

Acute and Sub lethal Effect of Potassium Cyanide on the Behaviour and ATPase Enzyme Activity in the Freshwater Fish, *Clarias gariepinus* (Catfish)

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ABSTRACT. The Present research studies the acute sub lethal toxicity of potassium cyanide (KCN) in the fresh water, *Clarias gariepinus* and the effects on its behaviour and the Na⁺ - K⁺ ATPase enzyme activity. Acute toxicity of potassium cyanide (free cyanide) to the freshwater fish was studied using static bioassay method over a period of 96 h. Different concentrations of the toxicant (KCN) were used and LC₅₀ value was found to be 361µg/L. Behavioural changes when exposed to lethal concentration of KCN showed increased feed intake, cannibalism, irregular swimming activity, rapid jerk movement, aggressiveness, loss of balance, opercula movement, surface behaviour, loss of equilibrium, change in body colour and convulsion. The effect of potassium on the Na⁺ - K⁺ ATPase of various physiological tissues which includes the gill, liver, muscles and intestinal over duration of 12 h- 35 days were also studied. Cyanide intoxication resulted in marked changes in ATPase, shown by significant decrease in the enzyme activities. The result shows that ATPase enzyme together could be employed as a sensitive and useful biomarkers for cyanide pollution while the behavioural changes could be an early signal of toxicity.

1. INTRODUCTION

Cyanide is an extremely destructive, suicidal, homicidal and chemical agent which kills both its target and non-target-organisms when released into the environment [Dube and Hosetti, 2011; Hariharakrishnan *et al.*, 2010). It has been reported to inhibit the mitochondrial enzyme, cytochrome oxidase in the respiratory electron transport chain, thus impairing both oxidative metabolism and the associated process of oxidative phosphorylation (Daya *et al.* 2000). It combines in an irreversible reaction with ferrocytochrome a/a₃ which leads to the inhibition of mitochondrial oxygen uptake, cellular respiration and reactions at the electron transport chain. Fernando *et al.* (1991) reported the effects of eight organochlorine pesticides on eels while also determining their 96 h LC values and behavioural changes of the effect of the pesticides. Observation made includes anxiety, swimming disorder pattern, loss of balance, excessive mucus secretion and lightening in fish colouration. Although the modes of function of these insecticides are markedly different than potassium cyanide, behavioural changes observed are similar to this study. Bradbury and Coast (1989) reported signs of fenvalerate poisoning in fish, which included loss of schooling behaviour, swimming near the water surface, hyperactivity, erratic swimming, seizures, loss of buoyancy, elevated cough rate, increased gill mucus secretions, flaring of the gill arches, head shaking and restlessness before death.

Adenosine triphosphates (ATPase) are a set of complex set of enzymes which requires Na⁺ - K⁺, Mg²⁺ and Ca²⁺ ions for their activity (Carfagna *et al.*, 1996; Praveen *et al.*, 2012). This enzyme have requirements for Na⁺ - K⁺, Mg²⁺ and Ca²⁺ ions activity and in the cleavage of ATP to ADP/AMP and inorganic phosphate (Begum, 2011), which is a vital metabolic process for the production of energy needed by cells for their metabolic processes. The major pathway for cyanide metabolism is the conversion of cyanide (CN⁻) to thiocyanate (SCN⁻), in the presence of a Sulphur

donor by the enzyme Rhodanese (thiosulfate sulfurtransferase; EC 2.8.1.1) (Isom *et al.*, 2010). Cyanide toxicity has been attributed to the presence of HCN derived from dissociation of the complexes that penetrate cells wall (Pablo *et al.*, 1996). Major effects of cyanide on fish had been observed to include; growth inhibition (Dixon and Leduc, 1981), behavioural alterations (Shwetha and Hosetti, 2009), hypoxia (Dube and Hosetti, 2011) and susceptibility to predation (Eisler, 1991).

