African Journal of Biotechnology Vol. 6 (20), pp. 2375-2385, 18 October, 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Distribution of cyanotoxins in aquatic environments in the Niger Delta

Lucky O. Odokuma[1*] and Joshua C. Isirima[2]

[1]Department of Microbiology University of Port Harcourt, Nigeria.
[2]Deparment of Pharmacology University of Port Harcourt, Nigeria.

Accepted 24 September, 2007

The presence and types of cyanotoxins in some aquatic environments in the Niger Delta were investigated. Water samples surveyed in the study were surface water of Sombreiro, Nun and New Calabar Rivers. Others were groundwater from Abonnema and Kiama and pond water from Ogboro. Sampling locations of Sombreiro and the New Calabar rivers and the groundwater at Abonnema are all in the Rivers State while other locations were in Bayelsa State all in Nigeria. Cyanotoxins were extracted using the rotary evaporator procedure. The toxins were intraperitoneally administered to mice. Pathological studies revealed that the extracts contained hepatotoxic peptides (microcystin and nodularin), cytotoxic alkaloids (cylindrospermopsin) and neurotoxic alkaloids (anatoxin-a, anatoxin-a(s) and saxitoxin). Cyanobacterial examination of the water samples revealed that *Anabena* was the most predominant cyanobacterium. *Anabena* and *Microcystis* were more predominant in the river and pond water while *Anabena* and *Cylindrospermopsis* were more predominant in the ground water. The nutrient load of water bodies influenced biomass (weight) of cyanobacteria. High nutrient load (BOD, COD, nitrates, sulphate, etc) produced high cyanobacterial biomass while low nutrient load produced correspondingly low cyanobacterial biomass. Nutrient load of river water were significantly higher than groundwater samples. The pond water produced intermediate values of most physicochemical parameters. The percentage hydrocarbon utilizing fungal counts (6.6 - 10.0%), total coliform (240 MPN/100 ml) and fecal coliform (92 to 160 MPN/100 ml) counts were greater than ground water samples (0%, 7.9 to 24 MPN/100 ml and 0.18 to 0.93 MPN/100 ml) respectively. These results suggested that though the conventional bacterial indicators were high, the presence of cyanobacteria and cyanotoxins in these aquatic systems may also contribute to rendering these drinking water sources unfit for domestic consumption.

Key words: Cyanotoxins, cyanobacteria, pathology, cytotoxic, hepatoxin, biomass, nutrients.

INTRODUCTION

Cyanotoxins are diverse group of natural toxins, which are produced by cyanobacteria. Although cyanotoxins are of aquatic origin, most of them, which have been found in recent times, are more hazardous to terrestrial mammals than to aquatic biota (Kaarina and Carry, 1999). Cyanotoxins are responsible for the intermittent but repeated widespread poisonings of wild and domestic animals and aqua cultured fish (Kaarina and Carry, 1999). Human poisoning, have, in the past been suspected but not confirmed due to a lack of information regarding vectors or circumstances that would confirm the presence of cyanotoxins in human food or water supplies as well as a shortage of appropriate methods of detection (Wayne et al., 2001). Because cyanotoxin poisoning occurs mostly, only when water blooms accumulate as thick surface scum's human do not experience acute intoxication. In addition, drinking water supplies usually receive a degree of treatment that prevents high concentrations of cyanotoxin from being present. This spares humans from severe poisoning episodes by the oral route (Wayne et al., 2001). Wayne et al. (2001) has also reported that acute liver failure, visual disturbance, nausea and vomit-

*Corresponding author. E-mail: luckyodokuma@yahoo.co.in.

ing occurred after routine haemodialysis treatment, which occurred in Caruaru Brazil in February, 1996. Animal studies confirmed that these effects were due to cyanotoxins (Wayne et al., 2001).

Classification of cyanotoxins can be based on their mechanisms of toxicity, which ranges from hepatotoxicity, neurotoxicity dermatoxicity and general inhibition of protein synthesis (Ksaarina and Gary, 1999). They can also be classified based on their chemical structure; which includes cyclic peptides, alkaloids and lipopolysaccharides.

Cyanotoxins are produced and contained within the actively growing cyanobacterial cells (Sivonen, 1990). Their release to the surrounding water to form dissolved toxin, occurs mostly during cell senescence, death and lysis, rather than continous secretion (Negri et al., 1997; Rapala et al., 1997). Studies have shown that in healthy log phase cultures of cyanobacteria less than 10 - 20% of microcystins/nodularins and saxitoxins were extracellular (Sironen, 1990; Negri et al., 1997). Microcystins bioaccumulation occurs in common aquatic vertebrates and invertebrates including fish (Carbis et al., 1997*),* mussel and zooplankton (Watainabe et al., 1997). In mussels, the highest microcystin concentrations are found in hepato-pancrease, and in vertebrates they are found in the liver.

Direct cyanobacterial poisoning of animals can occur by two routes, through consumption of cyanobacterial cells from water or indirectly, through consumption of other animals that have themselves fed on cyanobacterial and accumulated cyanotoxins (Carbis et al., 1997).

Cyanotoxins are of great concern to human health. The microcystins and the nodularins are specific liver poisons in mammals. Chronic exposure to comparatively low concentrations of these toxins in drinking water has caused death (Teixera et al., 1993). Acute exposure to high concentrations has caused death from liver haemorrhage or liver failure (Yu, 1995). The alkaloids cyanotoxins and saxitoxins have shown only acute effect in mammals (Yu, 1995). Cylindrospermopsis causes histopathocogical changes in various tissues. Gastroenteritis has been implicated by some cyanobacterial blooms in some rivers (Texera et al., 1993). Hepato-enteritis has been reported, in a total of 140 children and 10 adults after drinking water from river containing blooms of *Cylinchrospermopsis raciborski* (Byth, 1980).

These studies have revealed that conventional bacterial pathogens in natural waters such as *Escherichia vibrio, Salmonella* and *Shigella* may no longer be the main culprits responsible for the diseases acquired during consuming contaminated water. Natural waters especially those in the Niger Delta, which are impacted by inorganic and organic substances resulting from petroleum related activities (Odokuma and Okpokwasili, 1993a, 1997) might actually promote cyanobacterial growth. Thus it becomes imperative to spread the dragnet in terms of detection of causative agents of diseases resulting from consumption of contaminated water and aquatic foods. The objective of this study was to investigate the presence and type of cyanotoxins in some aquatic systems in the Niger Delta. The aim was to determine whether the predominant cyanotoxins in these aquatic systems way cause pathological conditions when consumed.

