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Potential Nephrotoxicity in African Mud Catfish (*Clarias gariepinus*) Following Exposure to Compost Derived Humic Acid

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Abstract: Influence of compost-derived Humic Acid (HA) on nephrotoxicity in juvenile African mud catfish (*Clarias gariepinus*) was evaluated in static water culture. Fish samples were exposed to different HA concentrations (0, 100, 250, 500 and 1000 mg L^{-1}) for 45 days at 5 samples per aquarium. Renal function was assessed spectrophotometrically via levels of serum albumin, measured using bromocresol green, creatinine by Jaffe method and urea by Nesslerization method. Results revealed that the mean value of albumin or urea in the exposed group (I), at each HA concentration, was lower than the value found in the control group (II). Creatinine was relatively higher in I relative to II. Significant ($p<0.05$) variations for I and II were obtained at 250 to 1000 mgHA L^{-1} for the three biomarkers. Relative to increasing HA concentration, decreasing albumin (0.84 to 0.43 g dL^{-1}: $r = -0.114$; $p> 0.10$), urea (5.21 to 1.95 mg dL^{-1}; $r = -0.586$; $p> 0.10$) and increasing creatinine (0.20 to 1.53 mg dL^{-1}: $r = +0.704$; $p> 0.10$) were recorded; r is correlation coefficient. Changes in urea were not predictive of nephrotic syndrome but alterations in albumin and creatinine revealed induced nephrotoxicity, especially at elevated HA concentrations (above 100 mg L^{-1}). Overall, the effect of humic acid was dose-dependent. Further studies at various humic acid concentrations, especially below 100 mg L^{-1}, are required to establish the actual nephrotoxic dose.

Key word: Compost, humic acid, nephrotoxicity, fish, environment

INTRODUCTION

Humic Substances (HS) are ubiquitous in natural environments, being found in soil, peat, sediments, composts and water. Among them Humic Acid (HA) which is that fraction not soluble in water under acidic conditions (<pH 2), but is soluble at higher pH, with typical average molecular weight of 2,000 to 3,000. By functioning as surfactants, HS can bind either hydrophobic compounds such as polynuclear aromatic hydrocarbons, chlorinated hydrocarbons and persisting organic pollutants or hydrophilic materials such as polar compounds. This function in combination with their colloidal properties, makes HA effective agent in transporting environmental xenobiotics (Stevenson, 1994; Balarezo *et al*., 2002).

Complexation of a variety of organic and inorganic contaminants with Dissolved Humic Substances (DHS) has been shown to affect chemical bioavailability and toxicity in addition to influencing the biodegradation and mobility of these contaminants in the environment. Humic substances in aquatic ecosystems have been shown to be important in influencing the bioavailability, toxicity and fate of organic xenobiotics, having the potential to either increase or reduce the bioconcentration of contaminants by aquatic organisms (Stevenson, 1994; Matthews *et al*., 1995; Balarezo *et al*., 2002).

In Nigeria, incremental food production is necessary to ensure national food security for the teaming human population. This can only be achieved by continual cultivation to exploit the dry season in addition to wet season farming. Consequently, the Federal Government of Nigeria initiated the small scale low cost farmer managed irrigation scheme to develop Fadama lands (flood plains). This gave rise to Fadama farming, prevalent along river banks and other wetland areas (Afolabi, 2010).

The use of organic fertilizer has gained global attention and composting of organic solid waste materials is seen as an economically feasible waste management option in Nigeria and the resulting products (solid and liquid organic fertilizers). Through leaching process, the transfer of organic matter containing humic substances, into the nearby fresh water body could add to dissolved humic substance fractions in the receiving waters. The concern, therefore, is the fate of aquatic organism when organic fertilizers are utilized in fadama farming scheme. In this regard, there is acute scarcity of scientific data.

Prognostic biomarkers of kidney disease include creatinine, urea and albumin. The kidneys are two bean-shaped organs that are located on either side of the

body, just underneath the ribcage. The main role of the kidneys is to filter out waste products from the blood before converting it into urine. However, the kidney is a preferential target for several environmental toxins and therapeutic agents. The interaction of these xenobiotics with the kidney, affect its central role in excretion, namely glomerular filtration and creatinine clearance (Spencer, 1986; Oduola *et al*., 2010). Such effects could lead to acute and/or chronic renal insufficiency after long term usage.

