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Effects of Some Heavy Metal Pollutants on Vitamin C and E Production In *Clarias gariepinus* (Burchell, 1822) In *In Situ* Bio-Assay In River Galma, Kaduna State, Nigeria

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Abstract
This research focuses on the effects of some heavy metals on production of Vitamin E and C in *Clarias gariepinus* in an *in situ* bioassay in River Galma.120 samples (40 per exposure) of 20-45g size range of juveniles were exposed to the river environment for fourteen days in a cage system at five different locations along the river course for three different periods of the year. Vitamins E and C were assayed for after the 14th day of exposure. Heavy metal (Pb, Cr, Cd, Zn and Mn) contents were also tested for in digested pooled samples of water, livers and gills of the fish.
The results indicate significant difference in the levels of Vitamin E and C production in the gill in all the months of exposure at $P<0.05$ level of significance but not in the liver. There were significant differences in the Chromium concentration in the gills and livers of the fish in all the exposures. Also, there was significant difference amongst the fish organs, water samples and Lead (Pb) concentrations. There were correlations amongst Vitamin E and C and the heavy metals. There was strong correlation between Vitamin E levels and Lead concentrations in all the exposure periods. This research also established the presence of heavy metals examined in varying degrees of concentrations in water samples and fish organs.
Vitamin E and C can be used as biomarkers of pollution in River Galma to give early warning on environmental pollution to the community and policy makers.
Keywords: Oxidative stress; Vitamin E and C; Reactive Oxygen Species; Heavy metals; Biomarkers

1. Introduction

The presence of toxic metals in environmental matrices is one of the major concerns of pollution control and environmental agencies in most parts of the world (Tay *et al*., 2009). Fish accumulate toxic chemicals such as heavy metals directly from water and diet, and contaminant residues may ultimately reach concentrations hundreds or thousands of times above those measured in the water, sediments and food (Labonne *et al*., 2001; Goodwin *et al*., 2003; Osman *et al*., 2007).

Exposure and ingestion of heavy metals can cause a myriad of physiological and neurological problems in both plants and animals and, ultimately deleterious effects in man and other higher consumers. For instance, exposure to lead has been associated with reduced IQ, learning dissabilities, slow growth, hyper-activity, anti-social behaviours and impaired hearing (Dahiya *et al*., 2005).

Among various causes of fresh water and riverine pollution, heavy metals are of considerable importance and consideration (Sthanadar *et al*., 2013). Edward *et al*. (2013) observed that though, heavy metals were below detectable level in the water samples, the levels of bioacumulation in fish part examined were beyond tolerable levels (WHO and FEPA recommendations) making the fishes unfit for human consumption.

"Heavy metals" are named after those metals between atomic number 21 (scandium) and atomic number 84 (polonium), except for aluminum, which has atomic number 13, but it is also considered a heavy metal (Schnoor, 1996). Heavy metals are thus commonly defined as those having a specific density of more than 5 g/cm^3. Heavy metals in the solid and liquid states are characterized by good heat and electrical conductivity, and are glossy and opaque. They have high melting and boiling points. They are malleable with usually monoatomic pairs and these include copper, cobalt, chromium, cadmium, iron, zinc, lead, tin, mercury, manganese, nickel, molybedmium, vanadium. Metalloids are antimony, arsenic, astatine, boron, germanium, silicon, tellurium and selenium. Heavy metals of note in environmental science literature include lead, mercury, cadmium, chromium, copper, manganese, nickel, zinc and silver.

Fish, on the other hand, are good organisms for the accumulation of heavy metals, and especially mercury (Dobrowolski and Skowronska, 2001). The process of bio-magnification, which involves the increase of metals concentration in organisms on the upper level of the trophic chain, is most visible in the aquatic environment (Dobrowolski and Skowronska, 2001). Heavy metals including both essential and non-essential elements have a particular significance in ecotoxicology, since they are highly persistent and all have the potential to be toxic to living organisms (Storelli *et al*., 2005). For the normal metabolism of the fish, the essential metals must be taken up from water, food or sediments (Canli and Atli, 2003).

Heavy metal contaminated food can seriously deplete some essential nutrients in the body that are further responsible for decreasing immunological defenses, intra-uterine growth retardation, impaired psycho-social faculties, disabilities associated with malnutrition and high prevalence of upper gastrointestinal cancer rates (Iyenger and Nair, 2000; Turkdogan *et al*., 2003; Arora *et al*., 2008). Heavy metal contamination may have devastating effects on the ecological balance of the recipient environment and a diversity of aquatic organisms (Ashraj, 2005; Vosyliene and Jankaite, 2006; Farombi *et al*., 2007). Various harmful effects including abnormal development of fetus, procreation failure, and immuno-deficiency has exhibited due to aquatic metal exposure. Also, the consumption of heavy metal contaminated food can seriously deplete some essential nutrients in the body that are further responsible for decreasing immunological defenses, intra-uterine growth retardation, disabilities associated with malnutrition and high prevalence of upper gastrointestinal cancer rates (Khan *et al*., 2008).

The most anthropogenic sources of metals are industrial, petroleum contamination and sewage disposal (Santos *et al*., 2005). Fresh water ecosystems are usually polluted from non-point pollutants. Examples of non-point source pollution include agricultural runoff (pesticides, pathogens, and fertilizers), storm-water and urban runoff, and atmospheric deposition _wet and dry deposition of persistent organic pollutants such as polychlorinated biphenyls (PCBs) and mercury (Ritter *et al*., 2002). The ultimate sink for many of these contaminants is the aquatic environment, either due to direct discharges or to hydrologic and atmospheric processes (Stegeman and Hahn, 1994). Also, heavy metals entering the aquatic ecosystem originate from different sources such as decay of plants and vegetation, atmospheric particulates, discharge of domestic and municipal wastes, etc (Abo *et al*., 2005; Fatima, 2008).

Heavy metals can also be gotten from preserved food materials which can eventually bioaccumulate in the body of organisms. For instance, Ckukwujindu *et al*. (2009) observed significant differences in the heavy metal levels in the different brands of canned sardines except for copper and chromium when they worked on characteristic levels of heavy metals in canned sardines consumed in Nigeria. Likewise, Korfali *et al*. (2013) demonstrated the presence of iron, zinc, manganese, selenium, molybdenum, copper, calcium, cadmium, magnesium and lead in dietary supplements imported into Lebanon.

Heavy metals can be bioaccumulated and biomagnified via the food chain and finally assimilated by human consumers resulting in health risks (Agah *et al*., 2009). Due to these, in the last 50 years, environmental conditions have changed at an unprecedented rate, impacting heavily on ecological processes (Diffenbaugh and Field, 2013).

Among the various toxic pollutants, heavy metals are particularly severe in their action due to persistent biological amplification through the food chain (Shah and Altindau, 2005). Transport of metals in fish occurs through the blood where the ions are usually bound to proteins. The metals are brought into contact with the organs and tissues of the fish and consequently accumulated to a different extent in different organs or tissues of the fish. Trace metals like copper, zinc and iron are readily concentrated in different fish tissues (Adewoye *et al*., 2005). Organo-phosphorus insecticides are widely used and harmful to non target organisms due to run off into the surrounding aquatic environment altering the normal activities of the ecosystem.