Table 1. Code for water samples.

Code	Source of Sample
SRNC	New Calabar River water
SRN	River Nun
SRS	River Sombreiro
SPD	Pond water from Ogboro
GWA	Ground water from Abonnema
GWK	Groundwater from Kiama

MATERIALS AND METHODS

Source of water samples

Surface water samples employed in this study were collected from river Sombreiro, River Nun, New Calabar River and Pond water from Ogboro. Ground water from Abonnema and Kiama were also employed. Sampling locations of River Sombreiro, New Calabar River and Abonnema are in Rivers State Nigeria while sampling locations in River Nun and Kiama are in Bayelsa State of Nigeria. Water samples from all three rivers were all brackish in nature while the pond water and ground water samples were fresh. The following codes employed to distinguish the samples are shown in Table 1.

Physicochemical parameters of water samples

Water temperature and conductivity were determined using a Jenuary 4010 conductivity and temperature meter. The pH was determined using PYE UNICAM PW 9418 pH and reference electrode (APHA, 1998). Dissolved oxygen and biochemical oxygen demand (BOD) were determined employing the azide modification of the Winkler method (APHA, 1998). Alkalinity was estimated using potentiometric titrimetric method (APHA. 1998). Phosphate and sulphate were estimated using the stannous molybdate and turbidometric methods (APHA, 1998) respectively. Nitrate was estimated using the Brucein method (APHA, 1998). Total Organic Carbon (TOC) and Chemical Oxygen Demand (COD) were determined by the carbon oxidation and dichromate method and dichromate reflux methods respectively (APHA, 1998). Total Suspended Solids (TSS) and total dissolved solids (TDS) by Gravimetric methods (APHA, 1998) respectively. Total hydrocarbon levels were determined using the photometric method (APHA, 1998). Ten millilitre of the sample was mixed with 10 ml of carbon tetrachloride solution. This mixture was stirred and allowed to stand. The CCL_4 phase was decanted into a clean conical flask. Enough Na_2SO_4 (anhydrous) was added and shaken vigorously to remove all traces of water that may have been present in the mixture. The resultant clear solution was analysed spectrophotometrically at 420 nm using CCL_4 solution as blank. Hydrocarbon (oil and grease) concentrations in the water sample were extrapolated from a standard curve obtained by preparing various concentrations of the crude oil (0.10, 1.0 and 10.0 mg/ml) with absorbance's (0.01, 0.1 and 0.3) at 420 nm and calculated using the relationship.

$$\% \text{ crude oil (ppm)} = \frac{\text{Conc. from graph X T.V.S.E}}{\text{Volume of sample (ml).}}$$

$$\text{Where T.V.S.E.} = \frac{\text{Tptal Volume of Solvent}}{\text{Extract (10ml)}}$$

Microbiological analyses

Total heterotrophic bacterial (THB) count was performed on nutrient agar using the spread plate technique (APHA, 1998). Plates were counted after 48 h of incubation. Mould and yeast (fungi.) counts were determined using aureomycin rose Bengal glucose peptone agar (APHA, 1998). Plates were enumerated after 48 and 96 h of incubation for yeasts and moulds, respectively.

Hydrocarbon utilizing bacterial and fungal population

Vapour – phase transfer method (Amanchukwu et al., 1989) was inoculated with water sample for bacterial number estimation. The medium contained (in g L^{-1}): NaCl, 10.0 g; Mg SO_4. $7H_20$ 0.42 g; KCl, 0.29 g: KH_2 $P0_4$. 0.83 g; NaHP04, 1.25 g: $NaN0_3$, 0.42 g; agar, 15.0 g deionised water, 1L; pH 7.2. The mineral salt medium was also fortified with 70 mg each of streptomycin and terramycin for fungal estimations. Sterile filter paper (Whatman No.1) saturated with crude oil was placed on the inside cover of each petri dish kept in an inverted position. These filter papers supplied the hydrocarbons by vapour – phase transfer to the inocula. Plates were counted after incubation at room temperature for 7 days. The percentage of hydrocarbon-utilizers within the heterotrophic population for each water sample was determined.

Coliform populations

Coliform counts were estimated using the method described by APHA (1998). The tests involved a series of three subsets of fermentation tubes, each containing different strengths (double strength and single strength) of lactose broth. Each subset contained five tubes, which held an inverted Durham tube to collect gas produced by fermentation. The three subsets were inoculated with water samples of 10, 1.0 and 0.1 ml respectively. The 10 ml of the water sample was inoculated in the tubes containing double strength lactose broth (DSLB) while 1 and 0.1 ml were transferred into the test tube containing single strength lactose broth (SSLB). The tubes were shaken and incubated at 37°C for 24 to 48 h after which they were observed for gas production. A positive test for gas formation is presumptive evidence of total coliforms and negative for gas production means the absence of total coliforms. A similar set – up was made but this time it was incubated 44.5 ± 0.2°C for 24 ± 2 h. A positive test for gas formation is presumptive evidence of fecal coliforms and negative for gas production indicating the absence of fecal coliforms. The number of positive tubes in each subset was tallied and this set of numbers was applied to a statistical table (APHA, 1998) to estimate the most likely or probable concentration of coliforms.

Enumeration of cyanobacterial populations

The Lugol's solution method (Ken-ichi et al., 1999*)* was employed to enumerate cells filaments and colonial forms of potentially toxic cyanobacterial populations in water samples. The Lugol's solution enhances sedimentation, because uptake of iodine increases the specific weight of the cells (Ken-ichi et al., 1999). Twenty millilitres of water sample was placed in a centrifuge tube and 0.1 ml of 1%hydrated aluminium potassium sulphate solution was added to enhance the solution (Ken-ichi et al., 1999). The tube was sealed and centrifuged at 360 rpm for 15 min. The supernatant was carefully removed and the pellets re-suspended in 0.5 ml-distilled water. The cyanobacteria were then counted as colonies of filaments using a haemocytometer under the microscope at a magnification of 400 X (Ken-ichi et al., 1999). Transect counting method was adopted for enumeration of cyanobacteria. Transect passed through the central point of the chamber. The horizontal and vertical sides of the counting grids were used to indicate the margin of transect. Enumeration was performed at x 400 magnification.

