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Anthracene-Induced Enzymatic Changes as Stress Indicators in African Catfish, *Heterobranchus bidorsalis* Geoffroy Saint Hilaire, 1809

[1]Taofik O. Sunmonu, [2]Olufemi D. Owolabi and [1]Oyelola B. Oloyede
[1]Department of Biochemistry, University of Ilorin, P.M.B. 1515, Ilorin 240003, Nigeria
[2]Department of Zoology, University of Ilorin, P.M.B. 1515, Ilorin 240003, Nigeria

Abstract: The impact of short term exposure to waterborne anthracene on the activities of Gamma Glutamyl Transferase (GGT), Alanine aminotransferase (ALT) and Alkaline aminotransferase (ALP) in the liver and stomach mucosa of juvenile African catfish, *Heterobranchus bidorsalis* Geoffroy Saint Hilaire, 1809 was investigated. Fish specimens weighing 73.00±2.50 g (n = 72) were grouped into six of twelve fishes each in 30 L aquarium. Each group was exposed to different concentrations (0 (control), 0.25, 0.50. 0.75, 1.00 and 1.25 g L^{-1}) of anthracene for 54 h. The results showed that there was a significant ($p<0.05$) inhibition of all the enzymes' (GGT, ALT, ALP) activities in both the liver and stomach of *H. bidorsalis* in relation to the control. Inhibition of each enzyme increased with increase in concentration of anthracene, with the highest inhibition of 79.96% (GGT), 89.74% (ALT) and 46.26% (ALP) and lowest inhibition of 13.98% (GGT), 22.80% (ALT) and 31.44% (ALP) recorded at the concentration of 1.25 and 0.25 g L^{-1}, respectively. The decrease in the activities of the enzymes could be due to their possible leakage into general blood circulation or could be as a result of organ dysfunction, thus indicating that anthracene could induce oxidative stress on *H. bidorsalis*. Percentage mortality ranged between 0 and 44.44%, with the highest mortality recorded at the highest tested concentration of anthracene. The results suggest that GGT, ALT and ALP can be used as potential environmental biomarkers for anthracene-induced hepatotoxicity and gastrotoxicity in *H. bidorsalis*.

Key words: *Heterobranchus bidorsalis*, anthracene, enzymes, oxidative stress, biomarkers, survival

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) comprise a large number of heterogeneous groups of organic contaminants which are formed and emitted as a result of incomplete combustion of organic material. Polycyclic aromatic hydrocarbons are found to be toxic and can induce toxic symptoms in experimental animals (Nicolas, 1999). A range of PAHs primarily enter the aquatic environment through oil well drilling operations, transportation and storage in the upstream industry, as well as refining, transportation and marketing in the downstream industry. PAHs pollution could also be from anthropogenic sources (Oberdorster and Check, 2001). In Nigeria, oil spill constitutes one of the most important sources of environmental pollution. Accidental oil pipeline breakages and vandalization occur frequently resulting in oil spillages, which have far reaching effects on aquatic biota. Between 1976 and 1996 alone, a total of 4,647 oil spills involving a loss to the environment of 1,820,410.50 barrels of oil were recorded (Asiodu, 2007). Short term toxicity in fishes includes lymphocytosis, epidermal hyperplasia, liver neoplasia and haemorrhagic septicaemia (Eisler, 1987; Beeby, 1993). This may lead to adverse effects on growth (Ostrander *et al*., 1990), reproduction (White *et al*., 1999; Monteverdi and Di Giulio, 2000) and survival (Collier and Varanasi, 1991; Hawkins *et al*., 1991).

Corresponding Author: Olufemi D. Owolabi, Department of Zoology, University of Ilorin, P.M.B. 1515, Ilorin 240003, Nigeria Tel: +2348034313927

Anthracene, also referred to as Para-naphthalene or green-oil is a colourless tri-cyclic aromatic hydrocarbon derived from coal tar; an important component of crude oil. It is a solid organic compound with a low molecular weight (178.2 g mL^{-1}) and has found considerable use in industry as an intermediate in dye production, in the manufacture of synthetic fibres and as diluents for wood preservations. It is also used in smoke screens, as scintillation counter crystals, in organic semiconductor research and in the synthesis of chemotherapeutic agent, Amsacrine (Wadler *et al*., 1986, Hawley, 1987). Anthracene is ubiquitous in the environment as a product of incomplete combustion of fossil fuel. It has been detected in surface water, ambient air, exhaust emission from internal combustion engine, smoke of cigarettes, tissues of fish and other food organisms (Baumard *et al*., 1998; Duke, 2008). Many PAHs have been found to originate reactive products after transformation that bind to DNA causing mutations or other alterations on the genetic material in animals (Marvin *et al*., 1995; Woodhead *et al*., 1999). For example, in fish, anthracene was found to alter gene expression in the mummichog, *Fundulus heteroclitus* (Peterson and Bain, 2004), while exposure of this species and steelhead trout, *Salmo gairdneri* to benzo[a]pyrene induced carcinogenic effects (Black *et al*., 1988). Polycyclic Aromatic Hydrocarbons (PAHs) have also been found to induce oxidative stress and caused lipid peroxidation in many fish species (Orbea *et al*., 2002; Reid and MacFarlane, 2003; Jifa *et al*., 2006).