Shwetha *et al.* (2012) reinstated the effects of cyanide on fish and the impact it might have on human health. Consumption of cyanide caught fish or fish exposed to cyanide poses health problems to human health. Also, chronic cyanide intoxication caused by consumption of cassava, a common food product in developing countries, is the main etiological factor in the debilitating tropical ataxic neuropathy (Egekeze and Oehme, 1980). Although, cyanide is known for its potent toxic nature, it still has large applications in variety of industrial processes such as metal mining (mainly gold and silver), electroplating, steel, automobiles, carbonisation, printed circuit board manufacturing and chemical industries which invariably leads to discharge of huge quantity of cyanide (HCN/CN-) containing effluents to water bodies (Prashanth *et al.*, 2011). During cassava processing in most communities in developing countries, large amounts of cyanoglycosides are released which are hydrolysed by plant borne enzymes, leading to cyanide concentrations in waste water as high as 200 mg/L (Siller and Winter, 1998). The peel, fibre, cassava juice and the residual water produced after the separation of starch and fibre during the fermentation and drying period rots and mixed with soil also contribute to the cyanide content of soil (Obboh *et al.*, 2003) and water bodies respectively. Although cyanide fishing is detrimental both to aquatic life and human health, it is still a practice that is common in rural communities. Fish are gaining interest as use in the assessment of the quality of aquatic environment making them a tool of bio-indicators of environmental pollution (Prashanth and Neelagund, 2007).

The present study was carried out to investigate the influence of potassium cyanide (KCN) on the behavioural activities of the freshwater fish, Catfish, *Clarias gariepinus* and on the activities of adenosine triphosphates enzyme (Na⁺/K⁺ - ATPase) within gills, liver, muscles and intestinal tissues of *Clarias gariepinus*.

2. MATERIALS AND METHODS

2.1. Fish specimen collection and maintenance

Cat fish, *Clarias gariepinus* were obtained from the fishery department of the University of Benin, Benin-Nigeria and acclimatized to laboratory conditions for one week before the start of the experiments. Only active specimens (6.5±0.4 cm, 10.9±0.5g) with no signs of stress and injury were used in the study. Dechlorinated tap water (temperature 25.9±1.0 °C, dissolved oxygen 6.5 ± 0.5 mg/l, carbon dioxide 6.3±0.4 mg/L, hardness 23.2±3.4 mg/l as CaCO₃, phosphate 0.38±0.005 µg/l, salinity 0.01 ppt, specific gravity 0.001, conductivity less than 10 µS/cm and a light period of 11 h/day) was used throughout the experimental period and water was renewed every 48 h. The experimental fish were reared in aquaria of dimension (45cm×30×25cm). Fish were fed regularly with commercial fish food pellets during acclimatization and test periods. Feeding was stopped two days prior to exposure to the test medium for acute toxicity test only. All water quality parameters (Dissolved oxygen, temperature, pH, salinity, alkalinity, conductivity) were monitored throughout the duration of the experiment except for minimal variation tolerated by the fish in its natural habitat. After acclimation, the following steps were carried out.

2.2. Preparation of potassium cyanide stock solution

Stock solution was prepared by dissolving KCN (99% purity) in double distilled water in standard volumetric flask. The required quantity of sodium cyanide was drawn from this stock solution using a micropipette. The concentration of test compounds used in short term definitive tests were between the lowest concentrations at which mortality was 100% and the highest the concentration at which mortality was 0% in the range finding tests.

2.3. Determination of LC_{50} of cyanide for *Clarias gariepinus* and observation of behavioural changes

Ten concentrations of cyanide (0.65, 0.55, 0.47, 0.44, 0.41, 0.368, 0.322, 0.2, 0.18, and 0.1mg/l) were prepared in 10 aquaria of different tanks of dimension (45cm×30×25cm) containing 20L of aerated dechlorinated tap water. A simultaneous control group was prepared with only aerated dechlorinated tap water after which ten healthy fish were stocked in each aquarium. Water with its fixed dose was changed daily within the period of the experiment (96 h). The average lethal concentration (LC_{50}) value of potassium cyanide to *Clarias gariepinus* exposed to different concentration was evaluated with consideration on the ecological importance of this specie and problems related to pollution of the aquatic environment. The mortality rate was determined at the end of 24, 48, 72 and 96 h and dead fish removed as and when observed. After 96 h from the exposure time, the percentage of fish mortality was calculated in each aquarium was according to Finney Probit Analysis (1971) and the data was evaluated using Dragstedt-Behrens equation (Carpenter, 1975). The experiment was repeated twice and LC_{50} value determined. After each exposure period of time (24h, 48h, 36h, 72h and 96h), behavioural changes were also critically observed and noted.

