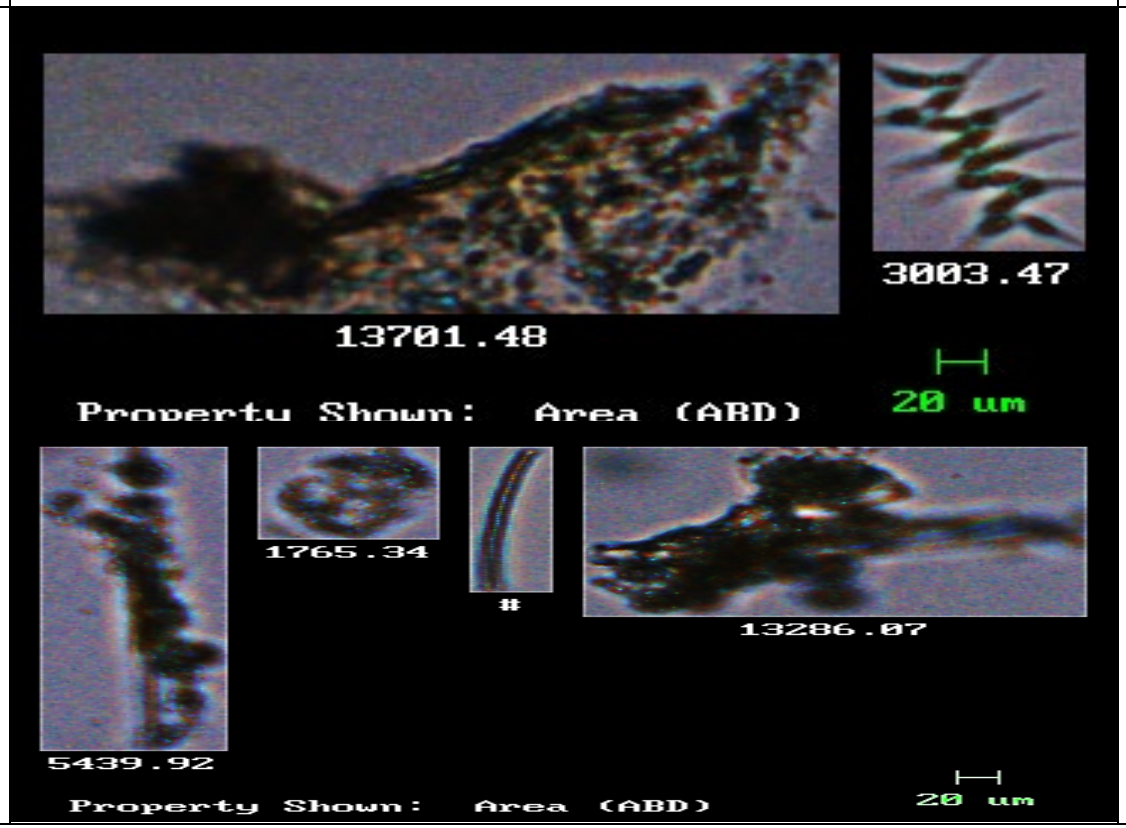
H2		*Microcystis*	Tshifura, 2018

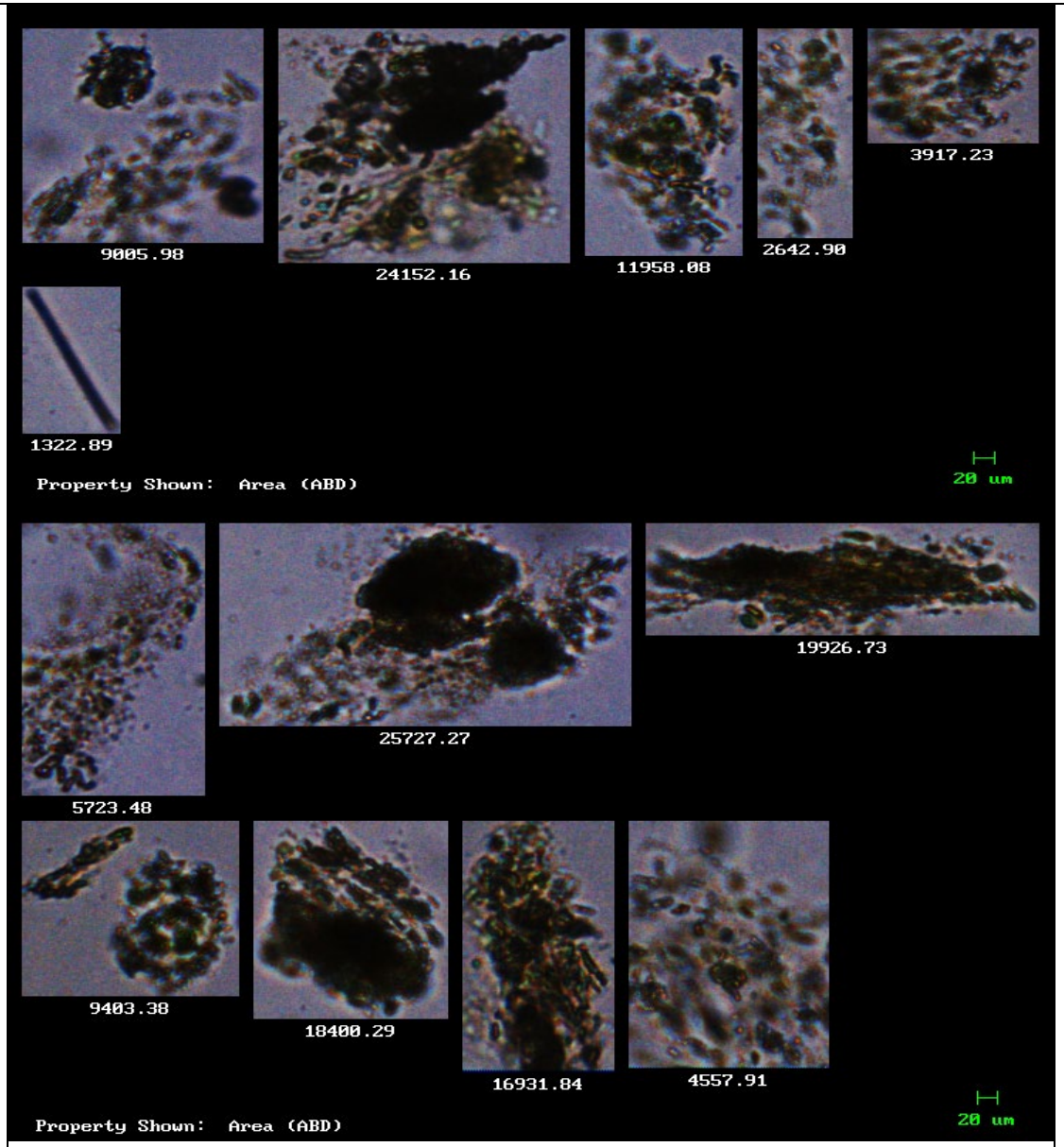
	 8709.57 8945.97 1480.9 2059.09 5570.33 7484.70 6928.71 8799.53 1882.9 12014.98 9647.34 5657.33 Property Shown: Area (ABD) 20 um		