Fish species have been used to assess the quality of water bodies and can serve as bio-indicators of environmental pollution (Lopes *et al*., 2001; Dautremepuits *et al*., 2004). The genus *Heterobranchus* is endemic to Africa and constitutes one of the main fish genera of economic value as food fish. In Nigeria *H. bidorsalis* are caught all year round in most freshwater and swamps and they command high market price either fresh or smoke dried. They are widely cultivated in Nigeria due to their fast growth rate and wide variability and adaptiveness in their natural diets (Fagbenro *et al*., 1991; Fagbenro, 1992). They are benthic feeders with euryphagous diets (Fagbenro, 1992), hence can easily be exposed, through direct contact, to xenobiotics in water and sediment. Therefore, the documentation of biological basis is not only necessary for proper management and conservation of this important resource but also the health implication on humans that consume fish become pertinent.

The toxic effects of PAHs are exerted on a variety of tissues and organs of animals. It has been suggested that the translocation of crude oil compounds in fish may be through the gills, gut or the intestinal walls (Roubal *et al*., 1977). Once ingested, anthracene appears to target the blood, stomach, intestine, liver and the kidney (Falkson *et al*., 1985; Faust, 1991). These organs are of great importance to fish as they perform various functions associated with the biotransformation and excretion of xenobiotics into bile (Jimenez and Stegeman, 1990; Landis and Yu, 1995). Therefore, in aquatic bio-monitoring these organs can be studied because of their high sensitivity to contaminants. Bioaccumulation of PAHs in fish organs have been reported to elicit the formation of Reactive Oxidative Stress (ROS), which may lead to environmental oxidative stress and cause changes in the activities of enzymes in these organs (Casillas *et al*., 1983). Various enzyme activities have been used as indicators or general biomarkers of stress in fish (Tejeda-Vera *et al*., 2007; Vutukuru *et al*., 2007; Ozmen *et al*., 2008; Simonato *et al*., 2008; Vieira *et al*., 2008). Gamma glutamyl transferase (GGT; EC. 2.3.2.2), Alanine aminotransferase (ALT; EC. 2.6.1.2) and Alkaline aminotransferase (ALP; EC. 3.1.3.1) are predominantly found in liver, stomach, cardiac cells and muscles where they play a very crucial role in transamination reaction. They have been employed in the diagnosis of liver, muscle and gill damages caused by pollutants in fish (Neff, 1985). The decrease in the levels of these enzymes in the liver and other organs indicates serious hepatotoxicity and gastrotoxicity, the extent of which depends on the type of toxicant, its mode of action and duration of exposure (Jacobson-Kram and Keller, 2001).

Although, several laboratory studies have been carried out to assess the toxicological effects of crude oil on fish species in Nigerian water, information on the individual effects of the various components of crude oil is scanty. The activities of GST, a detoxification enzyme was observed to increase in the presence of PAHs (Stien *et al*., 1998; Van Der Oost *et al*., 2003). Achuba and Osakwe

(2003) reported that a dose-dependent increases in the activity of catalase, an antioxidant in the liver and other organs of *Clarias gariepinus* exposed to crude oil were found after 14, 21 and 28 days. Sunmonu and Oloyede (2006) reported a reduced activity of ALT in the liver of *C. gariepinus* exposed to different concentrations of crude oil. Working on *H. bidorsalis* juveniles, Nwamba *et al*. (2006) observed increased activities of amylase and creatinine kinase enzymes as the concentration of crude oil to which they were exposed increased. Ugwu *et al*. (2007) also indicated that alterations in the morphometric status of the digestive tract of *H. bidorsalis* were dependent on the concentration of crude oil to which the fishes were exposed. However, dearth of information exists on the impact of anthracene on the activities of GGT, ALT and ALP in both the liver and stomach mucosa of *H. bidorsalis*. Histological analyses have been employed by many workers to study the effects of toxicants on selected organs in fish and other animals (Susithra *et al*., 2007; Van Dyk *et al*., 2007; Sunmonu and Oloyede, 2007; Simonato *et al*., 2008). This method as good as it is, is however faced with a drawback as it requires expertise, long time with attendant drudgery in preparation of histological slides and errors that may occur in interpreting results. Simple but reliable biochemical assay of enzymes provides a rapid assessment of cellular damage in animals exposed to xenobiotics within few hours. Therefore, the aim of this study was to determine the levels of GGT, ALT and ALP in response to anthracene toxicity; as potential bio-indicators of hepatotoxicity and gastrotoxicity due to oxidative stress.