2.4. Determination of adenosine triphosphate (ATPase)

To study the effect of cyanide on the enzyme activity of ATPase within the liver, muscle, gills and intestinal tissues, 40 healthy fish were stocked in each aquarium (10 fish/aquarium) and subjected to $1/3 LC_{50}$ of cyanide ($120.33\mu\text{g/L}$) for a time duration of 24h, 7D, 14D, 21D, 28D and 35D (where D means day's). Likewise, 10 fish were also stocked in an aquarium containing only 20L of aerated dechlorinated tap water each for the same time period to act as control. The fish were sacrificed to collect muscle, liver, gill and intestinal tissues. Total ATPase activity was measured as the rate of release of inorganic phosphate (Samson and Quin, 1967). Each tissues was crushed with a mortar and pestle and extraction was prepared with pre cold 0.35 M sucrose buffer pH 7.5 (1:10 W/V) and then centrifuge. Each assay was formed from 25 μl of the tissue sample

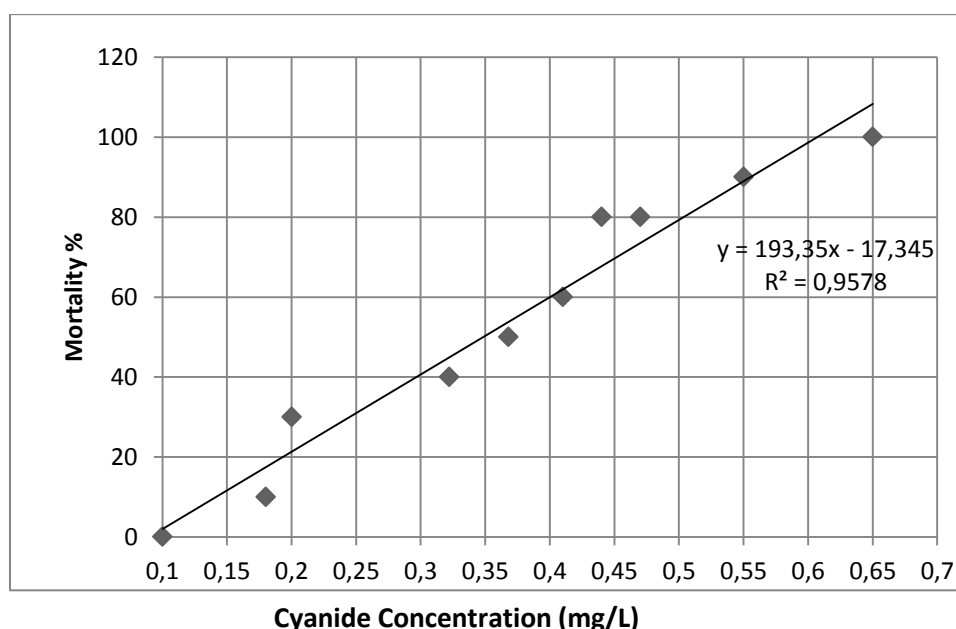


Fig. 1. Determination of LC_{50} of potassium cyanide in Cat Fish (*Clarias gariepinus*) homogenate and the supernatant was used to determine the enzyme activity in treated cyanide fish tissues by determination of inorganic phosphate in the supernatant which is liberated during the hydrolysis of the substrate adenosine triphosphate at 37°C. The method of Lowry and Lopez (1946) was used for inorganic phosphate assay.

The detailed ATPase activities were recorded and subjected to one way analysis of variance (ANOVA) and one Duncan's significant difference test was used for mean separation. Significance level was set at $p < 0.05$ and $p < 0.01$.

3. RESULTS

Toxicity Studies: Mortality was not observed in the control experiment; however, mortality increased with the increase in the concentration of the toxicant (KCN) and the duration of exposure. The concentration at which there was zero percent mortality was 100 µg/L and hundred percent mortality was 650 µg/L (Table 1). LC_{50} value of cyanide was found to be 361µg/L. Fig. 1 shows the percentage of fish mortality that were exposed to selected concentration of cyanide in aquatic environment following the linear equation ($193.35x-17.345$) with $R^2=0.9578$.

Behavioural Manifestation: The behaviour and condition of fishes in both control and test experiment was observed at 12h at first and 24h intermittently afterward up to 96 h (Table 1, 2). There were marked behavioural changes when the test samples were exposed to potassium cyanide. Erratic swimming activity, increased surface activity, loss in equilibrium, hyperactivity, rapid swimming and opercula movement were some observed behavioural changes. In lower concentration of potassium cyanide (100µg/L), the schooling behaviour of the fish was slowly disrupted with the fish showing rapid swimming than control. The fish died eventually at acute toxicity and 72 h-96 h exposure to the toxicant with opercula wide opened.