The eight most common pollutant heavy metals listed by the Environment Protection Agency (EPA) are: As, Cd, Cr, Cu, Hg, Ni, Pb, and Zn (Athar and Vohola, 2001). Zinc deficiency causes anaemia and retardation of growth and development. Its deficiency also results in poor immunity since it is known to have a role in the immune system. Even Cr (VI) is taken up in amount of 150 µg/day that are required for collagen, but it leads to irritation of the stomach, leading to ulcers and kidney and liver damage at higher concentration (Dayan and Paine, 2001). In humans, higher concentration of lead and mercury is associated with autoimmune disease such as rheumatoid arthritis as well as interfere with the proper functioning of the kidney and circulatory system that leads to injury of central nervous system (Patrick, 2006). Effects of cadmium on aquatic organisms are analogous to those in humans, and include skeletal deformities and impaired functioning of kidneys in fish. Skeletal deformities in fish can result in an impaired ability of the fish to find food and avoid predators; hence, this sub lethal effect becomes a lethal effect (Landis and Yu, 2003). Hexavalent chromium is mobile in the environment and is acutely toxic, mutagenic, teratogenic and carcinogenic to aquatic organisms (Palmer and Puls, 1994)

Fish are at high trophic level of the food web and may accumulate large amounts of some metals from the water and often in concentrations several times higher than in the ambient water. Fish come into contact with multiple contaminants that are dissolved in the water or incorporated in the food chain, and so fish are not only prone to endure negative toxicant-related health effects but also to bioaccumulate pollutants; fish may therefore, be used as bioindicators of environmental contamination (Whitefield and Elliot, 2002). Since fish often respond to toxicants in a similar way to higher vertebrates, they can be used to screen for chemicals that are potentially teratogenic and carcinogenic in humans (El-Shehawi *et al*., 2013).

Metals are well known inducers of oxidative stress, and assessment of oxidative damage and antioxidant defenses in fish can reflect metal contamination of the aquatic environment (Livingstone, 2003). Fish

tissues are endowed with an antioxidant defense system to protect them from oxidative stress caused by metals (Atli *et al*., 2006). Rajagopalan *et al*. (2010) demonstrated how constant production of free radical resulted in increased exploitation of the antioxidants leading to their depletion in which the levels of non-enzymatic antioxidants such as reduced glutathione, vitamins C and E were significantly reduced in the alcohol and PUFA (poly unsaturated fatty acid) treated livers of rats because of their complete utilization due to the oxidative stress.

Environmental stress as well as variety of physical conditions may lead to the production of certain protein in fish. Some of these proteins are capable of protecting the cells against damages that may result from such environmental perturbations; while others are involved in the regulation of various genes. Cellular antioxidant defense systems in biological systems are impaired when exposed to environmental pollutants, but the levels of antioxidants in living organisms can increase in order to restore the imbalance caused by oxidative damage. There is substantial evidence that environmental pollution increases oxidative stress (Olivia *et al*., 2012). Also, environmental stress has been demonstrated to cause an increase in the oxidative stress, an imbalance in the antioxidant status (Yildirim *et al*., 2010). A number of pollutants including heavy metals have been linked with the presence of free radicals which may induce oxidative stress in biological systems (Osuala, 2012).

A disturbance in the balance between the pro-oxidants and antioxidants leading to detrimental biochemical and physiological effects is known as oxidative stress. Oxidative and nitrative stress results from increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) mediated by pollutants (Scoullos *et al*., 2007). For example, a recent meta-analysis showed that oxidative stress increased with an increase in the duration of physiological stress, while acute exposure mostly resulted in up-regulation of the antioxidant response (Costantini *et al*., 2011a). Also, Oxidative stress is the result of an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses in living organisms (Nishida, 2011). Non-enzymatic antioxidants are represented by ROS (Reactive Oxygen Species) scavengers (both hydrophilic such as low-molecular mass thiols, glutathione (GSH), metallothioneins (MTs), ascorbic and uric acids, as well as lipophilic ones such as vitamin E and carotenoids (Viarengo *et al*., 2007). Reactive oxygen species are induced by substances such as transitional metal ions, pesticides, and petroleum pollutants (Slaninova *et al*., 2009; Lushchak, 2011). Similarly, Nilantika and Sengupta (2013) demonstrated how sub-lethal concentration of lead acetate has the capacity to bio-accumulate, thereby altering the normal functional activities of freshwater fish *C. punctata*. Furthermore, the association of oxidative stress including variation in its anti-oxidant profile suggests that the defense system of *C. punctata* is significantly compromised upon metal exposure at low concentrations.

The body fights the stress caused by excessive cadmium content by increasing the production of antioxidants such as glutathione, metallothionein, flavonoids and other chemical compounds (Moniuszko-Jakoniuk *et al*., 2005; Michalak, 2006; Jurczuk *et al*., 2006). Vitamins C and E also have antioxidant properties (Jurczuk *et al*., 2005). Tocopherol is a useful indicator of exposure to metals and organic contaminants that generate oxidative stress (Palace *et al*., 2005).

Non enzymatic antioxidants- These are biological molecules that can act as antioxidants by either quenching a free radical directly or indirectly by promoting a process responsible for radical scavenging indirectly (Irshad and Chaudhary, 2002). Some of these non-enzymatic antioxidants include metallothionein, glutathione, uric acid, ascorbic acids, tocopherol or vitamin E, carotenoids or vitamin A, etc. Antioxidants such as ascorbic acid, α-tocopherol (vitamin E), endogenous glutathione peroxidase and pineal hormone melatonin, have all been shown to be effective in defending the system against free radical mediated tissue injuries. For instance, Elevated level of TBARS (33.33%), Ascorbate (25%) and Glutathione (55%) and activity of SOD (87%) and CAT (54%) and APX (19%) as compared to control indicate that pollutants present in the Yamuna water like heavy metals could elevate the oxidative stress which in turn further elevates the activities of non-enzymatic and enzymatic antioxidants to overcome the oxidative stress induced by ROS (Elahi *et al*., 2011).

Ascorbate (vitamin C) is an important water-soluble antioxidant, and may additionally serve as a cofactor for enzymes involved in collagen biosynthesis or neuro-transmitter conversions (Stegeman *et al*., 1992; Lopez-Torres *et al*., 1993). Studies addressing the feasibility of ascorbate as a biomarker are very scarce. The utility of ascorbate as a biomarker is limited to plants and animals that can synthesize it.

Tocopherol is a useful indicator of exposure to metals and organic contaminants that generate oxidative stress (Palace *et al*., 2005). Antioxidants such as metallothionein, selenium, α- tocopherol, reduced glutathione (GSH), ascorbic acid and carotenoids share a common property that manifest protective influences by scavenging free radicals. Layachi and Kechrid (2012) demonstrated how oral exposure to Cd caused reduction in LPO and antioxidant enzyme activities in rat's liver, and vitamin C or vitamin E may have partial ameliorative effects on these disturbances, whereas vitamin C and vitamin E together assured a more efficient protection of the organ against the noticed oxidative stress. Thakur and Kanshere (2014) demonstrated how Vitamin C and Vitamin E supplementation played a positive role in detoxification of mercury toxicity specially the low dose. Ognjanovic *et al*. (2008) observed higher concentrations of LPO, Vitamin C and Vitamin E in the liver in

comparison with white muscle and the concentration of LPO in both tissues was higher, while that of Vitamin E was lower at Valdanos compared to Platamuni because oxidative stress are partly due to differences in temperature and the concentrations of nitrites, nitrates, and detergents in the waters of Valdanos compared to Platamuni. Ascorbic acid is well known for its antioxidant activity, acting as a reducing agent to reverse oxidation in liquids (McGregor and Biesalski, 2006). Vitamin E is a strong inhibitor of apoptosis and a stabilizer of biological membranes (Gago-Dominquez and Castelao, 2006). These vitamins have synergistic actions against damaging effects of reactive oxygen species. For instance, Vaziri *et al*. (2000) demonstrated how administration of Vitamin E and C ameliorated hypertension, improved urinary nitrate-plus-nitrite excretion, and mitigate nitrotyrosine accumulation in rats. Likewise, Sunmi and Soo (2002) demonstrated how α-tocopherol supplementation was beneficial in decreasing blood lipid peroxide concentrations without altering antioxidant enzyme activities in Korean patients with type 2 diabetes treated with CSII (continuous subcutaneous insulin infusion).