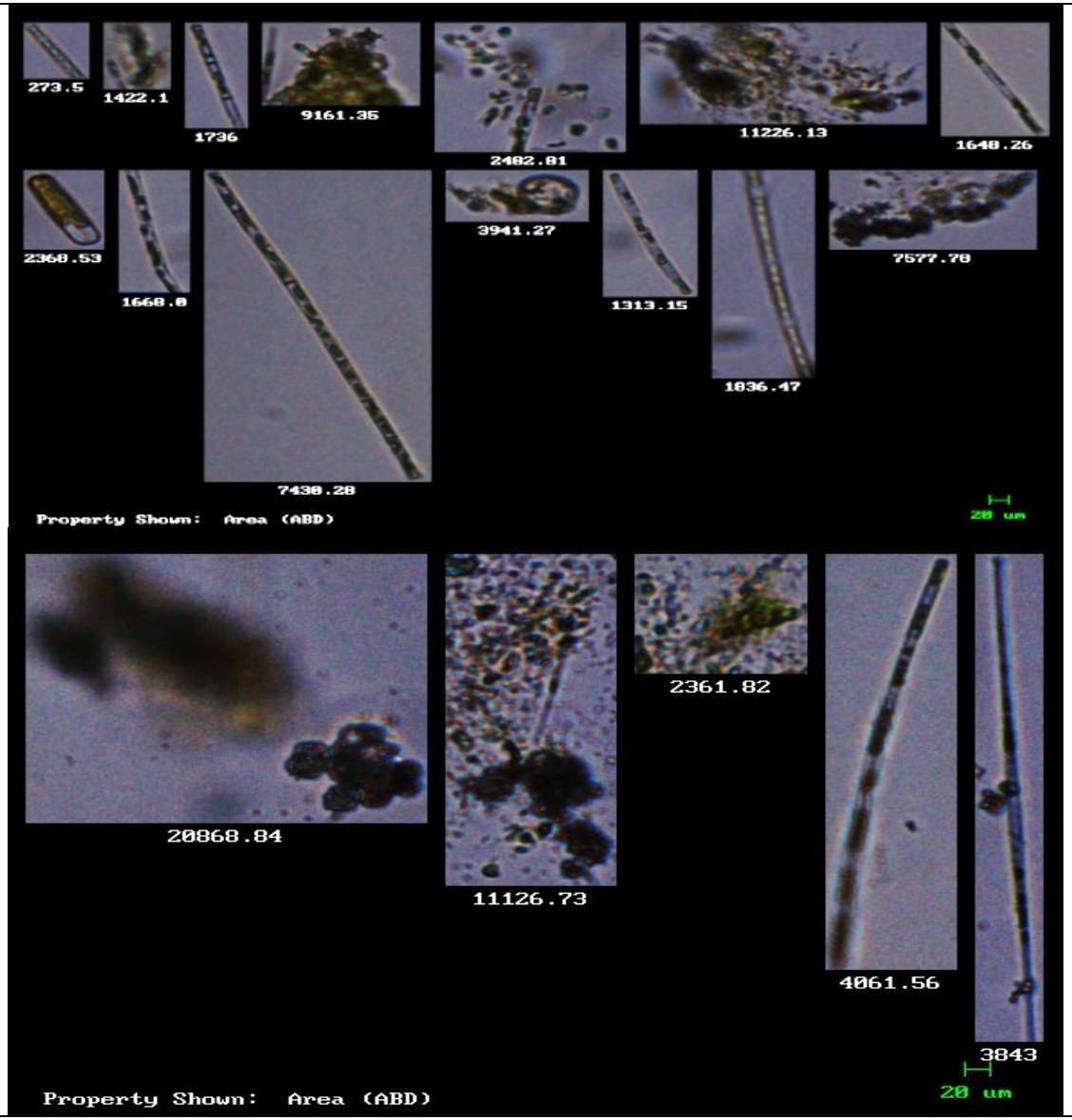
H3		(8147.52) *Microcystis*	Tshifura, 2018

H4		*Microcystis*; (301.8) *Anabaena*; (442.81) *Oscillatoria*	Tshifura, 2018. Janse van Vuuren *et al.*, 2006

APPENDIX D. THE DIFFERENT COMPOSITION OF HARMFUL CYANOBACTERIA IN IRRIGATION CANALS AND FARM DAMS IN ROODEPLAAT SITES

Site	Images of harmful cyanobacteria	Species	References
R1		(13701.48; 13206.07) *Microcystis*; (3003.47; 5439.92; 1765.34) *Anabaena*	Janse van Vuuren *et al.*, 2006 Tshifura, 2018

R2		(9005.90; 24152.16; 11968.88; 2642.90; 9917.23; 5723.40; 25727.27; 19926.73; 9403.38; 18400.29; 16931.04; 4557.91) *Microcystis*; (1322.89) *Oscillatoria*	Tshifura, 2018

R3		(1422.1; 9161.35; 2482.81; 11226.13; 11126.73; 2361.82; 20068.84) *Microcystis*; (273.5; 1736; 1648.26; 1668.0; 7430.28; 3941.27; 1313.15; 1836.47; 4861.56; 3843) *Anabaena*; (2368.53) *Oscillatoria*	Janse van Vuuren *et al.*, 2006 Tshifura, 2018

APPENDIX E. KRUSKAL-WALLIS TEST FOR MANGANESE IN *B. OLERACEA* AFTER 5 DAY EXPOSURE

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.6530, 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

Group	Number of Points	Sum of Ranks	Mean of Ranks	
T1	8	90.000	11.250	
T2	5	67.000	13.400	
T3	5	52.000	10.400	
T4	6	91.000	15.167	