Extraction of cyanotoxins

A concentration step was carried out for the determination of cyanobacterial distribution in these environments as described by Ken-ichi et al. (1999). The method employed was filtration technique. This method allows concentration of cells by several orders of magnitude and enables the weight of the cell mass to be determined.

Glass fibre filters were placed in desiccators under vacuum, then removed at intervals and weighed until constant weight was obtained. Each weighed filter was placed in a separate Petri dish with the weight recorded on the dish. Inverting the bottle several times mixed the water sample and then a known volume was measured using a measuring cylinder. The water sample was prescreened using a 0.1 - 0.2 mm sieve to remove large particles such as zooplanktons or feathers from waterfowl in the water sample, because these could affect the dry weight of the filters after reweighing. The water sample was then filtered using one of the preweighed filters and the filter was returned to the labelled Petri dish. While leaving the filter in the Petri dish, the dish was placed in an oven set at 45°C. Immediately the filter was dry, it was returned to the desiccators and weighed to a constant weight. The dry weight of the cells collected was calculated by subtracting the initial weight of the filter from the final weight of the filter. Extraction of the cyanotoxins was carried out immediately for some while others were stored in a freezer.

The cyanotoxin extraction procedure was adopted from Ken-ichi et al. (1999). Filter containing cells was placed into a glass beaker containing 20 ml of 75% methanol. Filter was allowed to extract for 1 h. The extract was decanted into a rotary evaporation flask and dried *in vacuo* at 45°C. A further 20 ml of extraction solvent was added into the beaker and allowed to extract as before. The process was repeated for three times, with each time decanting the extract into the same rotary evaporation flask. Pure methanol (0.5 ml) was added to the dry extract in the rotary evaporation flask, and was properly mixed and the suspended extract was transferred into a glass vial. This process was repeated with a further 0.5 ml of methanol. This aliquot was blown to dryness and stored in a deep freezer. After extraction the filter was also transferred into methanol acidified with hydrochloric acid (HCl) for further extraction and the same procedure of evaporation was followed. This was done to extract more cyanotoxins not extracted by the 75% methanol. The same process was followed for recovery of toxins from the rotary evaporation flask. This aliquot was combined with that of 75% methanol and was then blown to dryness. The dry sample was redissolved in a physiological saline because the bioassay technique requires the use of mice and, methanol on its own, is toxic to biological tissues.

Mouse bioassay

The male Swiss albino mice were the animals used for the toxicity testing. This is the most suitable animal (method) for detection of all cyanotoxins using a single dose assay (Ken-ichi et al., 1999). Toxicity was tested by intra-peritoneal (i.p.) injection of 0.5 ml of the

re-dissolved sample (Ken-ichi et al., 1999). The mice were observed for 24 h for neurological signs and killed by giving a knock on the head (Falconer, 1993). At the end of the observation period, post-mortem examination of tissue injury was performed. The observed symptoms and the results of the post-mortem examination were the parameters used for deducing the types of cyanotoxins present (Ken-ichi et al., 1999). A control animal (mouse) was injected with 0.5 ml of physiological saline, and similar observation and analysis was carried out.

Tissue preparation for microscopic examination

The methods employed were adapted from Culling et al., (1985). The processes include: fixation, infiltration/impregnation, embedding, sectioning, staining and mounting.

Fixation

The organs were fixed by putting them in a large volume of 10% formalin (ten volume of fixative for one volume of tissue).

Dehydration

After a few brief washes in distilled water, the tissues were dehydrated through a series of alcohol as shown in the schedule; (i) 50% concentrated alcohol for 5 min and changed twice (a total of 10 min); (ii) 70% concentrated alcohol for 5 min and changed 3 times (a total of 15 min); (iii) 95% concentrated alcohol for 5 min and changed 3 times (total of 15 min); (iv) absolute alcohol (100%), for 10 min and then changed 3 times (a total of 30 min).

Cleaning

The tissues were placed in xylene solution and allowed for a period of 30 min.

Infiltration or impregnation

The tissues were infiltrated or impregnated by using molten paraffin wax at little above the melting point of the wax on a hot plate for a period ranging from 1 - 2 h.

Embedding

This was done by using fresh molten paraffin wax, embedding mould, embedding pot and hot plate. The molten wax was poured into the mould and left for few seconds before the specimen was placed or embedded into the mould. This was kept for at least 1 h to solidify, before microtomy or sectioning.

Sectioning

The microtome was the instrument used for cutting thin section of the tissues in the paraffin block, for microscopic studies. Sliced tissues were then place on a clean microscopic slide, and then stained.

Staining

The following method was employed for staining the tissues.

Deparaffinization of tissue: the processes followed include leaving the slide in a solution of; i. Xylene for 5 min; ii. Absolutes alcohol for 5 min; iii. 95% alcohol for 5 min. iv. 75% alcohol for 5 min. v. 50% alcohol for 5 min; vi. washing the slide for 5 minutes with distilled water.

Staining proper: the processes followed include the following, i. The slides were stained with haematoxylin for 30 min. ii. Excess stain was then washed off with water. iii. Tissues were then differentiation with acid alcohol for a few seconds

Removal of excess stain: excess stain was removed using the following processes. Slides were place in 50% alcohol for 5 min. i) Slides were place in 70% alcohol for 5 min. ii) Slides were place in 90% alcohol for 5 min. iii) Slides were place in 100% alcohol for 5 min. iv) Slides were place in pure xylene for 5 min.

Mounting

Permanent mounts were made by using Canada balsam dissolved in xylene (mountant). The stained slides were removed from the xylene and excess xylene was wiped off from the slide. One drop of the mountant was placed at the middle of the section. A clear cover slip was lowered onto the section by resting the cover slip against a finger and leaving it down gently with a mounting needle. Excess mountant was wiped off, from the edge of the cover slip with care and the slide was left to dry up in an oven at 37^{o}C. These slides were then examined microscopically.

Statistical analysis

The analyses of variance (ANOVA) and the least significant difference (LSD) tests (Finney, 1978), at 95% confidence levels were employed to determine if there was a significant difference between the treatment options (physiochemical and microbiological parameters of water samples and effect of the cyanotoxin extracts on the test mice). All tests (physiochemical and microbiological parameters of water samples and effect of the cyanotoxin extracts on the test mice) were conducted in triplicate.