At longer duration of exposure to sub lethal dose of toxicant (35 days at $1/3LC_{50}$), it was also observed that fish exposed to cyanide showed also showed stress response which includes; gulp for air, cannibalism, swim in circle with their snout out, disorientation and increase water flow over their gills. Stunted growths were also observed. Increased in feed consumption compared to the control was observed. The increased feed intake had been reported to be an adaptation to compensate for the shortfalls in ATP production as a result of aerobic metabolism by cyanide (Okolie and Osagie, 1999). Also, Aslihan (2002) attributed membrane potential, intracellular volume and nutrient uptake to be regulated by proper function of the sodium pump. This invariably implies that disruption of the enzyme activity ($Na^+ - K^+ ATPase$) responsible for the proper function of sodium pump will have effect on the nutrition of the fish which might be a reason for this increase feed intake.

Table 1: The mortality rate of the Fresh water fish (*Clarias gariepinus*) in 96-h at different potassium cyanide concentrations and Probit values

Concentration (mg/L)	Log Conc.(µg/L)	No. of Fish exposed	No. of dead Fish	Mortality %	Probit Mortality
0.100	2.000	10	0	0	0.52
0.180	2.256	10	1	10	1.22
0.200	2.301	10	3	30	1.45
0.322	2.508	10	4	40	4.21
0.368	2.566	10	5	50	5.55
0.410	2.613	10	6	60	6.71
0.440	2.643	10	8	80	7.43
0.470	2.672	10	8	80	8.04
0.550	2.740	10	9	90	9.13
0.650	2.813	10	10	100	9.71

Table 2: Impact of Potassium cyanide (361µg/L) on the behavioural patterns of *Clarias gariepinus* at different time duration

Parameters	Potassium cyanide (361µg/L)				
	Control	24h	48h	72h	96h
Hyperactivity	-	-	+	++	+++
Rate of opercula activity	-	+	++	++	++
Loss of balance	-	-	-	+	++++
Rate of swimming	-	-	+	++	++++
Surface activity	-	++	+	+	++
Convolutions	-	-	-	-	+++

Increase or decrease in the level of behavioural parameters is shown by numbers of (+) sign while (-) indicates normal behavioural conditions.

ATPase Enzyme Activity: Figure 1 shows the pattern of changes in $\text{Na}^+ - \text{K}^+$ ATPase enzyme activity in the gills, liver, muscle and intestine of *Clarias gariepinus* exposed to potassium cyanide solution. Enzyme activity decreased significantly in cyanide exposed tissues compared to the control

Table 3. The mean activities of $\text{Na}^+/\text{K}^+ - \text{ATPase}$ ($\mu \text{mol Pi liberated/mg tissue}$) and % inhibition in different tissues of *Clarias gariepinus* in different times

	Exposure periods in days						
	1/3 LC_{50} (120.33µg/L)						
	Control	24 hrs.	7 days	14 days	21 days	28 days	35 days
Gills	16.56	18.74	15.24	13.74	11.34	10.34	6.32
% Inhibition		13.16	-7.97	-17.03	-31.52	-37.56	-61.84
SD	0.50	0.05	0.10	0.25	0.45	0.11	0.24
Liver	12.65	15.33	12.11	11.55	9.89	9.20	6.08
% Inhibition		21.19	-4.27	-8.71	-21.81	-27.27	-51.94
SD	0.55	0.10	0.21	0.10	0.43	0.01	0.90
Muscle	13.43	15.83	13.02	12.75	10.72	10.06	8.65
% Inhibition		17.87	-3.05	-5.06	-20.18	-25.09	-35.59
SD	1.12	0.88	0.55	0.10	0.21	0.78	0.40
Intestine	10.09	13.56	9.98	9.64	8.41	7.87	7.35
% Inhibition		34.39	-1.10	-1.15	-16.65	-22.00	-27.16
SD	0.11	0.21	0.10	0.10	0.19	0.20	0.22