Kruskal-Wallis Statistic KW = 1.628 (corrected for ties)

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

Summary of Data

Group	Number of Points	Median	Minimum	Maximum	
T1	8	47.185	44.063	93.162	
T2	5	65.887	41.916	70.317	
T3	5	48.305	39.616	83.199	
T4	6	68.568	40.957	84.645	
	*	*	*		

APPENDIX F. KRUSKAL-WALLIS TEST FOR STRONTIUM IN *B. OLERACEA* AFTER 5 DAY EXPOSURE

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.1973, 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 exact calculations would have taken too long.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean of Ranks
T1	4	50.000	12.500
T2	5	64.000	12.800
T3	5	59.000	11.800
T4	6	37.000	6.167

Kruskal-Wallis Statistic KW = 4.673

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

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
T1	4	155.27	131.84	197.71
T2	5	163.70	125.49	178.45
T3	5	154.52	103.79	198.32
T4	6	123.61	99.604	149.66

* * *

APPENDIX G. KRUSKAL-WALLIS TEST FOR ALUMINIUM IN *B. OLERACEA* AFTER 5 DAY EXPOSURE

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.8514, 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 exact calculations would have taken too long.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean Ranks
T1	4	51.000	12.750
T2	5	50.000	10.000
T3	5	47.000	9.400
T4	6	62.000	10.333

Kruskal-Wallis Statistic KW = 0.7919

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

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
T1	4	0.2451	0.1690	0.3231
T2	5	0.2133	0.1808	0.2324
T3	5	0.2055	0.1713	0.3244
T4	6	0.2163	0.1430	0.4283

* * *

APPENDIX H. KRUSKAL-WALLIS TEST FOR MANGANESE IN *B. OLERACEA* AFTER 20 DAY EXPOSURE

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.3194, 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 exact calculations would have taken too long.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean Ranks
T1	5	50.000	10.000
T2	3	48.000	16.000
T3	7	84.000	12.000
T4	6	49.000	8.167

Kruskal-Wallis Statistic KW = 3.511

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

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
T1	5	54.460	42.970	76.130
T2	3	91.440	52.660	94.630
T3	7	63.030	48.220	86.140
T4	6	50.235	30.240	114.34

* * *

APPENDIX I. KRUSKAL-WALLIS TEST FOR STRONTIUM IN *B. OLERACEA* AFTER 20 DAY EXPOSURE

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.7807, 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 exact calculations would have taken too long.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean Ranks
T1	5	49.000	9.800
T2	3	43.000	14.333
T3	7	75.000	10.714
T4	6	64.000	10.667

Kruskal-Wallis Statistic KW = 1.085

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

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
T1	5	119.09	90.990	177.33
T2	3	139.82	138.10	146.06
T3	7	129.91	70.730	155.57
T4	6	121.80	94.390	157.23

* * *

APPENDIX J. KRUSKAL-WALLIS TEST FOR ALUMINIUM IN *B. OLERACEA* AFTER 20 DAY EXPOSURE

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.0315, 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

Group	Number of Points	Sum of Ranks	Mean of Ranks
T1	5	68.000	13.600
T2	3	57.000	19.000
T3	7	61.500	8.786
T4	6	44.500	7.417

Kruskal-Wallis Statistic KW = 8.838 (corrected for ties)

Dunn's Multiple Comparisons Test

Comparison	Mean Rank Difference		P value
T1 vs. T2	-5.400	ns	P>0.05
T1 vs. T3	4.814	ns	P>0.05
T1 vs. T4	6.183	ns	P>0.05
T2 vs. T3	10.214	ns	P>0.05
T2 vs. T4	11.583	*	P<0.05
T3 vs. T4	1.369	ns	P>0.05

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
T1	5	0.1600	0.1600	0.2500
T2	3	0.2100	0.2000	0.2300
T3	7	0.1400	0.1000	0.1800
T4	6	0.1350	0.1200	0.1700

* * *

APPENDIX K. KRUSKAL-WALLIS TEST FOR MANGANESE IN *S. TUBEROSUM* AFTER 20 DAY EXPOSURE

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.4043, 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 exact calculations would have taken too long.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean Ranks
T1	4	33.000	8.250
T2	6	71.000	11.833
T3	6	89.000	14.833
T4	6	60.000	10.000

