Occurrence of Cyanobacteria and Microcystin variants in Musina Raw Water Supply and Limpopo River Sediment, South Africa

Gumbo JR, Netshambidi LK and Mavhunga P

***Abstract*—** The nutrient enrichment of freshwater dams, rivers and lakes leads to propagation of cyanobacteria which are a major concern due to microcystins in South Africa and worldwide. The presence of microcystins may be a source of natural organic matter (NOM) in raw water supply which stimulates the growth of cyanobacteria. Here we report on the occurrence of cyanobacteria and diatoms in Musina raw water supply and Limpopo river sediments. The study showed that source of cyanobacteria and diatoms were the river sediments. The FlowCAM and scanning electron microscope (SEM) identified three cyanobacteria genera: *Oscillatoria, Microcystis* and *Planktothrix.* The physico-chemical analysis of the river sediment and water samples showed low levels of clay particles, high levels of nitrates, inorganic phosphorus and total phosphorus. The UV-Vis spectrophotometric method and total organic carbon (TOC) analyzer confirmed presence of specific ultra-violet absorbance (SUVA) and dissolved organic carbon (DOC) levels in the water samples. The high DOC levels and nutrients in the raw water and sediments stimulate the growth of cyanobacteria and the production of microcystins. High pressure liquid chromatography with a photodiode array detector (HPLC-PDA) then confirmed the presence of microcystin LR and YR in the water samples.

***Keywords*—** river sediments; dissolved organic carbon; *Microcystis*; raw water supply

I. Introduction

The nutrient enrichment of freshwater dams, rivers and lakes leads to propagation of cyanobacteria which are a major concern because of production hazardous cyanotoxins in South Africa and worldwide [1-4]. Natural organic matter (NOM) is a complex organic material that is found in natural surface water sources as a result of the biodegradation of plants by microbes [5].Thus the water resources that are used for domestic and industrial purposes commonly contain natural organic matter (NOM) and is called dissolved organic carbon (DOC) [6]. The NOM has the potential to encourage bacterial formation which may on the long term affect human health [7].

The occurrence of cyanobacteria, mostly *Microcystis* species, in sediments even in deeper layers has been observed microscopically [8]. The presence of cyanobacteria both in water column and sediments may cause an adsorption of their toxins in sediment layers, because 65 to 85% of the benthic stock may be subjected to decay [9]. As reported by Morris et al. [10], clay minerals appear to be very effective in microcystin adsorption from water solution. The microcystins released into water during cyanobacteria bloom decay are comparatively persistent in aquatic environment [11]. They can be degraded by exposure to ultraviolet radiation or microbial activity [12-13]. During unfavourable environmental conditions, a few planktonic cyanobacteria species persist as resting/dormant stages that subsist in the sediments [13]. Characteristics such as texture, surface area, pore size distribution, and pH are the important factors that affect the extent of adsorption capacity of cyanotoxins onto the sediment [14].

The source of drinking water for Musina town is Limpopo River. The raw water is pumped from 19 boreholes that have been drilled in the Limpopo River and then subjected to chlorination before distributed to the town. The main objective of the study is to determine occurrence of cyanobacteria and microcystins in the Musina raw water supplies and the Limpopo river sediments. The specific objectives were: to determine the physical-chemical parameters of the Musina raw water and river sediments; to determine the dissolved organic carbon in Musina raw water supply; to identify cyanobacteria species that were present in Musina raw water supply and river sediments and to determine the levels of microcystins that were present in the Musina raw water.

II. Materials and Methods

A. The Study Area

The water and sediment samples were collected from abstraction point (Fig 1).

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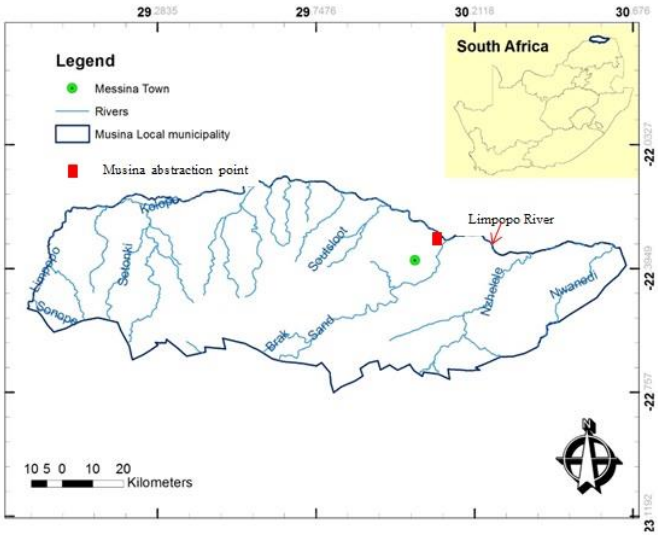


Fig 1. The location of raw water supply point and Musina town.