Serum creatinine and urea are some waste metabolic products excreted exclusively via the kidneys so provide useful information about the health status of the kidney (Panda, 1999). Nephrotoxicity manifests as renal failure with rise in serum creatinine and urea levels (Uboh *et al*., 2009). High levels of creatinine usually indicate that the kidneys are malfunctioning. Creatinine is produced naturally by the body, being a break-down product of creatine phosphate, which is found in muscle. It is freely filtered by the glomerulus. There is little-to-no tubular reabsorption of creatinine. If the filtering of the kidney is deficient, blood levels rise.

Urea is a break down product of amino acid catabolism. Similar to the case of creatinine, if kidneys are not able to remove urea from the blood normally, the level rises, predicting nephrotic dysfunction (Spencer, 1986; National Kidney Foundation, 2002). Serum albumin is the most abundant plasma protein in humans and other mammals, being essential for maintaining the osmotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues. It also acts as a plasma carrier by non-specifically binding several hydrophobic steroid hormones and as a transport protein for hemin and fatty acids. Albumin (when ionized in water at pH 7.4, as found in the body) is negatively charged. The glomerular basement membrane is also negatively charged in the body; some studies suggest that this prevents the filtration of albumin in the urine. According to this theory, that charge plays a major role in the selective exclusion of albumin from the glomerular filtrate (Spencer, 1986; National Kidney Foundation, 2002). A defect in this property results in nephrotic syndrome leading to albumin loss in the urine.

The objective of this study was to assess the nephrotoxicity potential of humic acid isolated from composted wastes (organic fertilizer) of Nigeria origin to African mud catfish (*Clarias gariepinus*) grown in static water culture using the three biomarkers: serum creatinine, urea and albumin.

MATERIALS AND METHODS

The research was carried out from June to December, 2008 in the Department of Environmental Management and Toxicology, University of Agriculture Abeokuta, Nigeria.

Compost preparation: Composting was carried out after the published procedure of Adekunle (2009), using source separated municipal solid wastes generated from Abeokuta city, Nigeria, consisting largely of vegetable matter, crop and food residues municipal organic waste materials, denoted as MSW. Green materials, which were soft and succulent, were first allowed to wilt for two to three days to remove excess moisture and waste paper materials were made into slurry before stacking. The MSW was mixed with sawdust (bulking agent) at organic waste to sawdust material ratio of 7:1 (w/w). The resulting bulk organics was then mixed with Cow Dung (CD) at a bulk organic/ CD material ratio 1:3 (w/w).

Turning schedule was adopted once in three days and systems were replicated three times in 30 L black composters. The composts matured within 42 days and were allowed further 35 days to stabilize. Stability of compost was established via color, physical form, C/N ratio, pH and temperature changes, which were determined using standard procedures. Phytotoxicity test was also carried out in a screen house to ascertain compost safety. Assessment of heavy metal levels were conducted on the compost as described in Adekunle (2009).

Extraction of humic acid from composted wastes: Humic acid was extracted from the cured product using alkaline method, purified and characterized using the method of Adekunle and Onianwa, (2001) and Adekunle *at al.* (2007a) with slight modification. The composted wastes was ground to fine powder and sieved through 2 mm mesh, decalcified by equilibrating with 0.1 M HCl for 24 h at room temperature (301±1 K). The dried residue was extracted with 0.5 M NaOH. The experiment was carried out in the presence of N_2 to minimize chemical changes due to autoxidation (Stevenson, 1994). The supernatant was separated from the residue by centrifugation (450 rpm for 5 min) and decantation techniques. The alkaline extraction was repeated several times until extraction was completed, as signaled by an almost colorless alkaline extract. The black humic solution was fractionated by pH adjustment to 2.0 using 10 mL of 5 M HCl.

The system was then allowed to stand for 24 h at room temperature (301±1 K) for the coagulated humic acid to settle at the bottom of the plastic ware. The black humic acid precipitate was separated by centrifugation and purified by re-dissolution in 0.1 M NaOH, re-precipitation by acidification (5 M HCl and 0.1 M HF) to remove

possible silicates. The humic acid precipitate was finally washed several times with deionized water until the wash water tested negative to chloride and hydroxide ions by means of silver nitrate and phenolphthalein tests. The purified humic acid was then dried, ground to powder using porcelain mortar and pestle and stored at room temperature prior to chemical analysis.

Exposure to humic acid isolated from composted wastes: Temperature, electrical conductivity, nitrate, phosphate, dissolved oxygen, total hardness and alkalinity of the process water were also analyzed using standard procedures according to the published work of Adekunle *et al*. (2007b). Twenty five juvenile fishes of average weight and length 43.2±0.5 g and 18.6±0.8 cm, respectively, fed with 2 mm copen fish feed, were utilized for the experiment at 5 per aquarium of 45 L capacity. After tolerance tests, fish samples exposed to different concentrations of HA (0, 100, 250, 500 and 1000 mg L^{-1}) in controlled water culture for 45 days.