Kruskal-Wallis Statistic KW = 2.919

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

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
T1	4	9.951	8.658	15.729
T2	6	15.342	8.570	24.986
T3	6	19.626	7.787	20.823
T4	6	11.238	7.762	23.891

* * *

APPENDIX L. KRUSKAL-WALLIS TEST FOR STRONTIUM IN *S. TUBEROSUM* AFTER 20 DAY EXPOSURE

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.5971, 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 exact calculations would have taken too long.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean Ranks
T1	6	59.000	9.833
T2	6	83.000	13.833
T3	6	93.000	15.500
T4	7	90.000	12.857

Kruskal-Wallis Statistic KW = 1.883

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

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
T1	6	3.261	2.388	56.053
T2	6	4.367	3.734	6.089
T3	6	4.345	3.858	5.900
T4	7	4.044	3.653	5.879

* * *

APPENDIX M. KRUSKAL-WALLIS TEST FOR ALUMINIUM IN *S. TUBEROSUM* AFTER 20 DAY EXPOSURE

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.1378, 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 exact calculations would have taken too long.

Calculation detail

Group	Number of Points	Sum of Ranks	Mean of Ranks
T1	6	88.000	14.667
T2	6	83.000	13.833
T3	6	89.000	14.833
T4	6	40.000	6.667

Kruskal-Wallis Statistic KW = 5.513

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

Summary of Data

Group	Number of Points	Median	Minimum	Maximum
T1	6	0.1315	0.09080	0.8230
T2	6	0.1656	0.06380	0.2424
T3	6	0.1703	0.08610	0.2398
T4	6	0.08358	0.06460	0.1995

* * *

APPENDIX N. UNPAIRED T TEST FOR MANGANESE UPTAKE IN TREATMENT 3I VS 3II IN *B. OLERACEA* AFTER 20 DAY EXPOSURE

Unpaired t test

Do the means of 3i and 3ii differ significantly?

P value

The two-tailed P value is 0.9324, considered not significant.

t = 0.08917 with 5 degrees of freedom.

95% confidence interval

Mean difference = 0.9983 (Mean of 3ii minus mean of 3i)

The 95% confidence interval of the difference: -27.787 to 29.784

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 = 1.306

The P value is 0.9225.

This test suggests that the difference between the two SDs is not significant.

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?
3i	Too few values to test.		
3ii	Too few values to test.		

Summary of Data

Parameter:	3i	3ii
Mean:	63.417	64.415
# of points:	3	4
Std deviation:	13.473	15.399
Std error:	7.779	7.700
Minimum:	48.220	50.330

Maximum:	73.900	86.140
Median:	68.130	60.595
Lower 95% CI:	29.945	39.915
Upper 95% CI:	96.889	88.915

* * *

APPENDIX O. UNPAIRED T TEST FOR MANGANESE UPTAKE IN TREATMENT 4I VS 4II IN *B. OLERACEA* AFTER 20 DAY EXPOSURE

Unpaired t test

Do the means of 4i and 4ii differ significantly?

P value

The two-tailed P value is 0.2401, considered not significant.

t = 1.379 with 4 degrees of freedom.

95% confidence interval

Mean difference = 29.847 (Mean of 4ii minus mean of 4i)

The 95% confidence interval of the difference: -30.257 to 89.950

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 = 9.887

The P value is 0.1837.

This test suggests that the difference between the two SDs is not significant.

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?
4i			Too few values to test.
4ii			Too few values to test.

Summary of Data

Parameter:	4i	4ii
Mean:	43.343	73.190
# of points:	3	3
Std deviation:	11.366	35.737
Std error:	6.562	20.633
Minimum:	30.240	49.940
Maximum:	50.530	114.34
Median:	49.260	55.290
Lower 95% CI:	15.107	-15.593
Upper 95% CI:	71.579	161.97

* * *

APPENDIX P. UNPAIRED T TEST FOR STRONTIUM UPTAKE IN TREATMENT 3I VS 3II IN *B. OLERACEA* AFTER 20 DAY EXPOSURE

Unpaired t test

Do the means of 3i and 3ii differ significantly?

P value
The two-tailed P value is 0.2802, considered not significant.

t = 1.211 with 5 degrees of freedom.