RESULTS

The physicochemical parameters of the water samples are presented in Table 2. Water temperature ranged from 21.4 to 25.0^{o}C. Geological position (whether it is surface river water or ground water) of the water source and size (river or pond) of the water body did not influence temperature. The pH of the water samples was neutral to slightly alkaline. It ranged from 7.0 to 7.64. Geological position and size of water body had no statistically significant effect on water pH. Surface river water conductivity values (480 – 3000 uSem/cm) were higher than ground water (310 – 810N uSem/cm) and the pond water 390 uSem/cm) value. Total dissolved solids (TDS) and total suspended solids (TSS) values showed similar trend with conductivity. Nitrate level. (4.9 – 6.0 mg/l) and phosphate levels (0.68 – 080 mg/l) showed no significant variation between geological position and size of water body. Biochemical oxygen demand (BOD) values of water samples ranged from 0.05 to 0.6 mg/l. Surface river water BOD values ranged from 0.3 to 0.06 mg/l. Groundwater BOD values ranged from 0.15 to 0-.2 mg/l while

Table 2. Physico-chemical parameters of water samples.

Sample	Temp (°C)	pH	Turb. (NTU)	Cond. (uSem/cm	SO_4^{2-} (mg/l)	NO_3^2 (mg/l)	PO_4^{2-} (mg/l)	TOC %	TSS (mg/l)	TDS (mg/l)	Alk. (mg/l)	BOD (mg/l)	COD (mg/l)
SRNC	23	7.64	0.25	1981	1	5.4	0.78	0.002	0.477	15.77	44	0.6	24.0
SRN	25	7.0	0.01	480	1	5.0	0.68	0.74	0.011	0.103	20	0.3	5.6
GWK	24.2	7.04	0.01	810	0.9	5.4	0.72	1.3	0.21	0.07	18	0.2	5.6
SPD	24.8	7.28	0.09	390	0.9	4.9	0.68	1.09	0.224	0.08	10	0.05	3.2
GWA	22.2	7.10	0.03	310	4.04	6.0	0.68	0.54	0.01	0.07	2.0	0.15	3.2
SRS	21.4	7.64	0.8	3000	6.8	5.2	0.80	0.002	1.279	25.54	104	0.43	32.5

Table 3. Microbial populations isolated from water samples.

Sample	THB x 10^3 (cfu/ml)	TF x 10^2 (cfu/ml)	HUB x 10^2 (cfu/ml)	HUB (%)	HUF x 10 (cfu/ml)	HUF (%)	MPN (Total coliform)	MPN (Fecal coliform)
SRNC	3.9	2	2.5	6.41	2	10.0	240	160.0
SRN	5.5	3	3.7	6.72	2	6.6	240	92.0
GWK	2.4	2	1.3	5.41	0	0.	7.9	0.18
SPD	3.5	2	1.0	2.85	1	5.0	28	2.3
GWA	2.7	1	1.0	3.70	0	0	24	0.93
SRS	1.5	1	1.1	7.33	3	7.5	240	92.0

TAB = Total Heterotrophic bacteria; HUB =Hydrocarbon utilizing bacteria; TF = Total Fungi; HUF = Hydrocarbon utilizing fungi.

Table 4. Weight of cyanobacteria filtered from water samples.

Sample	Volume of water used for filtration (ml)	Final weight of filter paper (g)	Initial weight of filter paper (g)	Weight of filtrate (g)	Weight of filtrate per volume of water used	Weight of filtrate (mg/ml)
SRNC	212	0.0940	0.0826	0.0114	0.0826/212	0.0570
SRN	300	0.0930	0.0835	0.0095	0.0835/300	0.0316
GWK	1700	0.0855	0.0825	0.0030	0.0825/1700	0.0017
SPD	400	0.0926	0.0822	0.0104	0.0822/400	0.0260
GWA	1700	0.0864	0.0834	0.0030	0.083/1400	0.0018
SRS	575	0.0939	0.0839	0.0100	0.0839/575	0.0174

pond water BOD value was 0.05 mg/l. Chemical oxygen demand values of water samples showed a similar trend with BOD values. Surface river water COD values ranged from 5.6 to 32.5 mg/l. Groundwater values ranged from 3.2 to 5.6 mg/l while the pond water COD value was 3.2 mg/l. Some microbial parameters of the water samples (Table 3) did not show the trend exhibited by physico-chemical parameters while some others did. The total heterotrophic bacterial (THB) populations of water samples ranged from 1.5 x 10^3 to 5.5 x 10^3 cfu/ml. There was no statistically significant difference at 0.05 probability levels between THB counts of river water samples groundwater samples and the pond water sample. This trend was also exhibited by the microbial parameters such total fungal (TF) population (1.0 x 10^2 to 3.0 x 10^2) and the percentage hydrocarbon utilizing bacterial (% HUB) population (2.85 TO 7.33%). However, the percentage hydrocarbon utilizing fungal (% HUF) population (0 to 10%) indicated that surface river water values (6.6 to 10.0%) were higher than ground water values (0%). The pond water recorded a value of 5.0%. This trend was apparent in % HUB populations only that it was not significant at 0.05 probability levels. The total coliform and fecal coliform counts exhibited the same trend as in % HUF. Surface river water total coliform counts (240 MPN/100 ml) and fecal coliform counts (92 to 160 MPN/100 ml) were higher than groundwater values of total coliform counts (7.9 to 24 MPN/100 ml) and fecal coliform counts (0.18 to 0.93 MPN/100 ml). The pond water total coliform count was 28 MPN/100 ml while its fecal coliform count was 2.3 MPN/100 ml.

In Table 4 the weight of cyanobacteria filtered from the water samples are presented results showed that surface river water samples weight of cyanobacteria (0.0174 to

Table 5. Populations of predominant cynobacteria.

Sample	Cyanobacteria	Average cyanobacterial counts per 100 ml
SRNC	*Microcystis* sp.	23.4
	Nodulania sp.	13.2
	Cylindrospermopsis sp.	11.4
SRN	*Anabaena* sp.	13.2
	Microcystis sp.	16.6
GWK	*Anabaena* sp.	13.2
	Cylindrospermopsis sp.	11.4
SPD	*Anabaena* sp.	16.2
	Microcystis sp.	11.2
GWA	*Cylindrospermopsis* sp.	13.2
	Anabaena sp.	11.4
SRS	*Anabaena* sp.	16.4
	Lyngbya sp.	13.2

Table 6. Pathological effects of cyclic peptides and alkaloids from water samples on mouse.