B. Sample collection and preparation

The water and sediment samples for this study were collected from the study area (Table 1). To collect sediments from such deeper depth a hand auger was used. The water samples were collected in two plastic 250 ml plastic containers of which one plastic container was sterilized prior to sampling. To the non-sterile plastic container, physico-chemical analyses were later performed. To the sterile plastic container, this was later incubated at room temperature (30 to 38 ^{o}C) for period of 30 days under continuous light conditions. The water samples were collected monthly from November 2012 to February 2013. All samples were stored in a refrigerator in the dark before use.

TABLE I: THE DESCRIPTION OF THE WATER AND SEDIMENT SAMPLE POINTS

Sample point	Sample description
Water tap 1	Tap outlet directly linked to one borehole located in the Limpopo riverbed
Water tap 2	Tap outlet from central reservoir, holds raw water from other all 19 boreholes
Sediment 1	Collected at the surface of riverbed (0m)
Sediment 2	Collected at a depth of 1.00m from the riverbed
Sediment 3	Collected at a depth of 1.68m from the riverbed

C. The physical-chemical and nutrient analysis of water samples

1) On-site physical analysis

The physical parameters, pH, EC, TDS were performed in triplicate for the water samples. The pH, EC and TDS were analyzed using a combination of pH and electrical conductivity probe from HANNA Instruments. The calibration of the pH and electrical conductivity was carried out with standards supplied by the manufacturer.

2) Nutrient analysis

The nutrients, phosphate and nitrate, were analyzed on the Ion Chromatography Metrohm 850 professional IC (Metrohm, Switzerland) was used and the procedure of Pereira et al. [15] was followed. The measurements were conducted in triplicates and the standard deviation and the mean of the concentration were calculated.

3) The analysis of Dissolved Organic Carbon

The same water samples were pre-filtered through a 0.45 µm filter (GVS Filter, USA) and then were also sent to University of Johannesburg who carried out the dissolved organic carbon (DOC) analysis. The DOC was measured with a total organic carbon (TOC) analyzer (Fusion instrument) according to procedure of Nkambule et al. [16]. The TOC analyzer was calibrated, prior to use, with a series of Potassium hydrogen phthalate (KHP) standards of 1 mg/l, 5 mg/l, 10 mg/l, 20 mg/l and 30 mg/l Carbon that were prepared in deionized water.

4) Ultraviolet visible (UV-Vis) spectrophotometric analysis

The waters samples were pre-filtered through a 0.45 µm filter (GVS Filter, USA) and were analyzed at University of Limpopo who carried out the UV-Vis spectrophotometric analysis in the following wavelengths: 214 nm; 254 nm; 272nm and 300 nm. The absorbances at 254 nm were used to calculate the Specific Ultra-Violet Absorbance (SUVA) as this equation (1).

$$SUVA\left(\frac{L}{mg.M}\right) = \frac{UV_{254}\,(Cm^{-1})}{DOC(\frac{mg}{L})}X100 \qquad \text{equation 1}$$

5) The analysis of microcystins in the raw water supply

The same water samples were pre-filtered through a 0.45 µm filter (GVS Filter, USA) and then were also sent to University of Johannesburg who carried out the analysis of microcystins. 100 mL of water sample was filtered through a 0.45 µm membrane filter and the filtrate divided into three 10 ml portions. Oasis® HLB 3 cc/60 mg was used. The Extracts were analyzed by the Surveyor Plus™ modular LC system and the ChromQuest™ data system, products of Thermo Fisher Scientific San Jose, on a 150 × 4.6 m, 5 µm column (waters) at 30 °C with a mobile phase composition of 60% water + 0.1% formic acid and 40% acetonitrile+0.1% formic acid at a flow rate of 1.0 mLmin^{-1}. The Surveyor Plus modular LC system consists of the Surveyor LC Pump Plus, the Surveyor Auto sampler Plus, and the Surveyor PDA plus Detector. Chromatograms at 238 nm were recorded with the Surveyor PDA plus Detector, and microcystin (MCYST) were identified by retention time and characteristic UV absorption spectra (200–300 nm). Quantification was based on external calibrations of MCYST-RR, -LR, LY and -YR, respectively. The injections were in triplicate.