95% confidence interval
Mean difference = 26.533 (Mean of 3ii minus mean of 3i)
The 95% confidence interval of the difference: -29.818 to 82.883

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 = 12.388
The P value is 0.0710.
This test suggests that the difference between the two SDs is not quite significant.

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?
3i	Too few values to test.		
3ii	Too few values to test.		

Summary of Data

Parameter:	3i	3ii
Mean:	109.64	136.17
# of points:	3	4
Std deviation:	42.853	12.175

Std error:	24.741	6.088
Minimum:	70.730	127.58
Maximum:	155.57	154.12
Median:	102.62	131.50
Lower 95% CI:	3.178	116.80
Upper 95% CI:	216.10	155.54

* * *

APPENDIX Q. UNPAIRED T TEST FOR STRONTIUM UPTAKE IN TREATMENT 4I VS 4II IN *B. OLERACEA* AFTER 20 DAY EXPOSURE

Unpaired t test

Do the means of 4i and 4ii differ significantly?

P value
The two-tailed P value is 0.3669, considered not significant.

t = 1.016 with 4 degrees of freedom.

95% confidence interval
Mean difference = -24.123 (Mean of 4ii minus mean of 4i)
The 95% confidence interval of the difference: -90.008 to 41.761

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 = 1.405
The P value is 0.8317.
This test suggests that the difference between the two SDs is not significant.

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?
4i	Too few values to test.		
4ii	Too few values to test.		

Summary of Data

Parameter:	4i	4ii
Mean:	136.74	112.62
# of points:	3	3

Std deviation:	31.419	26.509
Std error:	18.140	15.305
Minimum:	100.57	94.390
Maximum:	157.23	143.03
Median:	152.43	100.44
Lower 95% CI:	58.688	46.763
Upper 95% CI:	214.80	178.48

* * *

APPENDIX R. UNPAIRED T TEST FOR ALUMINIUM UPTAKE IN TREATMENT 3I VS 3II IN *B. OLERACEA* AFTER 20 DAY EXPOSURE

Unpaired t test

Do the means of 3i and 3ii differ significantly?

P value
The two-tailed P value is 0.7152, considered not significant.

t = 0.3863 with 5 degrees of freedom.

95% confidence interval
Mean difference = 0.01083 (Mean of 3ii minus mean of 3i)
The 95% confidence interval of the difference: -0.06127 to 0.08294

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 = 1.110
The P value is 0.8715.
This test suggests that the difference between the two SDs is not significant.

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?
3i	Too few values to test.		
3ii	Too few values to test.		

Summary of Data

Parameter:	3i	3ii
Mean:	0.1367	0.1475
# of points:	3	4

Std deviation:	0.03786	0.03594
Std error:	0.02186	0.01797
Minimum:	0.1100	0.1000
Maximum:	0.1800	0.1800
Median:	0.1200	0.1550
Lower 95% CI:	0.04261	0.09032
Upper 95% CI:	0.2307	0.2047

* * *

APPENDIX S. UNPAIRED T TEST FOR ALUMINIUM UPTAKE IN TREATMENT 4I VS 4II IN *B. OLERACEA* AFTER 20 DAY EXPOSURE

Unpaired t test

Do the means of 4i and 4ii differ significantly?

P value
The two-tailed P value is 0.0335, considered significant.

t = 3.182 with 4 degrees of freedom.

95% confidence interval
Mean difference = -0.03000 (Mean of 4ii minus mean of 4i)
The 95% confidence interval of the difference: -0.05617 to -0.003828

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 = 7.000
The P value is 0.2500.
This test suggests that the difference between the two SDs is not significant.

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?
4i	Too few values to test.		
4ii	Too few values to test.		

Summary of Data

Parameter:	4i	4ii
Mean:	0.1533	0.1233
# of points:	3	3
Std deviation:	0.01528	0.005774

Std error:	0.008819	0.003333
Minimum:	0.1400	0.1200
Maximum:	0.1700	0.1300
Median:	0.1500	0.1200
Lower 95% CI:	0.1154	0.1090
Upper 95% CI:	0.1913	0.1377

* * *

APPENDIX T. UNPAIRED T TEST FOR MANGANESE UPTAKE IN TREATMENT 3I VS 3II IN *S. TUBEROSUM* AFTER 20 DAY EXPOSURE

Mann-Whitney Test

Do the medians of 3i and 3ii differ significantly?