Vitamin E comprises eight naturally occurring fat-soluble vitamins of which the most predominant, essential and with the highest biological activity is α-tocopherol (Chen and Tappel, 1995). Vitamin E is a major antioxidant in biological systems acting as a powerful chain-breaking agent through the scavenging of peroxyl radicals (Beyer, 1994). Vitamin E terminates the chain reaction of lipid peroxidation in membranes and lipoproteins. Vitamin E also attenuates lipid peroxidation and protein oxidation, and increases antioxidant defense mechanism in diabetic rat treated samples (Shirpoor *et al*., 2007).

Biomarkers are measurements in body fluids, cells or tissues indicating biochemical or cellular modifications due to the presence and magnitude of toxicant, or of host response (NRC, 1987). Peakall (1994) defined biomarkers as changes in biological response (ranging from molecular through cellular and physiological responses to behavioural changes) which can be related to exposure to, or toxic effects of environmental chemicals. Fish biomarkers are promising for ERA (Environmental Risk Assessment) as supplements to existing chemical measures (Vander Oost *et al*., 2003). In addition, systematic use of multiple biomarkers has been found as most useful in the assessment of pollutants' effects (Tsangaris *et al*., 2010). Despite the considerable understanding of their links with contaminant exposure, the use of biomarkers is often limited by their strong variability due to natural biological and environmental cycle (Nahrgang et al., 2010a).

Although many research have been conducted to assess the toxicity of heavy metals in algae, however, the number of studies dealing with the toxic effect of heavy metal on aquatic animals including fish are limited (Harmon *et al*., 2005). Similarly, unlike with molluscs (Viarengo *et al*., 2007), for fish, the studies on the response of caged specimens are scarce. Thus, this investigation attempted to bridge the gap and bring to fore the biochemical responses of fish to heavy metal loads in River Galma. The River Galma is currently loaded with run-offs from agricultural and municipal activities of the surrounding communities along its course at various adjoining tributaries (most of which are seasonal); and contain myriads of toxic pollutants (heavy metals inclusive). There may also be contribution of toxic pollutants from the few industrial activities in Chikaji and Dakace areas. Viarengo *et al*. (2007) suggested that bio-monitoring programmes should use caging studies to obtain highly sensitive early warning signs of the effects of exposure to environmental pollution. Caging allows the exposure of individual fish to conditions at a certain site, for known time (Almroth, 2008). Caging studies often utilize farmed fish with known age and nutritional background, though wild captured fish can also be used. Caged forage fish can provide results useful for guiding and defining priorities for more thorough effects-driven assessments (Munkittrick and McMaster, 2000; Munkittrick *et al*., 2000) for determining cumulative impacts of multiple contaminant stressors.

Also, Yousafzai *et al*. (2010) showed that omnivorous fish may bioaccumulate more heavy metals than carnivorous fish in natural habitats. In this regard, the test sample, *Clarias gariepinus* is an omnivorous species. River Galma receives variable levels of pollution from different sources of anthropogenic activities along its banks (Butu and Bichi, 2013). Thus, the use of *Clarias* in *in-situ* investigation on the possible effects of xenobiotics in River Galma would possibly provide early warning to the users of the river, farmers and policy makers.

The knowledge of the levels of heavy elements in our environment is necessary for the purposes of setting background values of these elements, monitoring their accumulation in the biota regularly and estimating the amount of the metals that may possibly get trans-located across the compartments in the entire ecosystem (Oyekunle *et al*., 2012). In this regard, fish are largely being used for the assessment of the quality of aquatic environment and as such can serve as bio-indicators of environmental pollution (Dautremepuits *et al*., 2004). In this investigation, heavy metals such as lead, cadmium, zinc, chromium and manganese were tested for in water and fish organs such as liver and gill so as to determine their concentrations in setting background values of these elements.

Falfushynska *et al*. (2011); Hansen *et al*. (2006a; 2006b) used 14 days in their separate research. Thus, the current investigation also used 14 days to determine the effects of pollutants on Vitamin E and C production in *Clarias gariepinus* in River Galma.

Common practices of fish farming, such as capture, confinement, transportation and water quality, varying temperature and climatic conditions, etc are stressful to fish and can lead to increase in the incidence of diseases, immunological imbalances, mortality and impairment of growth and survival. For instance, Adeyemo *et al.* (2009) demonstrated significant difference in the values of neutrophil and lymphocytes of the stressed fish relative to the baseline data obtained from the control.

2. Materials and Methods

2.1 *Description of Study Area*

Galma River is one of the main tributaries of River Kaduna. It has its headwaters near the north western edge of the Jos Plateau and falls near the Magami village into Kaduna plains. The main tributaries of Galma River are Shika River in the middle course and the Rivers Kinkiba and Likarbu in its lower course. The Galma reservoir which is popularly called Zaria dam was constructed across the Galma River in 1975. The major land use in the catchment areas is farming and animal rearing. There are also some industrial and municipal activities (in the surrounding towns and villages such as Chikaji, Dakace and Sabon Gari areas) that produce myriads of wastes that ultimately get to the river either in the short- or long-run through run-offs and seepages. The few industries are located in Chikaji and Dakace. The main tributaries of the river in the sampling areas are all located in the Sabon Gari Local Government Area of Kaduna State (Fig. 1).