Tissue	Pathologic condition	Water sample						
		SRNC	SRN	GWK	SPD	GWA	SRS	N.S
Liver	Loss of sinusoidal structure	+	+	+	+	-	+	-
	Disruption of liver cells	+	+	+	+	-	+	-
	Congested liver	+	+	+	+	-	+	-
	Hemorrhage	+	+	+	+	+	+	-
	Vascular degeneration	-	+	-	+	+	-	-
	Necrosis	-	+	-	+	+	-	-
Kidney	Congestive capillaries	+	+	+	+	+	+	-
	Hyper-cellular glomeruli	+	+	+	+	+	+	-
Lungs	Pulmonary cell necrosis	+	+	+	+	-	+	-
	Pulmonary cell necrosis	+	+	+	+	-	+	
Small Intestine	Inflammatory mucosal cells	+	+	+	+	-	+	-
Thymus	Hemorrhage	-	+	-	+	+	-	-
	Necrosis of thymus cells	-	+	-	+	+	-	-
Heart	Inflammation	-	+	-	+	+	-	-
	Necrosis	-	+	-	+	+	-	-
Spleen	Congested spleen	-	+	-	+	+	-	-
	Necrosis	-	+	-	+	+	-	-

Organs affected by cylindrospermopsin: liver, kidneys, thymus, spleen and heart (Ohtani et al., 1992); microcystins: liver, kidneys, lungs and intestines (Falconer, 1993; Hooser et al., 1990); and nodularin: liver, kidneys, lungs and intestines (Runnegar et al., 1988).

0.0570 mg/ml) were significantly greater than ground water weights (0.0017 to 0.0018 mg/ml). The pond water level was 0.026 mg/ml. This is a similar trend with some physicochemical and some microbial parameters (% HUF, TC and FC)

Anabena was the most predominant cyanobacterial genera (Table 5) in all the water samples. *Microcystis* and *Anabena* were the most predominant cyanobacteria genera in both the surface river water samples and the pond water. *Anabena* and *Cylindrospermopsis* were the most predominant cyanobacterial genera in the ground water samples.

Table 6 shows the result of pathological effect of cyclic peptides and alkaloids from various water samples. Re-

Table 7. Neurological effects of neurotoxic alkaloids in water samples.

Neurological effect	Water sample						
	SRNC	SRN	GWK	SPD	GWA	SRS	N.S
Asphyxiation	-	+	-	-	-	+	-
Convulsion	-	+	+	+	+	-	-
Paralysis	-	+	-	-	-	+	-
Respiratory distress	-	+	+	+	+	-	-
Respiratory depression	-	-	-	-	-	+	-
Respiratory failure	-	-	-	-	-	+	-
Death	-	20 min	5 min	1 h	1 h	5 min	-

Effects produced by anatoxin-a: asphyxiation, paralysis and death anatoxin-a(s): respiratory distress, convulsion and death (Mahmood and Carmichael, 1986; Matsunaga 1989); and saxitoxins: respiratory depression, paralysis, respiratory failure and death (Easthaugh and Shepherd, 1989).

sults showed that extracts from SRNC affected the liver, kidneys, lungs, small intestine, thus showing the presence of microcystins and nodularins. Extracts from SRN affected all the tissues examined: liver, kidneys, lungs, small intestines, thymus, heart and spleen, thus suggesting the presence of both cylindrospermopsin and microcystins in this sample as shows in Table 6. Similarly, extracts from SPD affected all the tissues examined: liver, kidneys, lungs, small intestine, heart and spleen, thus suggesting the presence of both cylindrospermopsin and microcystins in this sample as can be seen in Table 6. The extracts from GWK affected the liver, kidneys, lungs, and small intestine suggesting the presence of microcystins. Similarly extracts from SRS affected the liver, kidneys, lungs, and small intestines, suggesting the presence of microcystins. Results in Table 6 shows that extracts from GWK affected the liver, kidneys, thymus, heart and spleen, indicating the presence of cylindrospermopsins in this sample. Table 6 showed that none of the tissues from the control animal was affected indicating the absence of any cyanotoxin.

Table 7 shows the results of neurological effects of neurotoxic alkaloids present in various water samples. Results showed that extract from SRNC did not produce any neurological effect on the experimental animal (mouse). Extracts from SRN caused asphyxiation, convulsion, paralysis and respiratory distress and caused the death of the animals in about 20 min, thus suggesting the presence of anatoxin-a and anatoxin-a(s) in this water sample. Extracts from GWK caused convulsion, respiretory distress and death of the animal in about 5 min suggesting the presence of anatoxin-a(s). Similarly, extracts from SRD and GWA caused convulsion, respiretory distress and death of the animals in about 1 h also suggesting the presence of anatoxin-a(s). Extracts from SRS caused asphyxiation, paralysis, respiratory depresssion, respiratory failure and death of the animal in about 5 min, thus suggesting the presence of anatoxin-a and saxitoxins in this water sample.

Pathological changes, which occurred in the liver, when the extracts from the various water samples, were administered to the mice (Table 8) showed that all liver tissues treated were affected except the one treated with normal saline. The toxic effects observed were loss of sinusoidal structure, disruption of liver cells, congested liver, haemorrhage, vascular degeneration and necrosis. Pathological changes produced on the kidneys by the extracts from the various water samples are presented in Table 8. It was observed that all the extracts affected the kidneys but normal saline did not cause any effect. The effects observed were congestive capillaries and hypercellular glomeruli. Pathological changes produced on the lungs are presented in Table 8. The observed effects were pulmonary congestion and pulmonary cell necrosis. All the extracts except GWA and normal saline produce these cytotoxic effects. The only toxic effect observed from the intestines was inflammatory mucosal cells. This cytotoxic effect was produced by all extracts except GWA and normal saline. This pathological change of the intestines (inflammatory mucosal cells) is presented in Table 8. The observed pathological changes of the thymus were haemorrhage and necrosis of the thymus cells. These changes are presented in Table 8. Observation of this tissue revealed that only extracts derived from SRN, SPD and GWA affected it. The neurotoxic cyanotoxins found in the water samples is presented in Table 9.