The two-tailed P value is 0.1000, considered not significant.
The P value is exact.

Regardless of what data you enter, it is impossible for this test to
yield $P < 0.05$ with so few data points.

Calculation details
Mann-Whitney U-statistic = 0.000
U' = 9.000
Sum of ranks in 3i = 6.000. Sum of ranks in 3ii = 15.000.

Summary of Data

Parameter:	3i	3ii
Mean:	14.515	20.169
# of points:	3	3
Std deviation:	6.045	0.5723
Std error:	3.490	0.3304
Minimum:	7.787	19.764
Maximum:	19.489	20.823
Median:	16.268	19.920
Lower 95% CI:	-0.5023	18.747
Upper 95% CI:	29.532	21.591

* * *

APPENDIX U. UNPAIRED T TEST FOR MANGANESE UPTAKE IN TREATMENT 4I VS 4II IN *S. TUBEROSUM* AFTER 20 DAY EXPOSURE

Mann-Whitney Test

Do the medians of 4i and 4ii differ significantly?

The two-tailed P value is 0.4000, considered not significant.
The P value is exact.

Regardless of what data you enter, it is impossible for this test to
yield P < 0.05 with so few data points.

Calculation details
Mann-Whitney U-statistic = 3.000
U' = 9.000
Sum of ranks in 4i = 15.000. Sum of ranks in 4ii = 13.000.

Summary of Data

Parameter:	4i	4ii
Mean:	35.183	12.303
# of points:	3	4
Std deviation:	34.282	4.739
Std error:	19.792	2.369
Minimum:	7.973	7.762
Maximum:	73.687	18.972
Median:	23.891	11.238
Lower 95% CI:	-49.984	4.763
Upper 95% CI:	120.35	19.842

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APPENDIX V. UNPAIRED T TEST FOR STRONTIUM UPTAKE IN TREATMENT 3I VS 3II IN *S. TUBEROSUM* AFTER 20 DAY EXPOSURE

Mann-Whitney Test

Do the medians of 3i and 3ii differ significantly?

The two-tailed P value is 0.7000, considered not significant.
The P value is exact.

Regardless of what data you enter, it is impossible for this test to
yield $P < 0.05$ with so few data points.

Calculation details
Mann-Whitney U-statistic = 3.000
U' = 6.000
Sum of ranks in 3i = 12.000. Sum of ranks in 3ii = 9.000.

Summary of Data

Parameter:	3i	3ii
Mean:	4.841	4.615
# of points:	3	3
Std deviation:	0.8795	1.119
Std error:	0.5078	0.6458
Minimum:	4.160	3.858
Maximum:	5.834	5.900
Median:	4.529	4.088
Lower 95% CI:	2.656	1.837
Upper 95% CI:	7.026	7.394

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APPENDIX W. UNPAIRED T TEST FOR STRONTIUM UPTAKE IN TREATMENT 4I VS 4II IN *S. TUBEROSUM* AFTER 20 DAY EXPOSURE

Mann-Whitney Test

Do the medians of 4i and 4ii differ significantly?

The two-tailed P value is 0.8571, considered not significant.
The P value is exact.

Regardless of what data you enter, it is impossible for this test to
yield P < 0.05 with so few data points.

Calculation details
Mann-Whitney U-statistic = 5.000
U' = 7.000
Sum of ranks in 4i = 13.000. Sum of ranks in 4ii = 15.000.

Summary of Data

Parameter:	4i	4ii
Mean:	4.319	4.434
# of points:	3	4
Std deviation:	0.5243	0.9909
Std error:	0.3027	0.4954
Minimum:	3.989	3.653
Maximum:	4.923	5.879
Median:	4.044	4.103
Lower 95% CI:	3.016	2.858
Upper 95% CI:	5.622	6.011

* * *

APPENDIX X. UNPAIRED T TEST FOR ALUMINIUM UPTAKE IN TREATMENT 3I VS 3II IN *S. TUBEROSUM* AFTER 20 DAY EXPOSURE

Mann-Whitney Test

Do the medians of 3i and 3ii differ significantly?

The two-tailed P value is 0.7000, considered not significant.
The P value is exact.

Regardless of what data you enter, it is impossible for this test to
yield P < 0.05 with so few data points.