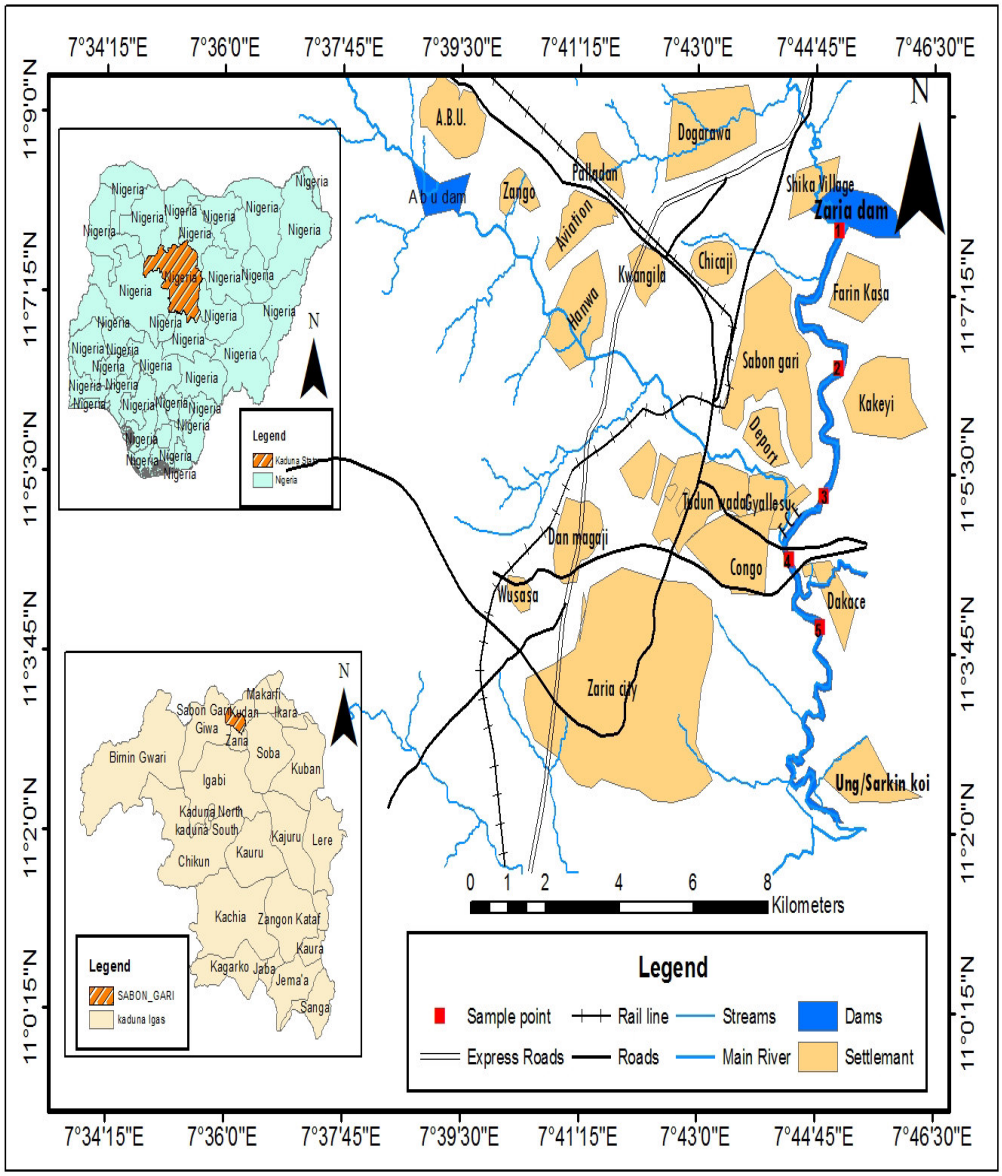


Fig 1 Map of Zaria and its Environment Showing Study Areas.
Source: Satellite Image (2013).

2.2 *Cage Construction*

Five cages were constructed manually using PVC (polyvinyl chloride) pipes of various sizes. One inch pipes were used to form the upper and lower frames of the cage. These were measured and cut to size (50cm). PVC of half inch were used as the side and basal bars. Each bar was 50cm long. Holes were made in each frame with the aid of red-hot iron rod wide enough to accommodate the bar conviniently. Holes were also made along the central and side axes of the two lower frames to accommodate the vertical and horizontal bars while the other PVC frames were punctured only on the central axis. A minimum of sixteen holes at regular interval per frame were created.

The PVC frames were connected with the aid of L-shaped elbow joints. For proper and firm fitting of the elbow joints, an adhesive was applied at each joint ready to be fitted into the frame. Shredded foams were placed in the PVC frame to ensure lightness and floatation. The adhesive was also applied on the bars for proper fitting into the holes. The length of each frame was 50cm in-to-in. Holes were also created at the joints to accommodate more bars to fill up the spaces. A lid was constructed at the upper end of the cage. An inch PVC pipes were used with the four sides connected to the frame at one side via T-shaped PVC connector joint after two holes were made at the side. A net of 2cm mesh size was attached to the inside of the cage with the aid of an adhesive and the free ends of the nets were tack on the side bars through the use of fish twine thread and needle. An iron mesh was used to cover the inner layer of the cage to avoid attack on the content of the cage from predators such as crabs.

Floaters in form of foams were attached to the cage on the outside through the use of thread at either sides of the cage. Another foam was attached on the lid to increase buoyancy and floatation capacity of the cage. And another PVC of two inch was used to cover the four edges of the upper frame of the cage in order to hold the nets firmly at the upper base.

2.3 *Acclimatization of Clarias gariepinus*

A total of one-hundred and twenty samples of *Clarias gariepinus* of 20-45g size range (in all the three *insitu* exposure that is, 40 per exposure) were purchased from the commercial fish farmer (Bagauda, Kano) and kept in concrete tank for a period of two weeks in which they were fed with Cupens, 2mm size feed morning and afternoon five days a week in each of the exposure period. The holding facility was aerated with Blagdon aerator (KOI Air, KA25). The same number and size range of fish were acclimatized as the replication samples during the wet and dry seasons when the first set of samples have been taken to the river. This helped the fish to be relieved of stress as a result of transportation and change of environment; as well as to shed off toxicity of its former environment.

2.4 *Sample Location and Collection*

Five (5) sites were selected along the river. The first site was located at Shika Reservoir (Zaria Dam). Shika reservoir was used as reference site because it served as the upper course of the river and relatively located far from the industrial areas and the municipal waste load was also relatively low compared with other sites. Other sites were located around Kakeyi village (which receives municipal wastes and effluents from the neighbouring villages and town such as Sabon Gari), two sites around FCE, Congo (FCEI and FCEII at about 500 metres apart and FCEI receives municipal wastes and effluents, agricultural run-offs from some parts of Sabon Gari, Chikaji, Kakeyi and Farin Kasa villages. FCEII site receives municipal wastes and effluents, agricultural run-offs from various parts of the town including Tudun Wada, PZ, Congo via the Kubani stream which adjoins the main stream at this point. Waters from Kakeyi along the stream also joins here); Dakace site at about 1000 metres apart from FCEII and receives municipal and agricultural wastes and effluents from Dakace village and the few industries located in the area (Fig.I). Farming and other agricultural activities such as animal rearing and the use of generator-powered irrigation especially during the dry season take place in both wet and dry seasons. The choice of these sites were made base on security reasons and ease of accessibility.

Water samples from each site were collected in 2L plastic containers from each site for physico-chemical parameters, nutrients and heavy metals (Cadmium, lead, zinc, chromium and manganese) analyses. Samples meant for Dissolved Oxygen were collected in 300ml BOD bottles and treated with 2ml manganous sulphate at the point of collection. These parameters were measured to ensure that the fish were in good condition during the period of exposure.

Forty samples of *Clarias gariepinus* (8 per site) were transported to the river in a special mesh using cage system for each sampling site and then suspended into the water in which the fish were fed once at dawn after 24 hours of exposure with little quantity of 2mm cupens feed for the remaining days of the fourteen (14) days. Three fish samples were harvested from each site after the first 7 days of exposure and then, 14 days after exposure and kept in a large plastic container and transported to the laboratory in each case. Each sample after the 14^{th} day was dissected and gills and livers removed and homogenized in 0.9% physiological saline solutions the same day and kept in the test tube meant for each organ from each fish and for each site and then refrigerated

prior to analyses for Vitamin C and E and heavy metals. For best results the samples were harvested from each sampling site the same day and in the early hours of the day. Prior to exposure, Vitamin E and C were tested for, serving as control from which comparison were made with the various sites.

The whole process (experiment) was replicated in October and in contrasting dry season (December) for comparison.

2.5 *Heavy metals Analyses*

The water samples from each site and the fish organs (gill and liver) from the seventh and fourteenth days of exposures in each period/month of exposure were digested separately and then, pooled. The samples were digested by adding 7.5 ml Nitric acid and 2.5m concentrated hydrochloric acid with 1ml of the water or homogenized fish sample in a 50ml test tube. This was then, subjected to heat in a fume chamber at 150ºC until near dryness. This was allowed to cool and then made up to 50ml with distilled water. The water samples from each site were pooled for a period of three months (August to October, 2014 and November, 2014 to January, 2015).While the gill and liver of fish from each site were pooled from the digested samples of the 7^{th} and 14^{th} day in each of the exposure period (September, October and December) respectively.

2.6 *Vitamins C and E Determination*

An isocratic liquid chromatographic method for the separation and simultaneous determination of ascorbic acid (vitamin C) and tocopherol (vitamin E) from homogenized samples of liver and gill of *Clarias gariepinus* from each site was as follow: Samples were analysed by means of a reverse-phase column (LiChrospher 100 RP-18), using methanol as mobile phase. The UV–Vis detector used was set at a wavelength of 300 nm and switch to 450 nm at 17 min. These vitamins were separated within 25 min and the detection limits ranged from 7 (β-carotene) to 65 ng ml^{-1} (ascorbic acid). Visible spectrophotometre (UV2550) was used for the analysis in NARICT (National Research Institute for Chemical Technology), Zaria, Kaduna State, Nigeria.

2.7 *Data Analysis*

Graphical representation of the various heavy metal concentrations were indicated using error bar charts. Same was also done for Vitamin E and C production levels in the various sites. Concentrations of the metals (mg/ml) were plotted against (vertical) sampling locations (horizontal).