D. The physical-chemical and nutrient analysis of river sediments

1) Total phosphorus (TP) analysis

Total phosphorus (TP) was determined by use of the perchloric acid digestion method as described by APHA [17]: 2 g of air dried sediment was acidified to methyl orange with concentrated HNO_3, another 5 ml concentrated HNO_3 was added and evaporated on a hot plate to 15 ml. 10 ml each of concentrated HNO_3 and $HClO_4$ was added and evaporated gently until the dense white fumes of $HClO_4$ appear. The

solution was then neutralized with 6N NaOH and made up to 100 ml with distilled water.

2) Total Inorganic phosphorus (TIP) analysis

Total inorganic phosphorus (TIP) was determined according to [18]: 1 g of air dried sediment was ignited in a muffle furnace at a temperature of 550 ^{o}C for 1 hour, and dissolved in 25 ml of 1 M HCl solution and determined as total inorganic phosphorus according to Strickland and Parsons [19].

3) Total nitrogen analysis

Total Nitrogen was determined according to Hilal and Alhaija [20] as ammonia: 1 g of each air dried sediment sample was treated with 2 ml of sulphuric acid. The sample was heated on a hotplate for 2 hours. Aliquots of 50 ml of deionized water were added to each sample. The sample was filtrated through No. 41 Whatman filter paper. The filtrate of each sample was made up to 250 ml with deionized water and 55 ml of 1 M sodium hydroxide solution.

4) *Particle size analysis*

A determination of particle size distribution was done with sieving method; 240 g of air-dried sediments was transferred directly into a sieve column. The sieve separation column was shacked for 1 hour. Mass retained on each sieve was recorded and presented as percentage.

E. The culture of cyanobacteria of river sediments

In the laboratory the BG11 medium was prepared as per procedure of Krüger and Eloff [21]. Under sterile conditions the 1.0 g river sediments were transferred in to 250 ml Erlenmeyer flasks containing 200 ml of BG11 medium and were incubated for 30 days under continuous light (1100 lux) of white florescent lamps at a room temperature.

1) *The presence of cyanobacteria species in raw water and river sediments*

The sterile plastic containers were incubated at room temperature (30 to 38 ^{o}C) for period of 30 days under continuous light conditions (1076 ± 204 lux). Later after 30 days, the water samples were subjected to flow cytometric analysis. A bench top FlowCAM (Model VS IV) was used to determine the composition of algae and cyanobacteria species that were growing in the samples. The FlowCAM was equipped with a blue (488 nm) laser for florescent and particle detection. For the analysis of algal composition in natural field samples a flow cell (FC300) was used with 4X objective and a cell size range of 20 to 300 µm. The water samples were transferred to the funnel with a pipette. The fluorescent particle/cell was digitally acquired and archived by the FlowCAM for latter processing. This instrument will capture images which were then used to identify the cyanobacteria image by comparison to literature. A confirmatory analysis based on scanning electron microscope will also be used as per procedure of Gumbo and Cloete [22]. The captured images were identified by comparison with published images from literature.

F. *Data analysis*

The Microsoft excel software was used to calculate the mean, standard deviation and carry out statistical analysis with single factor ANOVA to assess any significance differences between microcystins concentrations across months, water tap and microcystin congeners at P<0.05 significance level.

III. Results and Discussions

A. The effects of physico-chemical parameters on cyanobacteria communities in the water samples

The water samples were then incubated at room temperature under continuous lighting to stimulate the growth of cyanobacteria. After incubation for 30 days, some of the plastic containers had a green colour (water tap 2) and others did not have a green colour (water tap 1). This may imply that the presence of nutrients, dissolved organic carbon and light may influence the growth of cyanobacteria in the raw water. The physical chemical analysis of raw water showed variation between the months from November to January (Table 2). The pH was slightly alkaline, with a range of 7.27 to 7.54 (water tap 1) and 7.12 to 7.73 (water tap 2). The alkaline pH is one of the factors that may promote the growth of cyanobacteria as shown by sample from water tap 2 [23].