Biochemical analysis: At the end of the exposure period, the fishes were removed from water and directly placed in clove oil to make them inactive. Blood samples were then taken from the caudal vein by heparinized plastic syringe, transferred to heparinized tubes and centrifuged at 4, 000 rpm for 8 min and the resulting plasma was stored by refrigeration at -18°C. Renal function was assessed spectrophotometrically and calculations were made from generated standard (calibration curves) according to standard methods described by Pratt (1996) and Aitken *et al*. (2003) but based on the use test kits. Creatinine and urea were determined using Cromatest kits while albumin was determined using Randox test kit.

Determination of albumin: Albumin was measured spectrophotometrically, using bromocresol green method described in Pratt (1996) and Aitken *et al*. (2003). Three test tubes were labeled blank (B), standard (S) and sample (T). Exactly 10 μL of distilled water was aspirated into the B, 10 μL of albumin standard into S and 10 μL of plasma into T. Exactly 3 mL of BCG reagent (R1) was added into each of the three tubes (B, S and T), followed by thorough mixing and incubation in the dark for 10 min at room temperature. The absorbance of the sample and standard was measured against the reagent blank at 630 nm. Albumin was expressed in g dL^{-1}.

Determination of creatinine: Creatinine was determined by Jaffe spectrophotometric method described in Pratt, (1996) and Aitken *et al*. (2003). The working reagent, samples and standard were prepared at room temperature. Two test-tubes labeled S for standard and T for sample and 1 mL of the working reagent was into both followed by the introduction of 100 μL of standard into S and 100 μL of sample into T. The contents of each tube was gently mixed, distilled water was used to zero the spectrophotometer and the absorbance values of the standard and sample were recorded at 500 nm after 30 and 90 seconds. Distilled water was used for blank test. Plasma creatinine was expressed in mg dL^{-1}.

Determination of urea: Urea was determined via Nesslerization method, described in Pratt, (1996) and Aitken *et al*. (2003). Three test-tubes labeled blank (B), standard (S) and sample (T) were used and from the Cromatest kit manual, 1 mL of working reagent was transferred to B, S and T. Exactly 10 μL of distilled water was added in each tube and each solution was thoroughly mixed and incubated for 10 min at room temperature. The absorbance values of the sample and standard were read against the reagent blank. Plasma urea was expressed in mg dL^{-1}.

Statistical analysis: Data obtained from study were subjected to one-way analysis of variance and Pearson correlation using SPSS 16.0 for Windos®.

RESULTS AND DISCUSSION

Humic acid characteristics: Quality parameters found in the process water used for aquaculture were 27°C for temperature, 6.4 for pH, 20 mg L^{-1} for nitrate, 154 g L^{-1} for hardness and 80 mg L^{-1} for alkalinity. Infrared absorption bands for the extracted humic acid were recorded in the region of 3320 to 3470 cm^{-1} for H-bonded OH, N-H; 2471-2320 cm^{-1} for C-N multiple bond; 1721 to 1631 cm^{-1} for carbonyl (C = O) of carboxylic acid, ketone, aldehyde and quinone; 2897 cm^{-1} for C-H of hydrocarbon skeleton; 1591 cm^{-1} for C-H of substituted benzene and 1102 cm^{-1} for C-O bonds. Acidity groups were obtained as 3,300 cmol kg^{-1} for total acidity, 2,860 cmol kg^{-1} for COOH and 440 cmol kg^{-1} for OH functional groups. Results thus showed that COOH comprised 86.67% of the total acidity while OH was 13.33%. The E_4/E_6 ratio was found to be 4.08.

Serum album level: Albumin level (Fig. 1) in the control group was 0.87±0.19 g dL^{-1} and ranged from 0.43 to 0.87 g dL^{-1} in the test groups. Correlations gave negative coefficients for albumin versus HA concentrations (-0.114; $p > 0.10$), indicating albumin depletion (Fig. 1) with increasing HA concentration in water.