DISCUSSION

Geological position (whether it is a river or groundwater) and size of the water body influenced nutrient load of aquatic system. Nutrient loads (physicochemical parameters) of surface river waters were generally higher than groundwater samples. The pond water sample showed intermediate values between river water and groundwater samples for most physicochemical parameters. Odokuma and Okpokwasili (1993a) attributed upstream – downstream variation of the New Calabar River water levels of conductivity, TDS, chloride, salinity increased nutrient

Table 8. Hepatotoxic and cytotoxic cyanotoxins in water samples

Sample	Organisms found in the sample that produce it	Tissue							Cyanotoxin present
		Liver	Kidneys	Spleen	Thymus	heart	Lungs	GIT	
SRNC	*Microcystis* sp. *Nodularia* sp.	+	+	-	-	-	+	+	Microcystins, Nodularins
SRN	*Cylindrospemopsis* sp. *Anabaena* sp. *Microcystis* sp.	+	+	+	+	+	+	+	Cylindro-spermopsin, Microcystin
GWK	*Anabaena* sp.	+	+	-	-	-	+	+	Microcystis
SPD	*Cylindrospemopsis* sp. *Anabaena* sp. *Microcystis* sp.	+	+	+	+	+	+	+	Cylindro-spermopsin, microcystin
GWA	*Cylindrospemopsis* sp. *Anabaena* sp.	+	+	+	+	+	+	+	Cylindro-spermopsin, microcystin
SRS	*Anabaena* sp. *Lyngbya* sp.	+	+	-	-	-	+	+	Microcystin

Table 9. Neurotoxic cyanotoxins in water samples.

Sample	Organisms found in the sample that produce it	Neurotoxic effect						
		Asphyxiation	Convulsion	Paralysis	Respiratory distress	Respiratory depression	Respiratory failure	Cyanotoxin present
SRNC	-	-	-	-	-	-	-	-
SRN	*Anabaena* sp.	+	+	+	+	-	-	Anatoixin-a, Anatoxin-a(s)
GWK	*Anabaena* sp.	-	+	-	+	-	-	Anatoxin-a(s)
SPD	*Anabaena* sp.	-	+	-	+	-	-	Anatoxin-a(s)
GWA	*Anabaena* sp.	-	+	-	+	-	-	Anatoxin-a(s)
SRS	*Anabaena* sp. *Lyngbya* sp.	+	-	+	-	+	+	Anatoxin-a, Saxitoxin

load with progress downstream resulting from inputs from industrial discharges, erosional and surface run-off. They also partly attributed this variation to nearness to the sea and to tidal influences, this bringing more nutrients to downstream sites. The presence of these factors in these rivers (New Calabar, Nun and Sombreiro) may be responsible for the higher nutrient load than those of the groundwater samples. The ground water samples are not exposed to these factors. The pond water at Ogboro is located in a seasonal swamp environment. It was still subject to nutrient input from run-off from soil around it.

Geological position and size of the water body did not significantly influence THB and TF values. This showed that the nutrient load of all three water sources (river water, ground water and pond water) could support similar population levels of these microbial types. However the % HUF, TC and FC counts indicated higher values for river water samples and lower values for ground water samples. The pond water sample was intermediate between these two extremes. The differences in nutrient load of these three water types may be responsible. One may assume that both TF and THB showed this trend only that it was not significant at 0.05 probability levels. This trend was apparent but not significant at 0.05 probability levels in % HUB. Odokuma and Okpokwasili (1993b) have observed increases in counts of THB, moulds yeasts, actinomycetes and cyanobacterial counts in the New Calabar River water as one progressed downstream. They attributed this to increased nutrient inputs resulting from higher BOD as a result of increased soil erosion and surface run-off as one progressed downstream. These factors were prevalent in the groundwater. High TC and FC counts in the river water samples may also be partly attributable to the activities of humans living by these rivers. In the rural areas of the Niger Delta human waste is usually disposed in these rivers thus contributing to the coliform load of such recipient systems. The population of coliforms in ground water in these areas are relatively lower than river water because of the near absence of septic and soak-away

systems. This human waste disposal system would have contributed to groundwater load of coliforms.

River water weight of cyanobacteria was greater than ground water weights. The higher nutrient load of river water samples may be responsible for this. Cyanobacterial organisms though autotrophic require mesotrophic to oligotrophic environments to proliferate. The three rivers studied presented such conditions. Odokuma and Okpokwasili (1993b) have reported similar observations. They observed that the cyanobacterial counts of the New Calabar River increased with increase in nutrient load of the river.

Anabena was the most predominant cyanobacterial genera in all the three water types sampled in this study. *Anabena* and *Microcystis* were the most predominant in the river and pond waters while *Anabena* and *Cylindrospermopsis* were more predominant in the groundwater.