MATERIALS AND METHODS

Experimental Set Up

Juvenile specimens of *H. bidorsalis* weighing 73.00±2.50 g (n = 72) were obtained from Yauri, an upstream section of Kainji Lake, Nigeria. The fish were transported to Fisheries and Hydrobiology Laboratory, Department of Zoology, University of Ilorin in a portable well-aerated white polythene bag containing water from the lake. The experiment was carried out in 2008. In the laboratory the water from the lake was gradually replaced by dechlorinated borehole water and the fishes were held in a large water bath of 120 L capacity at 25.6-27.3°C and acclimatized for 1 week. The fishes were fed ad libitum and aerators were used to supply oxygen throughout the experimental period. Feeding was stopped 24 h before the commencement of biochemical assay (Solsbe and De, 1993). The fishes were grouped into 6 of 12 fishes each in 30 L plastic aquarium. Group 1 served as the control and fishes in this group were introduced into borehole water free of any toxicant, while those in groups 2 to 6 were introduced into different concentrations (0.25, 0.50, 0.75, 1.00 and 1.25 g L^{-1}) of anthracene-contaminated borehole water respectively; with two replicates each. Also, the water in each plastic aquarium was changed once in every two days. The experiment lasted 54 h based on preliminary studies, which revealed that the fishes are liable to stiffen up and die after 54 h following exposure to 1.25 g L^{-1} anthracene-contaminated water. Hence, fishes could be maintained alive for biochemical assessment.

Biochemical Assay

At the expiration of 54 h, the experiment was terminated and the fishes were removed from both the control and test aquaria. They were thereafter dissected and portions of the liver and stomach were removed and cut separately into ice-cold sucrose solution. Known weight of each tissue was sliced into small pieces and homogenized using pre-cooled pestle and mortar in a bowl of ice chips. The homogenates were further diluted with 0.25 M sucrose solution to obtain a dilution factor of 60 before they were kept in freezer prior enzyme assay. Total protein in the liver and stomach homogenates was determined according to the method of Henry *et al*. (1974). The method described by Szasz (1974) was employed to assay for Gamma Glutamyl Transferase (GGT). Alanine transaminase (ALT) was assayed using the procedure described by Schmidt and Schmidt (1962), while the method of Wright *et al*. (1972) was employed in the assay of alkaline phosphatase (ALP).

Estimation of Mortality

Mortality was estimated by counting the number of dead fish in each aquarium and this was expressed as percentage.

Statistical Analysis

The data obtained were analysed statistically using Analysis of Variance (ANOVA) by employing the method of Steel and Torrie (1960). Significant difference between treatment means was determined at 5% confidence limit using Duncan Multiple Range Test (Duncan, 1955). Relative activity of the enzymes was determined as control corrected values using the formula:

$$\left(\frac{C-T}{C+T}\right)\times 100$$

Where:
C = Control value
T = Treatment value

RESULTS

The specific activity of GGT in the liver of *H. bidorsalis* exposed to various concentrations of anthracene polluted water revealed that there was a significant ($p<0.05$) inhibition of GGT activity in the fish relative to control (Table 1). The results shows that the inhibition increased with increase in concentration of anthracene, with the highest (79.96%) and lowest percentage (13.98%) inhibition recorded at the concentration of 1.25 and 0.25 g L^{-1}, respectively. The specific activities of ALT (Table 2) and ALP (Table 3) in the liver of *H. bidorsalis* exposed to anthracene contaminated water were also significantly ($p<0.05$) inhibited compared to the control. The inhibition of the two enzymes followed a similar trend as those recorded for GGT, with the highest inhibition of 89.74% at the concentration of 1.25 g L^{-1} and lowest inhibition of 22.8% at the concentration of 0.25 g L^{-1} for ALT. Highest inhibition of ALP (46.26%) was also found at 1.25 g L^{-1} concentration of anthracene, while the lowest (31.44%) was recorded at 0.25 g L^{-1}. Percentage mortality ranged from 0 to 44.44% and increased with corresponding increase in the concentration of anthracene between 0.75 and 1.25 g L^{-1} with the highest mortality recorded at the highest tested concentration of anthracene (Table 4).