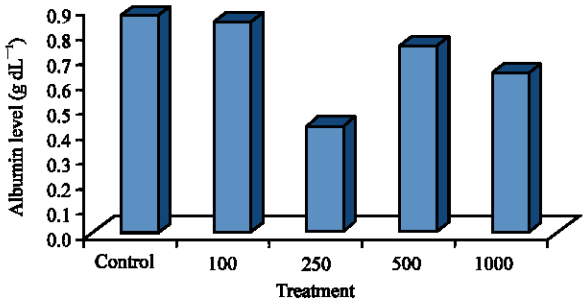

Fig. 1: Changes in albumin concentrations in *C. gariepinus* in relation to humic acid concentrations (mg L^{-1}) in water

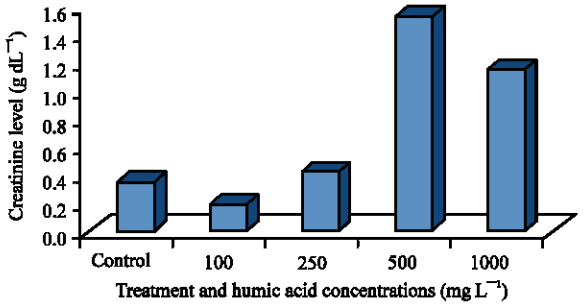

Fig. 2: Changes in creatinine concentrations in *C. gariepinus* in relation to humic acid concentrations (mg L^{-1}) in water

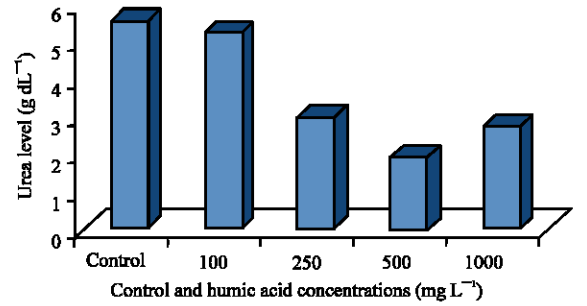

Fig. 3: Changes in urea concentrations in *C. gariepinus* in relation to humic acid concentrations (mg L^{-1}) in water

Creatinine and ureal levels: The creatinine level (Fig. 2), in the control group was 0.36±0.09 mg dL^{-1} and ranged from 0.20 to 1.53 mg dL^{-1} in the test groups. Positive correlation was obtained for creatinine and HA concentrations (+ 0.704; p> 0.10), indicating rising blood levels of creatinine (Fig. 1) with increasing HA concentration in water. The urea level (Fig. 3), in the control group was 5.61 ± 0.07 mg dL^{-1} and ranged from 1.95 to 5.21 mg dL^{-1} in the test groups. Correlations gave negative coefficients for urea versus HA concentrations

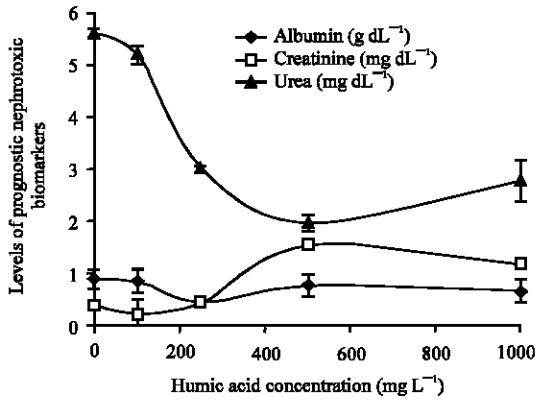

Fig. 4: Comparative evaluation of albumin, creatinine and urea concentrations in *C. gariepinus* in relation to humic acid concentrations (mg L^{-1}) in water

Table 1: Inter-biomarker ratios of the nephrotoxic biomarkers

HA	Biomarker ratios					
(mg L^{-1})	Ur-Cr	Cr-Ur	Alb-Cr	Cr-Alb	Alb-Ur	Ur-Alb
0	15.58	0.064	2.42	0.414	0.155	6.45
100	26.05	0.038	4.20	0.238	0.161	6.20
250	7.00	0.143	1.000	1.000	0.143	7.00
500	1.27	0.785	0.375	2.040	0.385	2.60
1000	2.39	0.419	0.552	1.813	0.231	4.328

Ur: Urea, Cr: Creatinine, Alb: Albumin

(-0.586; p > 0.10), indicating a decreasing trend (Fig. 1) with increasing HA concentration in water.

Comparative evaluation of the biomarkers of nephrotoxicity: Figure 4 showed that the levels of the three biomarkers in the control fish population relatively decreased in the order: Urea>albumin> creatinine but this order was altered on exposure to humic acid. At 100 mgHA/L, the trend was maintained but with increased humic acid concentration, Urea>creatinine> albumin was obtained.

Results also revealed that the inter-biomarker ratios found in the control fish group was altered by exposure to the humic acid. For instance, urea to creatinine ratio recorded as 15.58 in the control became 1.27 to 26.05 in the test groups. Similarly, albumin to creatinine ratio that was 2.47 in the unexposed group (control) ranged from 0.375 to 1.0 in the exposed fish groups (Table 1).