One way Analysis of Variance (ANOVA) followed by Duncan Multiple Range test was used to determine the differences between the reference site samples and the samples from other sites at the different periods of exposure using a computer program of IBM SPSS Inc. (version 20.0 for Windows) at $P<0.05$ level of significance.

The relationship between heavy metals concentration and Vitamin C and E production levels were determined by carrying-out Correlation using Spearman's Correlation Coefficient.

3. Results

3.1 *Sample Collection and General Observations*

Generally, three field exposures were carried out with varying out-come. The first exposure in September, 2014 ended with four sites as there was case of escape of the fish into the wild due to activities of crabs that tore the net mesh of the cage at Dakace site. The second exposure in October, 2014 ended with three sites with the same reason in FCEII and Dakace sites. Similarly, the third exposure in December, 2014 ended with three sites with the same reasons in Dakace site and stagnant water and flushing of the embankments due to strong wind current in Shika site that led to the death of all the fish kept in the cage after six days of exposure. An attempt was made to avoid the previous challenges of the field exposure by replacing the net mesh with finer iron mesh. This only proved effective in three sites. There was also constant problem of theft from Dakace site because of its proximity to the public. The security at this site was unable to check- mate this.

During the harvesting of the fish from the various sites for the analyses in all the exposure periods, priorities were given to collection of samples for heavy metals, Vitamin E and C analyses. The extra samples in each site and in each exposure were taken as collateral in case something happen during the 14 days exposure.

3.2 *Vitamin E and C Production*

From the One Way Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) there was no significant difference in the production levels of Vitamin E and C amongst the sites (locations) and the fish organs. The level of Vitamin E production ranged from 4.93±2.98 to 47.44±39.35 (Shika Dam to FCEI) in the gill samples in all the exposure while the range was 6.93±3.50 to 23.56±14.45 (Shika Dam to FCEI) in the liver samples. Likewise, the level of Vitamin C production ranged from 0.24±0.12 to 3.02±2.74a (Shika Dam to FCEI) in the gill samples in all the exposure while the range was 0.21±0.13 to 1.18±0.95 (Shika Dam to FCEI) in the liver samples (Table1).

Table1 Vitamin E and C production in *Clarias gariepinus* in an *insitu* exposure in River Galma amongst the sites (mg/ml)

Location	Vitamin E		Vitamin C	
	Gill	Liver	Gill	Liver
Control	13.58±0.00a	13.68±0.00a	0.98±0.00a	1.39±0.00a
Shika Dam	4.93±2.98a	6.93±3.50a	0.24±0.12a	0.21±0.13a
Kakeyi	42.58±36.32a	17.27±11.45a	3.00±2.60a	1.18±0.95a
FCE I	47.44±39.35a	23.56±14.45a	3.02±2.74a	1.04±0.78a
FCE II	40.50±31.32a	14.18±7.53a	2.85±2.65a	0.77±0.57a
Dakace	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a
Total	**24.84±9.87**	**12.60±3.37**	**1.68±0.72**	**0.76±0.23a**
P value	**0.643ns**	**0.465ns**	**0.715ns**	**0.463ns**

There were significant differences in the levels of Vitamin E and C production in the gills in all the *insitu* exposure in all the months while there was no significant difference in the levels of Vitamin E and C in the liver of the exposed specimens in all the months. The range of Vitamin E production amongst the month is 7.30±2.38 (October) to 59.52±24.87 (December) in the gills. The range of Vitamin E production amongst the month is 5.91±2.20 (October) to 21.92±8.73 (December) in the liver. The result also indicate that the range of Vitamin C production amongst the month is 0.31±0.15 (October) to 4.30±1.79 (December) in the gill. The range of Vitamin C production amongst the month is 0.29±0.22b (October) to 1.49±0.53(December) in the liver. (Table 2. Fig. 2.)

Table 2 Vitamin E and C production in *Clarias gariepinus* in an *insitu* exposure in River Galma in the various months of exposure (mg/ml)

Months	Vitamin E		Vitamin C	
	Gill	Liver	Gill	Liver
September	7.70±2.99b	9.98±2.60a	0.43±0.13b	0.52±0.19ab
October	7.30±2.38b	5.91±2.20a	0.31±0.15b	0.29±0.22b
December	59.52±24.87a	21.92±8.73a	4.30±1.79a	1.49±0.53a
Total	**24.84±9.87**	**12.60±3.37**	**1.68±0.72**	**0.76±0.23**
P value	**0.034***	**0.128ns**	**0.024***	**0.063ns**

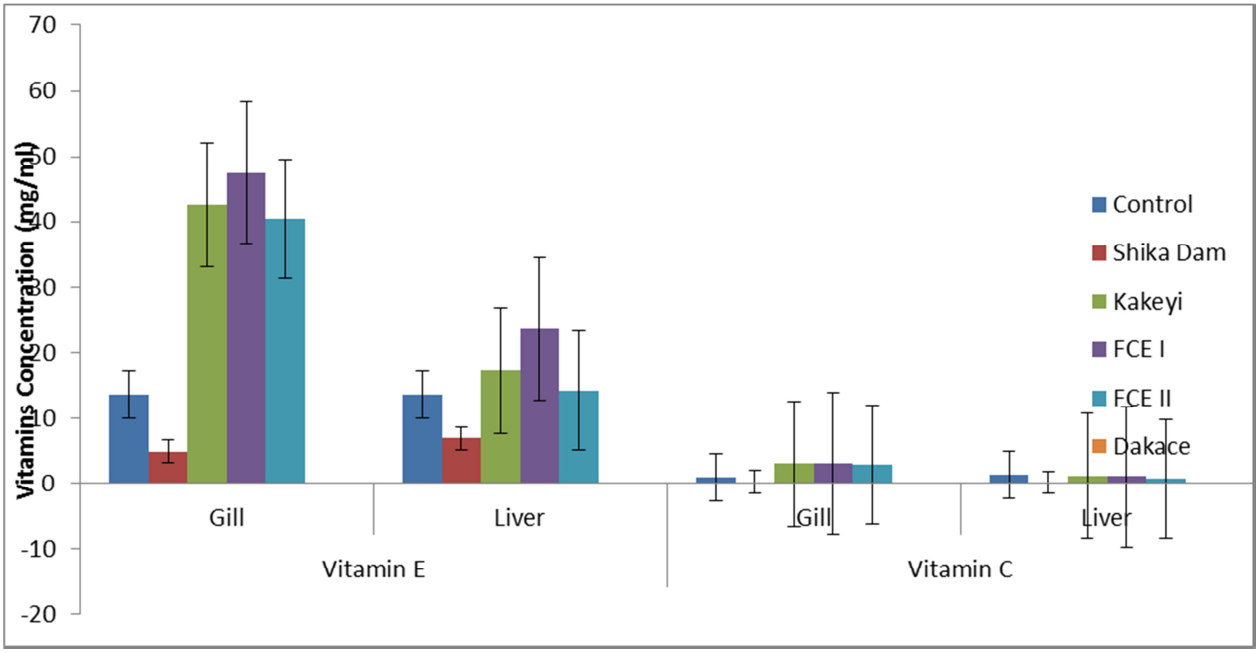


Fig 2 An Error bar indicating the various Vitamin concentrations in each site from all the 14 days *insitu* bio-assay of *Clarias gariepinus* in River Galma

3.4 *Heavy Metals*

From the ANOVA and DMRT of the results the comparison of heavy metals in the gill and liver samples and that of water samples indicate: that there was significant difference ($P < 0.05$) amongst the fish organs, water samples and Lead (Pb) concentrations. While there were no significant differences amongst the fish organs,

water samples and the remaining heavy metals (Cr, Cd, Zn and Mn). (Table 3).