Table 1: Specific activity and percentage inhibition of Gamma Glutamyl Transferase (GGT) in the liver of *H. bidorsalis* exposed to various concentrations of anthracene polluted water

Concentration of anthracene (g L^{-1})	Specific activity (U L^{-1})×10^2	Inhibition (%)
0 (Control)	48.86±2.71^a	0.00
0.25	36.87±1.05^b	13.98
0.50	33.99±0.41^c	17.94
0.75	25.61±2.98^d	31.22
1.00	16.06±1.46^e	50.50
1.25	5.44±0.56^f	79.96

Values are Mean±SEM for 12 fishes. Values with different superscripts are significantly different ($p<0.05$)

Table 2: Specific activity and percentage inhibition of alanine transaminase (ALT) in the liver of *H. bidorsalis* exposed to various concentrations of anthracene polluted water

Concentration of anthracene (g L^{-1})	Specific activity (U L^{-1})×10^2	Inhibition (%)
0 (Control)	1.48±0.15^a	0.00
0.25	0.93±0.04^b	22.80
0.50	0.73±0.11^c	33.90
0.75	0.44±0.19^d	54.16
1.00	0.38±0.01^e	59.13
1.25	0.08±0.01^f	89.74

Values are Mean±SEM for 12 fishes. Values with different superscripts are significantly different ($p<0.05$)

Table 3: Specific activity and percentage inhibition of alkaline phosphatase (ALP) in the liver of *H. bidorsalis* exposed to various concentrations of anthracene polluted water

Concentration of anthracene (g L^{-1})	Specific activity (U L^{-1})×10^2	Inhibition (%)
0 (Control)	191.45±1.67[a]	0.00
0.25	99.86±0.85[b]	31.44
0.50	89.66±1.57[c]	36.21
0.75	87.43±0.93[d]	37.29
1.00	70.92±0.38[e]	46.26
1.25	70.33±0.28[e]	46.28

Values are Mean±SEM for 12 fishes. Values with different superscripts are significantly different ($p<0.05$)

Table 4: Percentage mortality of *H. bidorsalis* exposed to various concentrations of anthracene polluted water

Concentration of anthracene (g L^{-1})	Mortality (%)
0 (Control)	0.00
0.25	0.00
0.50	0.00
0.75	2.13
1.00	22.07
1.25	44.44

DISCUSSION

The destruction of aquatic ecosystem inform of sub-lethal pollution by man usually lead to chronic stress of aquatic biota. The transfer of energy from one trophic level to another within the food chain may be disrupted resulting in an ecological imbalance and dwindling fish production. This may ultimately have a serious consequence on man who depends on fish as means of livelihood. The data obtained from this study show that anthracene-contaminated water causes a stress-inducing effect on *H. bidorsalis* during exposure as reflected by the inhibition of the enzymes investigated. In such cases, the fish could become more susceptible or predisposed to any invading pathogens and/or be more vulnerable to predators. Hence, it becomes imperative to take cognizance of the toxicity of this petroleum derivative to aquatic biota. The concentration of anthracene tested in this study can be considered as being ecologically important due to the fact that it falls within the lower or upper ranges of anthracene concentrations detected in sediment, surface waters and fish from water bodies polluted by PAHs (Duke, 2008; Vieira *et al*., 2008). It stands to reason therefore that the percentage inhibition of the enzymes (GGT, ALT and ALP) between 13.98 and 89.74% is considerably high indicating that 0.25-1.25 g L^{-1} seemed to be the ecological relevant concentration that are lethal to the survival of *H. bidorsalis* in water bodies polluted with PAH anthracene. This suggests that these enzymes may be suitable for use as environmental biomarkers to identify metabolic disturbances in *H. bidorsalis* from aquatic ecosystems polluted with anthracene and other derivatives.

Sotherton (1991) stated that exposure of organisms such as fish to hazardous chemicals caused cell injury and death to even non-target organisms. This situation is exemplified in this study by the high percentage mortality (approximately 69%) recorded throughout the experimental period, suggesting that prolonged exposure more than 54 h could be more disastrous. This explains the termination of the experiment after 54 h in order to ensure a drastic reduction in the mortality of the test organism; so as to maintain appreciable number of the organism alive for biochemical assessment. Casillas *et al*. (1983) reported that in acute exposure of organisms to pollutants, changes in concentration and enzyme activities reflect cell and organ damage in specific organs. The liver contains numerous enzymes which play important metabolic roles in the detoxification of many xenobiotics. GGT catalyses the transfer of gamma glutamyl group of glutathione to acceptors and has been observed to be critical in detoxification process (Meister, 1988; Keillor *et al*., 2005; Shaw *et al*., 2005). It increases the antioxidant status of the liver by recycling nutrients and amino acids, as well as conjugating toxins with glutathione to detoxify harmful substances (Dickson and Forman, 2002). ALT and ALP also provide an indication of the degree of inflammation as well as possible causes of hepatocellular damage.