The cyanotoxoins extracted from SRNC caused loss of sinusoidal structure, disruption of liver cells, congested liver and haemorrhage in the liver, congestive capillaries, and hyper-cellular glomeruli on the kidneys pulmonary cell necrosis on the lungs and inflammatory mucosal cells on the intestines. Falconer, (1993) reported that microcystins when administrated to mouse intraperitoneally produced the above mentioned effects on liver, kidneys, and lungs. Hooser et al. (1990), reported that infections of microcystins on the gastro-intestinal tract has caused the inflammation of mucosal cells. Runnegar et al. (1988), reported that the toxicity and liver pathology induced by nodularin is similar to that caused by microcystins. Carmichael et al (1988) has also reported that Nodularin is isolated from *Nodularia spumigena*. The result of microscopic examination revealed that the samples contained *Microcystis* sp. and *Nodularia* sp. Thus the effect produced by the extract in the tissues may be associated with presence of microcystins and nodularins. Extract of cyanotoxins from SRN affected all the test tissues producing the above stated effects in the liver, kidneys, lungs, intestine and also caused haemorrhage necrosis of thymus cells on the thymus inflammation and necrosis on the heart and congestion, spleen and necrosis on the spleen. Falconer (1993) and Hooser et al. (1990) reported that microcystins when administered to mouse intraperitoneally affect the liver, kidneys and large intestines. Ohtani et al. (1992) have shown that cylindropermopsin produced by *Cylindrospermopsis reciborskii* caused vascular degeneration and necrosis on the liver congestive capillaries and hypercelular glomeruli on the kidneys, haemorrhage and necrosis of thymus cells, inflammation and necrosis of the heart and congested spleen after intraperitoneal administration of the cytotoxic alkaloid. Furthermore, the result of microscopic examination revealed the presence of *Cylindrosper-mopsis, Microcystis* and *Anabaena* sp. Microcystins and Cylindrospermopsin no doubt cause the effect of this extract of these organs. After the intraperitoneal administration of the extract, it was observed that the animals experienced asphyxiation, convulsion, paralysis, and respiration distress that led to the death of the animals in 20 min. Carmichael et al. (1988), reported that intraperitoneal administration of the neurotoxin anatoxin-a, caused asphyxiation, paralysis and death. This toxin is also produced by *Anabaena flosaqae* and *Anabaena lemmermannii*. On the other hand anatoxin-a has been known to cause convulsion respiration distress and death (Carmichael et al*.,* 1988; Matsunage et al., 1989). Thus these observed effects may be as a result of a combined effect of the two neurotoxins anatoxin-a(s) on the nervous system of the animals. Cyanotoxins extracted from GWK affected the liver, kidneys, lungs and intestines in much the same way as stated above. Thus it could be deduced that the effects seen on the tissues could have been produced by the presence of microcystins as well as nodularin. However, the result of microscopic examination of this sample showed the presence of *Anabaena* sp. *Anabaena* is known to produce microcystins (Park et al., 1993) not nodularins, hence the effects on the liver, kidneys, lungs and intestines must have been caused by microcystins. The observable pharmacodynamic (side) effects after the intraperitoneal (ip) administration of the extract from this water sample revealed respiratory distress, convulsion and death after 5 min which are known to be produced by anatoxin –a(s) after an ip administration of this toxin (Matsunaga et al., 1989). The water sample showed the presence of *Anabaena* sp and it has also been reported that *A. flosaquae* (Matsunaga et al., 1989) and *A. limmermannii* (Onodera et al., 1997) are known to produce anatoxin-a(s). Thus the presence of *Anabaena* sp in the water sample supported the conclusion that anatoxin-a(s) is responsible for these effects, hence anatoxin-a(s) may be present in this water sample. Pathophysiological changes associated with the ip administration of the extract derived from the water sample SPD revealed a generalized damage on all organs tested, (liver, kidneys, lungs, intestines, thymus and spleen) suggesting the presence of microcystins and cylindrospermopsins as reported by Falconer (1993), Hooser et al. (1990) and Ohtani et al. (1992). This was further supported by the presence of *Cylindrospermopsis* sp, *Anabaena* sp and *Microcystis* sp in the water sample as revealed by microscopic studies. Observable neurological effects on the organisms revealed respiratory distress, convulsion and death after 1hr. These effects are known to be produced by the presence of anatoxin-a(s), Matsunaga et al, (1989) and Onodera et al. (1997) report of anatoxin-a(s) production by *A. lemmermannii* and thus justified the deduction that, the observed neurological effects on the organism is due to the presence of anatoxin-a(s). Intraperitoneal administration of the extract derived from GWA sample revealed some pathophysiological changes on the liver, kidneys, thymus, spleen, heart, lungs and intestines, which are

summarized in 6. These effects are known to be produced by *Cylindrospermopsin* (Ohtani et al., 1992) and microcystin (Falconer, 1993). Microscopic examination of this water sample revealed the presence of *Cylindro-spermopsis* sp (which produces cylindrospermopsin; Hawkins et al., 1997) and *Anabaena* sp. These findings justify the deduction that cylindrospermopsin and microcystins were present in the water sample. Neuropathological observable responses after the (ip) administration were respiratory distress, convulsion and death after 1 h that are known to be caused by anatoxin-a(s), (Matsunaga et al., 1989). As stated earlier, the water samples contained *Anabaena* sp which are known to produce anatoxin-a(s) (Onodera et al., 1997). Thus it could be concluded that the observable neurological changes was due to the presence of anatoxin-a(s). Hepatological and cytological changes after an (ip) administration of the extract derived from SRS sample were loss of sinusoidal structure, disruption of liver cells, congested liver, liver haemorrhage and congestive capi-llaries, hyper-cellular glomeruli, pulmonary congestion, pulmonary cell necrosis, and inflammatory mucosal cells of the intestines. These effects are produced by microcystins (Falconer, 1993; Hooser et al., 1990). Microscopic examination revealed the presence of *Anabaena* sp which have been known to produce microcystins (Kaya and Watanabe, 1990) and *Lyngbya* sp. Thus the presence of *Anabaena* sp suggests that the observed hepatological and cytological changes were due to the presence of microcystins. Observable neurological effects of the administered extract were asphyxiation, paralysis, respiratory depression, respiratory failure and death after 5 min. These effects suggest the presence of anatoxin-a and saxitoxin (Easthaugh and Shepherd, 1989). Anatoxin-a is produced by *A. flos-aquae, A. lemmermannii,* and *Anabaena planktonica* (Carmichael et al., 1988). *Anabaena circinalis* has been reported to produce saxitoxin (Lehane, 2000). On the other hand *Lyngbya wollei* also produces saxitoxin (Onodera et al., 1997). Thus, it could be deduced that the observed effects were the results of both anatoxin-a and saxitoxin, which are produced, by *Anabaena* sp and *Lyngbya* sp.

Conclusion

Pathological studies on the tissues and neurologic symptoms produced by the animals revealed six types of cyanotoxins. These were cylindrospermopsin, microcystin, nodularin, anatoxin-a, anatoxin-a(s) and saxitoxin. Microcystin and nodularin occurred in SRNC. Cylindrospermopsin, microcystin, anatoxin-a and anatoxin-a(s), were found in SRN. GWK was found to contain microcystin and anatoxin-a(s). Cylindrospermopsin, microcystins and anatoxin-a(s) occurred in SPD. GWA contained cylindrospermopsin and anatoxin-a(s) while microcystin, anatoxin-a and saxitoxin were found in SRS.