Table 3 Heavy Metals Comparison in Gill and Liver samples of *Clarias gariepinus* from *insitu* bio-assay and Water Samples of River Galma (mg/ml)

Source	Pb	Cr	Cd	Zn	Mn
Gill	0.15±0.05b	0.45±0.10a	0±0.00a	0.15±0.04a	0.13±0.08a
Liver	0.13±0.05b	0.32±0.10a	0±0.00a	0.12±0.03a	0.13±0.08a
Water Sample	0.34±0.07a	0.47±0.11a	0±0.00a	0.16±0.06a	0.14±0.04a
Total	**0.19±0.03**	**0.41±0.06**	**0±0.00**	**0.14±0.02**	**0.13±0.04**
P value	**0.022***	**0.530ns**	**0.379ns**	**0.837ns**	**0.984ns**

There were no significant differences in the heavy metals amongst the sites (locations).

Pb concentration had its lowest in Kakeyi with 0.091±0.06 while the highest was 0.536±0.02 from Shika Dam.

Cr concentration ranged from 0.379±0.29 to 0.834±0.17 (Shika Dam).

Cd was not detected in any of the sites except in water sample from Kakeyi.

Zn concentration ranged from 0.095±0.05 (Shika Dam) to 0.339±0.30 (FCEII).

Mn concentration ranged from 0.119±0.09 (Kakeyi) to 0.178±0.15 (Shika Dam). (Table 4)

Table 4 Heavy metals Results for Pooled Water Samples from River Galma amongst the sites (mg/ml)

Location	Pb	Cr	Cd	Zn	Mn
Shika Dam	0.536±0.02a	0.834±0.17a	0.00±0.00a	0.095±0.05a	0.178±0.15a
Kakeyi	0.091±0.06a	0.379±0.29a	0.005±0.01a	0.11±0.07a	0.119±0.09a
FCE I	0.277±0.243a	0.379±0.29a	0.00±0.00a	0.11±0.09a	0.132±0.10a
FCE II	0.449±0.07a	0.379±0.29a	0.00±0.00a	0.339±0.30a	0.149±0.11a
Dakace	0.363±0.16a	0.379±0.29a	0.00±0.00a	0.14±0.08a	0.14±0.11a
Total	**0.343±0.07**	**0.47±0.11**	**0.001±0.00**	**0.159±0.06**	**0.143±0.04**
P value	**0.308ns**	**0.694ns**	**0.486ns**	**0.761ns**	**0.996ns**

There were significant differences in Chromium concentrations from the gills and livers of the fish from the various sites of exposure. (Table 5; Figs 3-7).

Table 5: Heavy Metals From pooled samples of Gill and Liver from the *in situ* exposures of *Clarias gariepinus* in River Galma

Location	Gill					Liver				
	Pb	Cr	Cd	Zn	Mn	Pb	Cr	Cd	Zn	Mn
Shika Dam	0.02±0.01b	0.44±0.22ab	0.00±0.00a	0.20±0.11a	0.03±0.01a	0.08±0.06a	0.00±0.00b	0.00±0.00a	0.19±0.10a	0.03±0.02a
Kakeyi	0.31±0.14a	0.78±0.11a	0.00±0.00a	0.26±0.08a	0.11±0.04a	0.31±0.10a	0.78±0.11a	0.00±0.00a	0.16±0.06a	0.09±0.05a
FCE I	0.31±0.10a	0.78±0.11a	0.00±0.00a	0.18±0.07a	0.41±0.38a	0.20±0.16a	0.56±0.29ab	0.00±0.00a	0.17±0.07a	0.42±0.37a
FCE II	0.12±0.06ab	0.25±0.21b	0.00±0.00a	0.10±0.08a	0.08±0.07a	0.06±0.04a	0.25±0.21ab	0.00±0.00a	0.11±0.08a	0.09±0.07a
Dakace	0.00±0.00b	0.00±0.00b	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00b	0.00±0.00a	0.00±0.00a	0.00±0.00a
Total	**0.15±0.05**	**0.45±0.10**	**0.00±0.00**	**0.15±0.04**	**0.13±0.08**	**0.13±0.05**	**0.32±0.10**	**0.00±0.00**	**0.13±0.03**	**0.13±0.08**
P value	**0.061ns**	**0.020***	**0.580ns**	**0.225ns**	**0.489ns**	**0.210ns**	**0.031***		**0.371ns**	**0.451ns**

Mean values with the same alphabet are not significantly different from each other.

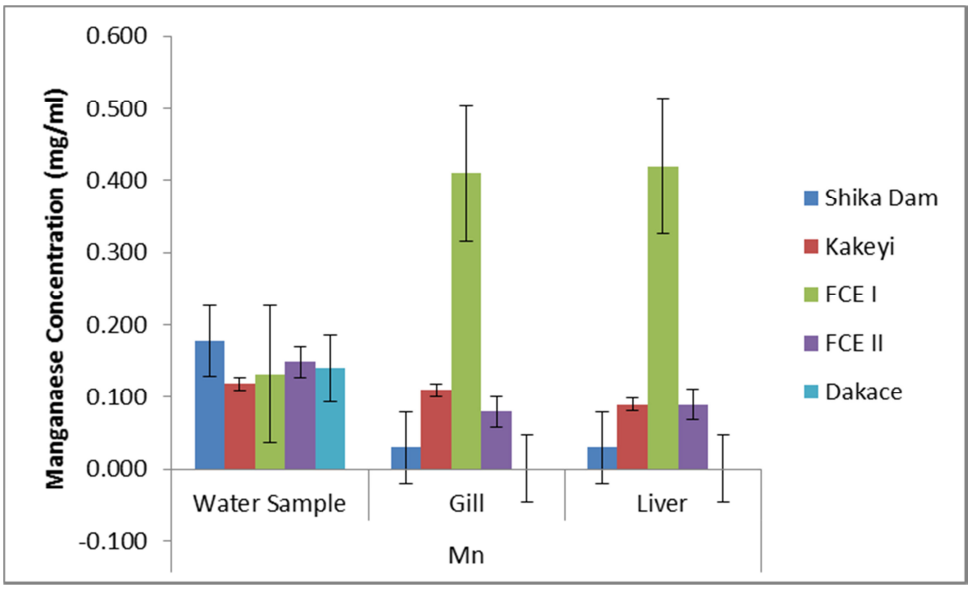


Fig. 3. An Error bar indicating Manganese Concentrations (mg/ml) in Water samples and Fish organs in all the sites.

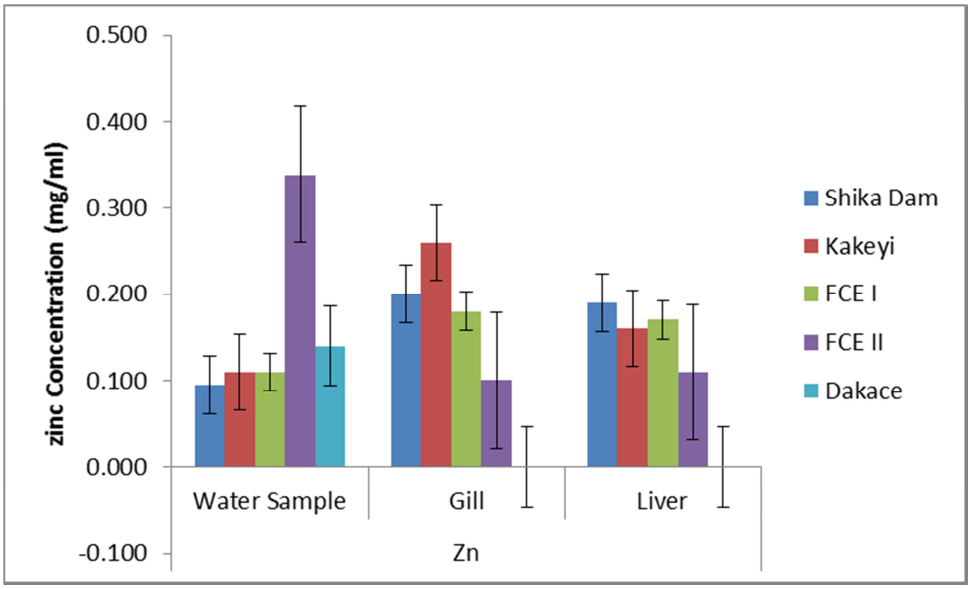


Fig. 4. An Error bar indicating Zinc Concentrations (mg/ml) in Water samples and Fish organs in all the sites.