TABLE II: The Physico-Chemical characteristics of Water Quality During The Study Period

Month		Nov 2012		Dec 2012		Jan 2013	
Sample point		WT1	WT2	WT1	WT2	WT1	WT2
Limpopo River flow		Zero*	Zero*	Moderate**	Moderate**	High ***	High ***
pH		7.54	7.73	7.57	7.34	7.27	7.12
Salinity mg/l		0.5	0.4	0.6	0.5	0.5	0.4
EC µS/cm		96.2	75.5	96.7	73.5	96.7	71.5
Nitrates mg/l		0.5	0.6	0.8	0.7	0.7	0.6
Phosphates mg/l		--	--	--	--	--	--
Absorbances	214 nm	0.495	0.196	2.287	0.248	2.295	1.798
	254 nm	0.134	0.106	0.155	0.151	0.160	0.104
	272 nm	0.114	0.093	0.132	0.136	0.136	0.093
	300 nm	0.083	0.071	0.099	0.111	0.101	0.074
DOC (ppm)		2.31	2.84	2.46	7.27	2.43	2.43
SUVA l/mg.M		5.81	3.73	6.30	2.08	5.85	4.28
Microcystin RR		--	--	--	--	--	$
Microcystin LR		6.60	46.78	21.27	20.53	14.47	$
Microcystin YR		--	27.26	9.77	--	--	$
Microcystin LY		--	--	--	--	--	$

*dry riverbed; **moderate flows; ***High floods; -- Not detected in the water samples; WT water tap; $ The water sample was lost during transit to the University of Johannesburg

The salinity results show that the salinity for each water taps 1 and tap 2 during the study period were high. The high salinity may promote the growth of cyanobacteria [23]. The levels of electrical conductivity (EC) and total dissolved solids (TDS) were found to be quite high with the range of 71.5 to 96.7 µS/cm and 476 to 587 mg/l respectfully. The high EC and TDS level may promote the growth of cyanobacteria [24]. A high nitrate level, ranging from 0.5 to 0.8 mg/L, this may provide nutritional food for cyanobacteria and promote their growth [25]. It was found that the phosphates were zero and this may imply that the phosphate is a limiting nutrient. Thus, only nitrogen fixing cyanobacteria may be available [26].

The results of dissolved organic carbon (surrogate for natural organic matter) showed that there were variations within the water taps and between the months and Limpopo River flows (Table 2). The significance of the absorbances results is as follows:

- 214 nm indicate the presence of nitrates and nitrites in water [27].
- 254 nm indicative of humic substances and aromatics [27].
- 272 nm the best predictor for Trihalomethane (THM) formation [27].
- 300 nm used by Rand Water and other treatment plants as a measure of DOC [27].

Thus at 214 nm wavelength for each water taps there were significant amount of (nutrients) nitrates and nitrites, and these nutrients may contribute to cyanobacteria growth in the raw water [25]. At 254 nm wavelength NOM absorbance was detected, this wavelength indicates that there are humic substances in water and may contribute to carbon sources for cyanobacteria growth. At 272 wavelengths NOM absorbance was high for each water tap 1 and tap 2 and this indicate that there is potential for trihalomethane formation when the raw water is chlorinated. At 300 nm wavelength DOC was also high, this implies that high amount of DOC in water contribute to cyanobacterial biofilm formation [16].

The SUVA result of < 2 litre/(mg.M) indicates that the water samples were mainly composed of non-humic substances while, a SUVA result of >4 litre/(mg.M) may indicate that the water sample was mainly composed of humic substances [28]. The raw water samples of Musina are mainly composed of humic substances based on the information (Table 3).The SUVA results ranges from 2.08 to 6.30 and this indicate that there were ample carbon sources (humic substances) in the raw water to contribute to the growth of cyanobacteria. This was further collaborated by the DOC levels which were in the range of 2.31 to 7.27 ppm. The humic substances may promote the growth of cyanobacteria species in the raw water supply [29]. The corresponding high levels of DOC in the raw water may imply a higher growth of cyanobacteria since there will be ample carbon sources. This may be shown by the high levels of SUVA in water tap 1 as compared to water tap 2, which influences the growth of cyanobacteria [30].

B. The presence of microcystins in the raw water supply

The water samples were assessed for the levels of different microcystin variants such as microcystin RR, microcystin LR, microcystin YR and microcystin LY (Table 2). The microcystins LR was the dominate microcystin congener [13]. These microcystins have been reported to affect human health and some of the symptoms may include weakness and anorexia [31]. The guideline value of 1.0 µg/L for microcystin LR for water intended for human consumption [32]. The microcystin LR levels that were found in Musina raw water supply were considerable higher than the WHO guideline value [32]. The occurrence of the microcystins LR and LY based on statistical mean was not significance different across the water taps 1 and 2 (P=0.24); marginal insignificance across the months (P=0.06) and highly significant across the microcystin congeners (P=0.03). This implies that the occurrence of the microcystins was dominated by the potent microcystin LR and the presence of microcystins throughout the months is worrisome event since these are toxins are harmful to humans on long term consumption of drinking water. The microcystin congeners, YR (which contains the Tyrosine and Arginine amino acids) and LY (which contains Leucine and Tyrosine amino acids) are currently not regulated by WHO and these microcystins are still considered as toxic and hazardous to humans[23].