Calculation details
Mann-Whitney U-statistic = 3.000
U' = 6.000
Sum of ranks in 3i = 12.000. Sum of ranks in 3ii = 9.000.

Summary of Data

Parameter:	3i	3ii
Mean:	0.1860	0.1603
# of points:	3	3
Std deviation:	0.08656	0.02409
Std error:	0.04998	0.01391
Minimum:	0.08610	0.1403
Maximum:	0.2398	0.1870
Median:	0.2320	0.1536
Lower 95% CI:	-0.02909	0.1004
Upper 95% CI:	0.4010	0.2201

* * *

APPENDIX Y. UNPAIRED T TEST FOR ALUMINIUM UPTAKE IN TREATMENT 4I VS 4II IN *S. TUBEROSUM* AFTER 20 DAY EXPOSURE

Mann-Whitney Test

Do the medians of 4i and 4ii differ significantly?

The two-tailed P value is 0.2286, considered not significant.
The P value is exact.

Regardless of what data you enter, it is impossible for this test to
yield P < 0.05 with so few data points.

Calculation details
Mann-Whitney U-statistic = 2.000
U' = 10.000
Sum of ranks in 4i = 16.000. Sum of ranks in 4ii = 12.000.

Summary of Data

Parameter:	4i	4ii
Mean:	0.3810	0.1058
# of points:	3	4
Std deviation:	0.5045	0.06285
Std error:	0.2913	0.03143
Minimum:	0.08695	0.06460
Maximum:	0.9635	0.1995
Median:	0.09245	0.07950
Lower 95% CI:	-0.8724	0.005761
Upper 95% CI:	1.634	0.2058

* * *

APPENDIX Z. ANOVA FOR TOTAL CHLOROPHYLL *B. OLERACEA* AFTER 20 DAY EXPOSURE TO THE 4 TREATMENTS

One-way Analysis of Variance (ANOVA)

The P value is 0.4484, 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) = 2.071
The P value is 0.5578.
Bartlett's test suggests that the differences among the SDs is not significant.

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	0.2338	>0.10	Yes
T2	0.2001	>0.10	Yes
T3	0.1688	>0.10	Yes
T4	0.2340	>0.10	Yes

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	3	38.122	12.707
Residuals (within columns)	19	261.44	13.760
Total	22	299.56	

F = 0.9235 =(MStreatment/MSresidual)

Summary of Data

Group	Number of Points	Mean	Standard Deviation	Standard Error of Mean	Median
T1	5	12.407	3.028	1.354	11.372
T2	6	11.108	2.745	1.121	10.701
T3	7	9.177	3.595	1.359	9.216
T4	5	11.987	5.233	2.340	10.195

Group	Minimum	Maximum	95% Confidence Interval From	To

T1	9.132	16.230	8.649	16.166
T2	8.164	15.560	8.227	13.989
T3	4.762	13.934	5.851	12.502
T4	5.514	17.936	5.491	18.484

* * *

APPENDIX ZA. ANOVA FOR TOTAL CHLOROPHYLL *S. TUBEROSUM* AFTER 20 DAY EXPOSURE TO THE 4 TREATMENTS

Total chlorophyll treatments potatoes

One-way Analysis of Variance (ANOVA)

The P value is 0.1471, 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) = 2.599
The P value is 0.4577.
Bartlett's test suggests that the differences among the SDs is not significant.

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?
Tmt 1	Too few values to test.		
Tmt 2	Too few values to test.		
Tmt 3	0.3151	0.0633	Yes
Tmt 4	0.2141	>0.10	Yes

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	3	55.755	18.585
Residuals (within columns)	16	144.92	9.057
Total	19	200.67	

F = 2.052 =(MStreatment/MSresidual)

Summary of Data

Group	Number of Points	Mean	Standard Deviation	Standard Error of Mean	Median
Tmt 1	4	21.776	1.915	0.9577	21.639
Tmt 2	4	20.652	1.703	0.8517	20.030
Tmt 3	6	17.284	3.565	1.456	19.060
Tmt 4	6	19.267	3.511	1.433	18.488

Group	Minimum	Maximum	95% Confidence Interval From	To
Tmt 1	19.886	23.938	18.728	24.823
Tmt 2	19.384	23.163	17.941	23.362
Tmt 3	11.426	20.052	13.542	21.027
Tmt 4	15.357	25.156	15.582	22.952

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