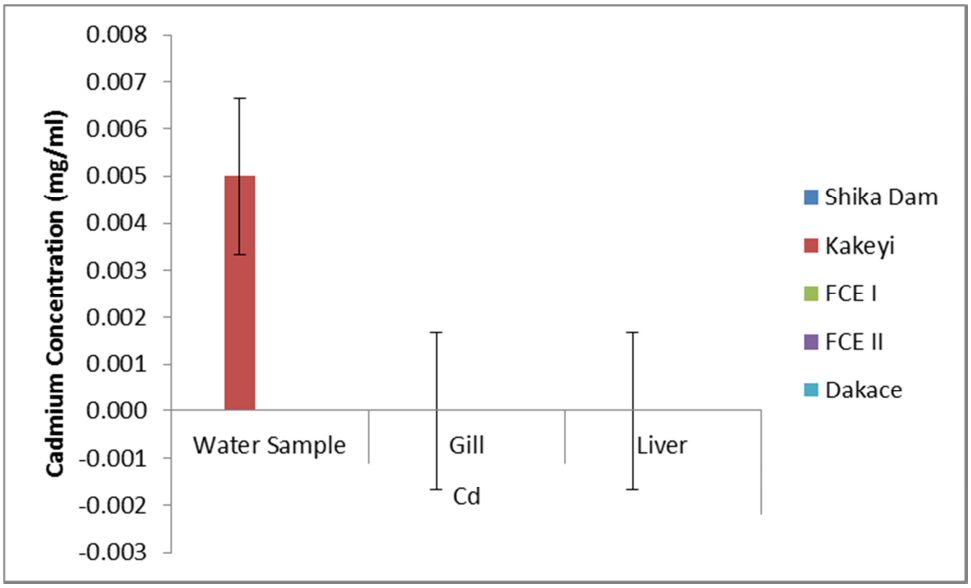


Fig. 5. An Error bar indicating Cadmium Concentrations (mg/ml) in Water samples and Fish organs in all the sites.

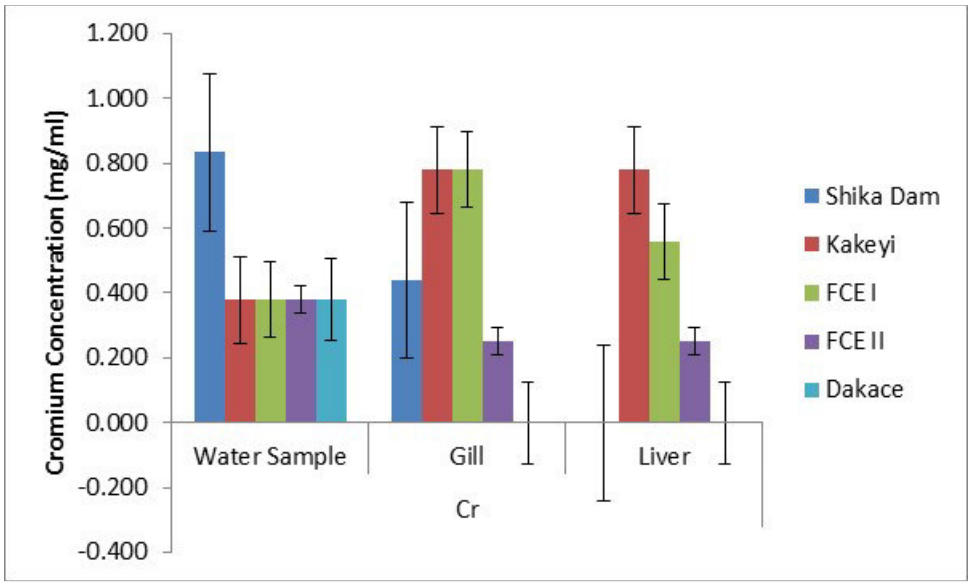


Fig. 6. An Error bar indicating Chromium Concentrations (mg/ml) in Water samples and Fish organs in all the sites.

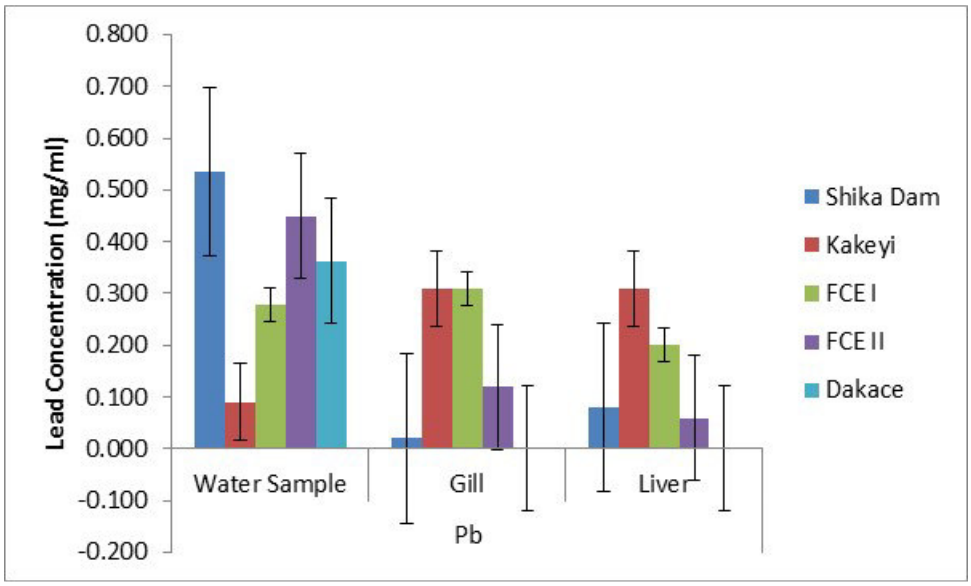
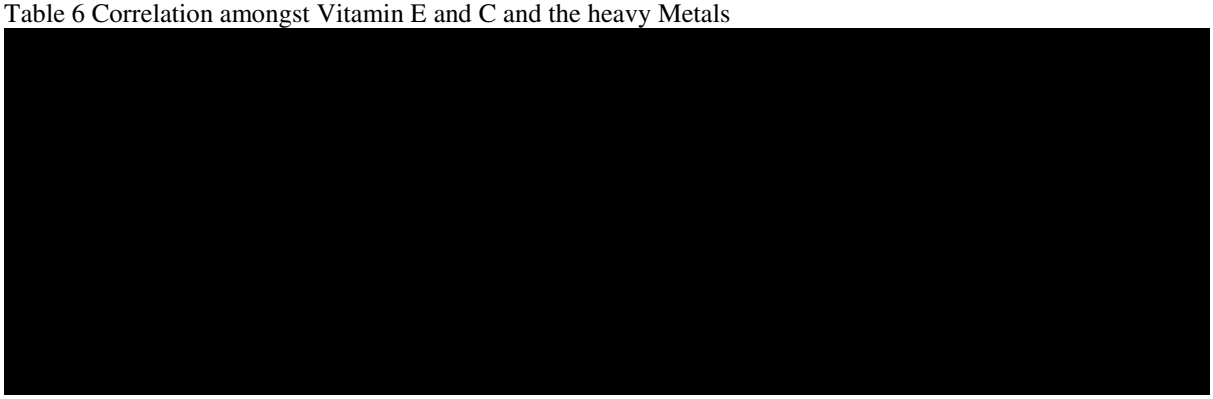


Fig. 7. An Error bar indicating Lead Concentrations (mg/ml) in Water samples and Fish organs in all the sites.