The process water used for aquaculture in this study was suitable for aquaculture and conforms to the reports of (Adekunle *et al*., 2007c). The decreasing albumin level, coupled with the increasing creatinine concentration with increasing humic acid concentration in water, obtained in this study, indicated nephrotic syndrome. However, based on changes in the decreasing urea levels obtained in this study, nephrotoxicity was not induced. It is reported in literature (National Kidney Foundation, 2002)

that creatinine is a more accurate marker of kidney disease than urea. High creatinine level implied that many waste products in the fish bloodstream would not be cleared, indicating that the kidneys were not functioning properly. Depletion of albumin showed that exposure to high concentration of HA impacted negatively on albumin synthesis or that it facilitated increased loss of albumin, which signaled nephrotic syndrome.

A more complete estimation of renal function can be made when interpreting the blood (plasma) concentration, using the ratios. This can indicate other problems besides those intrinsic to the kidney. As an illustration, the ratio of urea to creatinine can indicate other problems besides those intrinsic to the kidney; for example, a urea level raised out of proportion to the creatinine may indicate a pre-renal problem such as volume depletion (Spencer, 1986; National Kidney Foundation, 2002).

The adverse impact of humic acid exposure on renal function was attributed to the influence of humic acid via the oxo-functional groups identified in the molecular fragment. Dissolved humic substances are taken up by organisms and interact on various molecular and biochemical levels (Steinberg *et al*., 2007). Infrared spectra and results from volumetric analyses showed oxygenated functional groups and hydrocarbon skeleton, indicating content of phenolic hydroxyl and carboxyl reactive sites similar to the humic acids properties reported in our previous published works (Adekunle and Onianwa, 2001; Adekunle *et al*., 2007a).

The compost-derived humic acid, on absorption of light could have initiated the generation of oxidizing species via the production of radicals such as peroxy radicals, hydroxyl radicals, hydrated electrons, hydrogen peroxide, singlet oxygen and superoxide, typical of humic substances (Cooper *et al*., 1989; Balarezo *et al*., 2002). The generation of the above mentioned oxidizing species could have provoked decreased renal antioxidant enzyme activity with enhanced lipid peroxidation and oxidative stress (Balarezo *et al*., 2002).

Heavy metals are known to induce organ damage in biological systems. The water used in this study was sourced from a borehole, used for aquaculture in the region and contained up to 5 mg L^{-1} of Pb. Mager *et al*. (2010) reported potential Pb-induced motor/behavioral impairment in fathead minnow (*Pimephales promelas*) in water containing elevated HA. Increased Zn levels by 11 to 30% ($p>0.05$) in the presence of HA compared to the control group was reported in the tissues of chicken broiler (Navratilova *et al*., 2009). These suggest that the humic acid-mediated nephrotoxicity could be associated with the dissolved cationic ions in water. The result from this study on humic acid mediated nephroyoxicity is consistent with the works of Bunnell *et al*. (2007) who worked on evaluating nephrotoxicity of high-molecular-weight organic compound in drinking water from lignite aquifers and reported that exposure of kidney tissues to these compounds such as humic acid produced excess cell death or proliferation depending on concentration and duration of exposure.

The Kidney is highly susceptible to toxicants for two reasons. A high volume of blood flows through it and it filters large amounts of toxins which can concentrate in the kidney tubules, which otherwise can result in systemic toxicity causing: decreased ability to excrete body wastes, inability to maintain body fluid and electrolyte balance and decreased synthesis of essential hormone (Uboh *et al*., 2009; Oduola *et al*., 2010) which could have been responsible for observed behavioural changes (erratic and uncoordinated swimming) and bleached body towards the end of the experiment.

Results from this study that humic acid concentration < 100 mg L^{-1} was relatively safe indicates that addition of organic matter from organic fertilizer to nearby freshwater, whose natural humic matter concentration are found in the range of 0.5 to 50 mg L^{-1} (Bittner *et al*., 2006), above 100 mg L^{-1}, poses health risk to the aquatic organisms such as fish.

CONCLUSIONS

The present results show that exposure of *C. gariepinus* to up to 100 mgHA/L did not evoke significant adverse effects on the three biomarker levels in the blood but beyond this HA concentration, from 250 to 1000 mg L^{-1}, significant nephrotic dysfunction was predicted from albumin and creatinine levels but based on urea levels, nephrotoxicity was not induced. Study showed that nephrotic syndrome was HA concentration dependent, indicating safety at levels $= 100$ mg L^{-1}.

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