Although, the activities of these enzymes in the serum were not investigated, their low levels in the blood are normally found (Ogueji and Auta, 2007). However, the accumulation of PAH component like anthracene in the liver, stomach and other organs might have caused serious pathological damage following exposure (Braunsbeck, 1994; Vutukuru *et al*., 2007). Burtis *et al*. (1996) and Vutukuru *et al*. (2007) reported that when the liver cell is damaged, tissue specific enzymes are released into the bloodstream, thus making the enzymes level in the blood to go up. Therefore, the significant decrease of the enzymes' (GGT, ALT and ALP) activities in the liver and stomach of *H. bidorsalis* exposed to anthracene-contaminated water could either be due to their possible leakage from the cytosol across damaged plasma membrane into the general blood circulation or decrease in their synthesis as a result of the organ dysfunction. The decrease which was concentration dependent could be considered to be manifestation of oxidative stress caused by anthracene. The peak inhibition of GGT and ALT observed at the highest tested concentration of anthracene (1.25 g L^{-1}) was three fold higher than that obtained at 0.25 g L^{-1} of anthracene, thus confirming the possibility of serious hepatocellular damage in the fish. Previous studies have also shown that ALT and ALP activities in fish organs decreased with increasing concentration of metals, pesticides and /or metabolic by-products of xenobiotics following malfunction of these organs. For instance, the activities of ALT showed a decrease following exposure to increasing concentrations of cadmium, copper and lead (Neff, 1985); suggesting that ALT can be used as a general biomarker (De LaTorre *et al*., 2000; Almeida *et al*., 2002). Similarly, the inhibition of ALP due to sub-lethal exposure of *Labeo rohita* fingerlings to cypermethrin (Das and Mukherjee, 2003) and that of African catfish, *Clarias gariepinus* to Lambda-cyhalothrin (Ogueji and Auta, 2007) have been reported.

However, more recent studies indicate that petrochemical products and/or PAHs caused inhibition of enzymes activities in some fishes. Wang *et al*. (2006) observed the inhibition of GST activity after exposure to benzene[a]pyrene in the rock fish, *Sebasticus marmoratus*. Sunmonu and Oloyede (2006) also reported a similar trend in the activity of ALT in the liver of *Clarias gariepinus* exposed to crude oil. A significant decrease of acetylcholinestarase (AChE) and glutathione-S-transferase (GST) at the highest tested concentration of anthracene, corresponding to 52 and 42% inhibition respectively were observed in the common goby, *Pomatoschistus microps* (Vieira *et al*., 2008). Therefore, the reduction observed in the activities of the enzymes in the organs of *H. bidorsalis* after exposure to anthracene appears to generally agree with the findings of these authors. The apparent decrease in GGT and ALT activities in the liver with concomitant reduction in the levels of ALP in the stomach suggest an adaptive and protective role of these enzymes against oxidative stress as the fish approach death due to the effects of the toxicant.

Since, environmental and human health are closely interrelated, the public health implication of this study is that the consumption of anthracene-contaminated *H. bidorsalis* may exert its effects on man through inhibition of synthesis, nerve synapse function, disruption of membrane transport system and damage to plasma membrane and bone marrow leading to haemorrhage (Prescott *et al*., 1995; Onwurah *et al*., 2007). As reported in many organisms, the genetic integrity may also be challenged resulting in carcinogenesis, mutagenesis and impairment of reproductive capacity (Short and Heintz, 1997).

In conclusion, this study shows that the accumulation of anthracene in the liver and stomach of *H. bidorsalis* led to the functional damage of these organs as reflected by the decrease in the activities of the enzymes. This biochemical dysfunction may interfere with the homeostatic processes which may ultimately affect the survival and rational exploitation of this fish in their natural environment. The decrease in the activities of the enzymes reinforces their important roles in the detoxification of toxic substances present in anthracene and further shows that GGT, ALT and ALP are potential biomarkers that can be used for anthracene-induced hepatoxicity and gastrotoxicity in *H. bidorsalis*.

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