From the Spearman's correlation coefficient analysis there were correlations amongst Vitamin E and C and the heavy metals (Pb, Cr, Zn and Mn) with the exception of Cadmium. There was strong correlation between Vitamin E levels and Lead (Pb) concentrations in all the exposure periods. (Table 6).

Table 6 Correlation amongst Vitamin E and C and the heavy Metals



4. Discussions

Fish is one of the chief protein sources for humans that play a role in lowering the blood cholesterol level and offers omega-3 fatty acids that minimize the danger of stroke and heart related disorders (Al- Busaidi *et al*., 2011). Of all aquatic species, fish are particularly sensitive to waterborne contamination and are recognized as bio-indicators for water quality monitoring. To attenuate the negative effects of Reactive Oxygen Species (ROS), fish possess an antioxidant defence system like other vertebrates that utilizes enzymatic and non-enzymatic mechanisms. The use of fish in environmental monitoring has become increasingly important in recent years in the investigation of natural variability, as well as anthropogenic substances, many of which function as prooxidants, accumulating in aquatic environments (Almroth *et al.*, 2008)

An *in situ* bio-assay was carried-out to determine the effects of xenobiotics on the production of Vitamin E and C (VE and VC) in *Clarias gariepinus* as non – enzymatic antioxidants in River Galma in different locations along the river course. Gills and livers of the fish were employed in the assay. Gills were chosen for being the first organ which gets in contact with the environment becoming a potential target for oxidative disruption. Liver is believed amongst other functions to be the organ of detoxification in vertebrates. Liver was chosen because it possesses high potential for ROS generation, which seems to be efficiently counterbalanced by powerful protective mechanisms to detoxify and repair damaged lipid and proteins (Oliveira *et al*., 2008; Lattuca *et al,*. 2009, Nahrgang *et al*., 2010). Also, measuring the same biomarker in different locations simultaneously gives us information about the pollution status of the region and provides a better comprehension of the mechanisms of response of the organisms to pollutants (Giarratano *et al*., 2010).

Amongst the locations there were no significant differences in the levels of VE and VC production. This may be as a result of the uniform distribution of the municipal waste being washed into the river which is the major source of pollutants in the river under study. However, lowest values of VE and VC were observed in Shika Dam which served as the reference point. It is the upper course of the river and is relatively located far away from the municipal discharge of the neighbouring towns and villages. While highest values of the antioxidants were obtained from FCEI site. This may be as a result of the municipal discharge from the neighbouring towns and villages from Sabon Gari, Kakeyi, Farin Kasa, Chikaji, Bakin Pipe, etc. Also, the decrease in VC levels in all the sites in comparison with the control may be as a result of response of the ascorbic acid to ROS generated due to xenobiotics (Oakes *et al*., 2004).

The levels of VE and VC production in all the months were significant in the gills of the fish but not in the liver. This may be as a result of the fact that the gills are the first point of contacts as noted earlier above. These results are in contrast with those found by Lattuca *et al*. (2009) who showed that the liver of *Odontesthes nigricans* exhibited a better control of the oxidative damage than the gills, allowing minimization of intracellular damage when it is exposed to environmental stressful conditions. The results are also in contrast to the work o Lattuca *et al*. (2013) on *O. nigricans.* This is also in contrast with the work of Sthanadar *et al*. (2013) when he observed that fish livers have comparatively more chemical affinity to bioaccumulate cadmium as compared to heavy metals like Zn, Cr and Ni. These differences in the organ most responsive to ROS stress may be due to differences in the aquatic habitat as this work was done in fresh water habitat and also, due to the fact that this was an *in situ* exposure but not an extraction of an already existing fish in the river. Also, lowest values of VE and VC were obtained in the month of October and highest values in December in gills and livers respectively. This may be as result of high volume of water and the dilution factor of the river in the month of October due to high rainfall during the period which was the peak of the rainy season. The highest values in the month of December may be as a result of low water volume and the concentration of the pollutants in the river body. It is important to note that whether ROS will act as damaging, protective or signaling factors depends on the delicate equilibrium between ROS production and scavenging at the proper site and the time (Gratao *et al*. 2005). Also, numerous low molecular weight antioxidants such as glutathione, β-carotene (vitamin A), ascorbate (vitamin C), and tocopherol (vitamin E) can participate in the process of eliminating oxy-radicals (Van Der Oost *et al*., 2003).

The correlations observed in the VE and VC levels and the heavy metals especially the strong correlation between VE and Pb indicates the presence of generation of oxidative stress from the river due to the presence of heavy metals and the consequent production of VE to counteract or at best attenuate the effects. This further buttresses the connection or linkage between non-enzymatic antioxidant (such as VE and VC) production levels and heavy metal pollutants (Pb, Cr, Zn and Mn) in the aquatic medium. The VE and VC production in *Clarias gariepinus* play significant role in the adaptability and survival of the fish against ROS in their environment. Plasma VE and VC have been reported to have certain protective effects on cells against certain doses of DDVP-induced (dichlorvos) oxidative stress (Eroglu *et al*., 2013). Likewise, supplementation of lycopene and vitamin E decreases the toxic effect of diazinon in the work of Ahmed and Mahdi (2014). Also, Galal *et al*. (2014) concluded that VE supplementation has beneficial impacts on DM neurotoxicity in rats through its antioxidant and anti-apoptotic properties.

The significant difference observed in the fish organs, water samples and Lead (Pb) concentrations; and

the significant differences in Chromium (Cr) concentrations observed in the gills and livers of the fish and may be as result of urban and agricultural run-offs into the river system. Lead is well known to be a very toxic heavy metal even in its lowest concentration. Its detection and other heavy metals in the fish organ can serve as biomarkers used in bio-monitoring to give biological information that is, the effects of pollutants on living organisms. A link between DNA damage and concentration of Cr and Mn in soft tissues was observed in mussels in the most polluted site (Dallas *et al*., 2013). The harmful effect of toxic chemicals on natural ecosystems has led to an increasing demand for early –warning systems to detect those toxicants at very low concentration levels (Durrieu *et al.,* 2006).

Lead, Chromium and Manganese had their highest values in Shika Dam. These heavy metals may have been gotten from River Kaduna since River Galma is one of its main tributaries or they may have been retained within the embankments of the reservoir over time and being washed off from time to time. Zinc however, had its highest in Kakeyi which is located downstream below Shika Dam where the river body is beginning to have its impact from the surrounding towns and villages.

5. Conclusions

This research investigated the possibility of using non-enzymatic antioxidants such as Vitamins C and E as biomarkers of oxidative stress generated due to some heavy metal pollutants in *Clarias gariepinus* in 14 days *in situ* exposure in River Galma. There are significant differences in the levels of VE and VC production levels in the gills in all the months of exposure but not in the liver of the fish. The production levels of the antioxidants are high in the dry season than in the wet season. There is also, significant difference amongst the lead (Pb) concentrations in the water samples and fish organs but not with the other heavy metals (Cr, Cd, Zn and Mn) studied.

There is strong correlation amongst the vitamins and lead (Pb) concentrations in the fish samples. There are also correlations amongst the vitamins and the other heavy metals except Cadmium. This research also established the presence of heavy metals examined in varying degrees of concentrations with the exception of Cadmium which was below detectable limit in fish organs.

VE and VC can be used as biomarkers of pollution in River Galma.

Recommendations

The out-come of this research can serve as invaluable information to the members of the immediate community and the policy makers on environmental issues. It is also an eye-opener to the scientific community.

Better caging system and security measures should be adopted to ensure greater success in future *in situ* exposure. Further research should be carried out to test the outcome of this research on the use of Vitamins E and C as biomarkers.

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