All the water samples analyzed where found to contain both cyanobacteria and harmful cyanotoxins probably in harmless concentrations (concentrations in water samples were not determined). This makes the water a potential health risk with regards to cyanotoxin poisoning. The river waters despite their lotic nature may not support algal blooms. However, the ground water (hand dug wells and bore holes) sources and the pond water despite having a relatively lower carbon flux compared with the river waters may promote algal blooms because of their lentic nature, thereby, increasing the potential of cyanotoxin poisoning. Thus if these water sources should continue to serve as domestic water sources, they should be properly treated to eliminate all cyanobacteria, cyanotoxins, and other conventional indices (THB, TF and coliforms etc) of water quality.

REFERENCES

Amanchukwu SG, Obafemi A, Okpokwasili GC (1989). Hydrocarbon degradation and utilization of a palm-wine yeast isolate. F. E. M. S. Microbiol. Lett. 151-154.

American Public Health Association (APHA) (1998). Standard Methods for the Examination of Wastewater and Water 19th ed. APHA Washington DC.

Byth S (1980). Palm Island Mystery disease. Med. J. Austr. 2: 40 –42.

Carmichael WW, Yu MJ, He ZR, He JW, Yu JL (1988). Occurrence of the toxic cyanobacterium (blue-green alga*) Microcystis aeruginosa* in Central China. Arch. Hydrobiol. pp. 21–30.

Carbis CR, Rawlin GT, Grant P, Mitchell GF, Anderson JW, Mclanley I (1997). A study of feral carp *Cyprinus carpio I*, exposed to *Microcystis aeroginosa* lake Mokoan, Australia and possible implication on fish health J. fish Dis. 20: 81-91.

Culling CFA, Allison RT, Barr WT (1985*)*. Cellular Pathology Techniques 4th edition London.

Easthaugh J, Shepherd S (1989). Infections and toxic syndromes from fish and shellfish consumption. Arch. Int. Med. 149: 173–1740.

Falconer IR (1993). Measurement of toxins from blue – green algae in water and foods stuffs In: Algae Toxins is Seafood and Drinking water Falconer IR (ed) Academic Press, London pp. 165–175.

Finney DJ (1978). Statistical Methods in Biological Assay 3rd. edition les Charles Griffin London pp. 1-252.

Hawkins PR, Chandrasena NR, Jones CJ, Humpage AR, Falconer IR (1997). Isolation and toxicity of *Cylindrospermopsis raciboriskii* from an Ornamental lake. Toxicology 35: 341–346.

Hooser SB, Beasley VR, Basgall EJ, Carmichael WW (1990). Microcystin –R- induced utratructural changes in rats. Vet. Pathol. 27(1): 9–15.

Kaarina S, Gary J (1999). Cyanobacteria toxins In: World Health Organization toxic cyanobacteria M water; Guide to public Health, consequences Monitoring and Management London.

Kaya K, Watanabe MM (1990). Microcystin Composition of an axenic clonal strain oif Microcystis viridis containing water blooms in Japanese freshwater. J. Appl. Phycol. 2: 173–178.

Matsunaga S, Moore RE, Niemczura WR, Carmichael W (1989). Anatoxin – a (s) a potent anticholmesterase from *Anabaena flosaquae.* J. Am. Chem. Soc. pp. 8021–8023.

Ken-ichi H, Fumio K, Linda L (1999). Determination of cyanobacteria in the laboratory In: Toxin cyanobacteria in water. A guide to their public Health Consequences. Monitoring and Management World Health Organization (1999) London

Negri AP, Jones GJ, Blackburn SI, Oshima Y, Onodera H (1997). Effects of culture and bloom development and of sample storage on paralytic shellfish poisons in the cyanobacterium *Anabaena circinalis* J. Phycol. 33: 26 – 35.

Odokuma LO, Okpokwasili GC (1993a). Seasonal Influences on inorganic Anion Monitoring oif the New Calabar River Nigeria.

Environ. Manage. 17(4): 491–496.
Odokuma LO, Okpokwasili GC (1993b). Seasonal Ecology of Hydrocarbon Utilizing Microbes in the surface waters of a River Environ. Monit. Assess. 27: 175–1991.
Odokuma LO, Okpokwasili GC (1997). seasonal influences of the organic pollution monitoring of the New Calabar River, Nig. Environ. Monit. Assess. 45: 43–56.
Ohtani I, Moore RE, Runnega MTC (1992). Cylindrospermopsin-a potent hepatotoxin from the blue-green alga *Cylindrospermopsis* raciborskii, J. Am. Chem. Soc. 114 (20): 7941–7942.
Onodera H, Oshima Y, Henrisksen P, Yasumoto T (1997). Confirmation of anatoxins-a(s) in the Cyanobacterium Anabaena lemmer mannii as the cause of bird kills in Danish lakes. Toxicology 35: 1645–1648.
Park HO, Watanaba MF, Harada KI, Nagai H, Suzuki M, Hayashi H (1993). Hepatotoxin (Microcystin) and neurotoxic (Anatoxin-a) contained in natural blooms and strains of Cyanobacteria from Japanese freshwaters. Nat. Toxins 1: 353–360.
Rapala J, Sivonen K, Lyra C, Niemela SI (1997). Variation of Microcystins Cyanobacterial hepatoxin, in *Anabaena sp* as a function of growth stimuli. Appl. Environ. Microbiol. 64: 206–221.
Runneger MTC, Jackson ARB, Falwner IR (1988). Toxicity of the Cyanobacterium *Nodularia spumigena mertens* Toxicology 26(2): 143–151.
Sivonen K (1990). Effects of light, temperature, nitrate, orthophosphate and bacteria on the growth and hepatotoxin production by *Oscillatoria agardhii* strains. Appl. Environ. Microbiol. 56: 2658–2666.
Teixera MGLC, Costa MCN, Carvalho VLP, Pereira MS, Hage E (1993). in aquatic Environment Bulletin of the Pan American Health Organization 27, Cyanotoxin: pp. 244–253.
Wayne W, Carmichael I, Sandra MFO, Azeredo J, Si An, Renato JR, Molica EM, Jochimsen SL, Kenneth L, Rinehart GR, Shaw R, Eaglesham GK (2001). Human Fatalities from Cyanobacteria: Chemical and Biological Evidence for cyanotoxins. Environ. Health Perspect. 109: 663–668.
Watanabe MM, Kaya K, Takamura N (1992). Fate of the toxin cyclic hepapeptides, the microcystins from blooms of microcystic (Cyanobacteria) in a hypertrophic lake. J. Psychol. 28: 767–769.