4. DISCUSSION

The study reveals that potassium cyanide was toxic to *C. gariepinus* at sub-lethal concentration (LC_{50} : 120.3µg/L) and highly toxic at lethal concentration (650 µg/L). Within the aquaria, the experimental results showed that when fish was subjected to varying concentration of cyanide (100µg/L-650 µg/L) for a period of 96h, the fish revealed different behavioural mode. While the swimming pattern of fishes in the control group remains normal, fishes in the aquaria exposed to cyanide became hyperactive and restless at sub lethal concentration. At acute concentration, fishes were seen moving sluggishly in an erratic rhythm, gulping for air, losses equilibrium and convulsing prior to death. The fish very often come to the surface to gulp for air as a result of the toxic environment which could be attributed to decreased respiratory surface area a drop in the metabolic rate of the fish as observed by Shwetha and Hosetti (2009). Prashanth *et al.* (2011)

reported the effect of sodium cyanide on the behavioural pattern of the freshwater fish, *Labeo rohita*, to be increased opercula movement, increased surface behaviour, loss of equilibrium, change in body colour, increased secretion of mucus, irregular swimming activity, rapid jerk movement, partial jerk and aggressiveness. Also, studies by Shwetha and Hosetti (2009) of the acute effects of zinc cyanide on the behaviour and oxygen consumption of the Indian major carp *Cirrhinus mrigala* reveals similar pattern similar to observation made in this study. Such effects may be due to osmotic stress, which affects the nervous system of the animal (Palanichamy *et al.*, 1985). Hindrance in the functioning of the enzyme AChE in relation to nervous system explains probable reason for hyperexcitability and aggressiveness of the fish at the earlier stage of exposure to lethal dose of the toxicant, an observation which agrees with previous report of Deva Prakasa (2000) and Prashanth (2003). There is accumulation of acetylcholine which might prolong excitatory post synaptic potential, leading to stimulation and a block in the cholinergic system (Prashanth *et al.*, 2011).

Enzymatic manifestation of the sodium pump is the Na^+ , K^+ -ATPase (Aslihan Aydemir, 2002). This membrane enzyme plays unique role in cellular function since it helps in preserving the ionic gradients, active transport of Na^+ and K^+ across cell membranes across cell membrane and thus the membrane potential and osmotic equilibrium of cell. Na^+ - K^+ -ATPase are cell membrane enzyme involve in the. Cyanide was found to have effect on ATPase enzyme activity. In the present study, Na^+ - K^+ -ATPase activity in tissue decreased significantly in liver, muscle, gills and intestine of sub lethal ($1/3 \text{ LC}_{50}$) exposure and this decrease was duration-dependent. Maximum inhibition was observed in gill (-37.56% and -61.84%), followed by liver (-27.27% and -51.94%), Muscle (-25.09% and 35.59%) and intestine (-22.00% and -27.16%) on day 28 and day 35. The effect of the toxicant was more pronounced on day 28 - 35. The inhibition observed in the exposed fish tissues might be as a result of disruptions of the Na^+ - K^+ pump, which results in erratic entry of Na^+ into cell along the concentration gradient and the water molecules along the osmotic gradient which might lead to the swelling the cell membrane and rupturing. Begum (2011) coincidentally revealed that the inhibition of this enzyme by cyanide exposure leads to build up high ion concentrations in the extracellular spaces which results in the obstruction of the movement of internal destructive extra ions towards the external medium via the leakage junctions. The reduction of Na^+/K^+ -ATPase may have a metabolic or ionic regulation when the fish is subjected to toxic exposure (Shwetha and Hosetti, 2012). Also, it can be said that decrease in the mean values of Na^+/K^+ -ATPase within the gill filaments, liver, muscles and intestine of fish are related to the metabolic activity of fish when it is subjected to sub-lethal concentration of sodium cyanide for a period of time. Further possible explanations for this observation might be due to inhibition of ATPase enzyme by the cyanide molecules, damage to cell membrane of the cyanide exposed fish which might have resulted in decrease ATP content in the respective tissues as a result of ATP leakages into the blood stream and pathological changes in tissues.

5. CONCLUSION

The result of this research work shows that potassium cyanide is toxic to the freshwater fish *Clarias gariepinus* even at both acute toxicity and sub lethal toxicity. The findings also indicate that cyanide exposure alters ATPase enzyme activity which was linked to some negative health response of the fish. Proper treatment, detoxification and disposal of cyanide containing wastes are essential for the sustenance of a clean and healthy environment. Hence, dysfunction in ATPase activity and behaviour can serve as a biomarker and index of potassium cyanide toxicity.

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