C. The effects of physico-chemical parameters on cyanobacteria communities in the sediment samples

The results shown in Fig.3 indicated that sediments were mainly composed of sand and minimal contribution of silt and clay. The study of Miller et al. [33] indicated that the loss of cyanotoxin, in the presence of soil particles, was due to adsorption processes onto the soil.

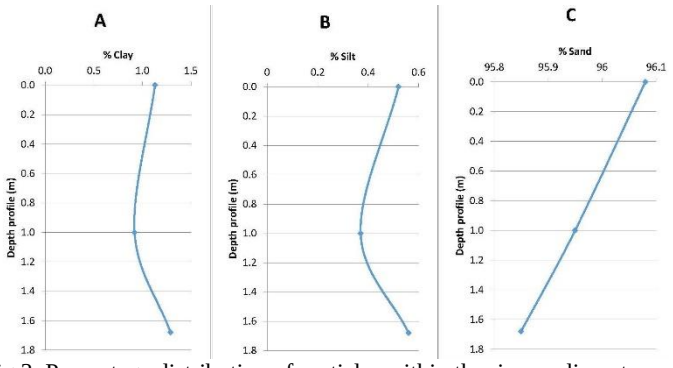


Fig 3: Percentage distribution of particles within the river sediment vertical profile

Several studies have demonstrated that high content of clay and silt are the important factor for adsorption of microcystins onto the sediments [12-13,33]. For this study, sediments from Limpopo River had low content of clay and silt and thus making the absence of the clay and silt leading to possible low level of adsorption of microcystins. Thus, riverbank filtration cannot be used as the candidate technique for the removal of microcystins in Limpopo River and this could explain the presence of microcystins in the raw water (Table 2).

The occurrence of cyanobacteria was also influenced by the availability nutrients in sediments. These nutrients would become available once the Limpopo River flowed again. The nutrients, phosphate and nitrate are the main nutrients that encourage the growth of cyanobacteria and were found (Figure 4). Although the water samples showed absence of soluble phosphates (Table 2), the sediment study indicated the richness of the sediments with the phosphates. The interesting observation was that the total nitrates increased downwards the river sediment profile (Fig 4B) and phosphates concentrations decreased with increasing depth (Fig 4A, C). Could perhaps explain the absence of phosphates in the borehole waters (Table 2)?

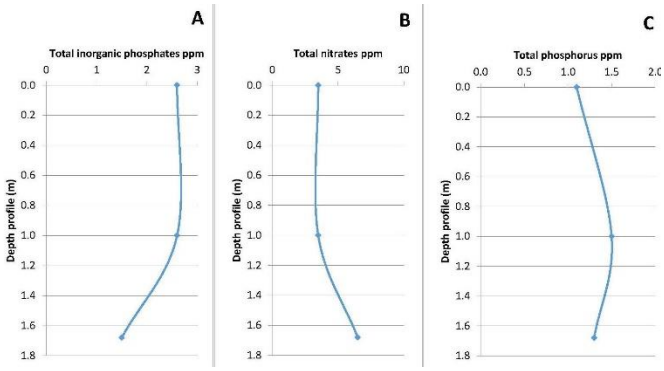
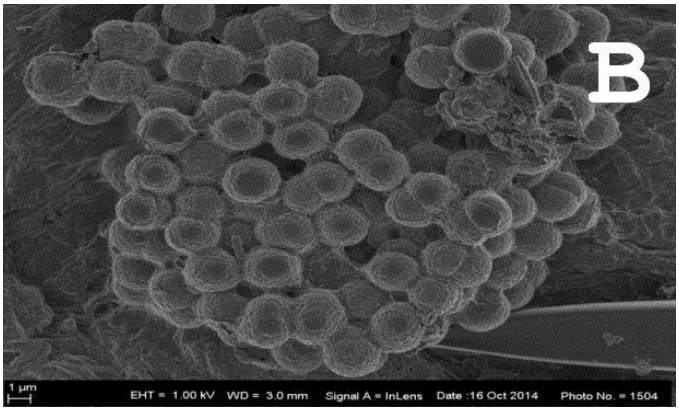


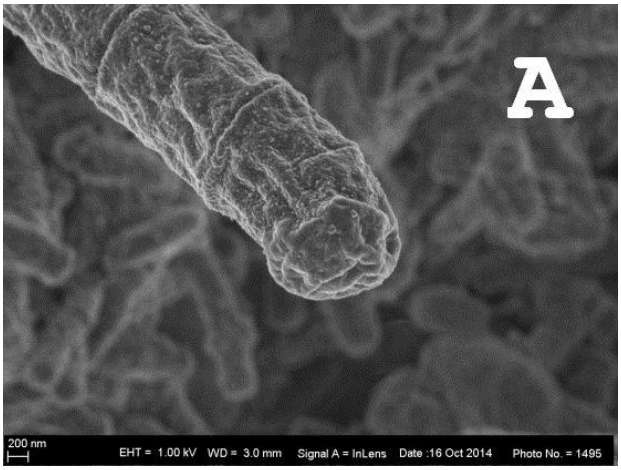
Fig 4. The distribution of total phosphorus (TP), total inorganic phosphorus (TIP) and total nitrate in the Limpopo river sediment profile.

The study showed that the level of total nitrate in sediments ranges from 3.5 to 6.2 mg/l (Figure 4). The highest content of total nitrate was measured in sediment sample 3 and was found to be 6.2 mg/l. The high content found for total nitrogen in the sediments; probably indicated that cyanobacteria accumulate nitrogen by taking it from the sediment and from water as well. Nitrogen is usually the limiting for factor for cyanobacteria growth [34], a relative accumulation in phosphorus also occurs, although to a smaller degree compared with nitrogen.

D. The composition of cyanobacteria communities in the samples

The water and sediment samples were also incubated at room temperature under continuous lighting to stimulate the growth of cyanobacteria. After incubation for 30 days, the glass containers showed a green colouring and were identified as *Microcystis and Planktothrix species* (Fig 5). This may imply that the cyanobacteria species were present in the river sediment. Thus, by providing favourable conditions for these resting stages of the cyanobacteria were able to spring to life. The study of Chen et al. [4] also confirmed that *Microcystis aeruginosa* cells were viable in the lake sediment. Then moving down the sediment profile to 1.68 m, the cyanobacteria species were also found and identified. These cyanobacteria species have been implicated in the production of microcystins (Table 2) [31] and may impact negatively on human health [32] and has been confirmed by this study. An interesting observation was the presence of diatoms in sediment profiles.





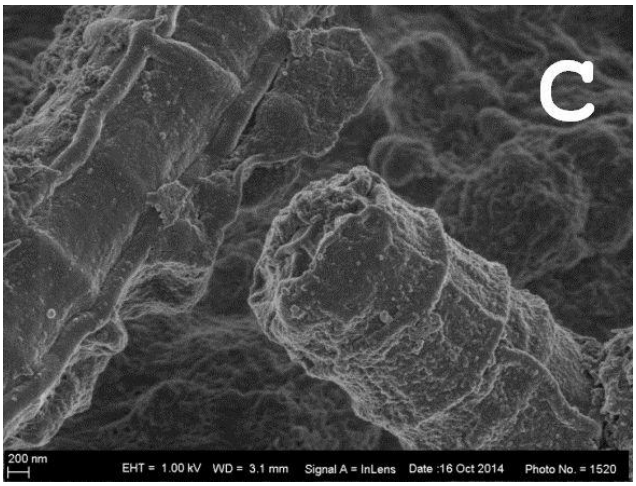


Fig. 5**.** The presence *of (a) Planktothrix spp (surface 0m), (b)Microcystis aeruginosa, Planktothrix spp* (at a depth of 1.0m) and (d) *Planktothrix spp* (at a depth of 1.68m).

IV. CONCLUSION

The study showed the presence of cyanobacteria species and their cyanotoxins n the raw water supply. The environmental factors such as pH, salinity, nutrients, dissolved organic carbon in the borehole water and sediments promote the proliferation of cyanobacteria. Further studies are required to determine the dissolved organic carbon, nutrients, diatoms, cyanobacteria and cyanotoxins in the sediment profile at greater depths of 30 m or more. The cyanotoxins showed the dominance of microcystin LR and likely impact on human health.

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