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Studies on the Injection of *Heterobranchus bidorsalis* Adults with Different Concentrations of Bonny-Light Crude Oil and its Effects on Total Bilirubin Enzyme Concentration

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ABSTRACT: The activity of birlirubin enzyme in *Heterobranchus bidorsalis* adults (mean weight , 130.80±0.36g), when injected with different concentrations of Bonny-light crude oil (BLCO) was studied within 4 days toxicity and 42 days recovery periods. Significant decreases ($P < 0.05$; $P < 0.01$) in the values of total bilirubin enzymes concentrations (BEC)(mg/100ml) were recorded in the fish liver serum as the BLCO concentrations increased from 10.00 to 50.00µl.g^{-1}. Fish samples injected with 10.000 µl.g^{-1} BLCO recorded the highest values of BEC than those injected with 20.00-50.00 µl.g^{-1}BLCO. Increases in BEC values during the recovery period at day 14(23%), day 28 (15%) and day 42(5%) suggested some measure of relief on the liver tissues from oil toxicity and were a reflection of the tremendous effects of the oil injections on the activities of the bilirubin enzymes within the liver. This result is consistent with the suggestions by other workers on the necessity for a comparative monitoring of biomarkers and pathological changes in liver tissues in order to use good enzymatic markers as indicators of organ dysfunction. The BEC values in this study: whether on decreases due to increasing BLCO concentrations or on increases due to fish recovery from oil injections point to the fact that bilirubin enzyme activity in the fish livers was dose dependent. This result shows that bilirubin enzymes has the potential of a biomarker for monitoring pollution levels.

Key Words: *Heterobranchus bidorsalis*, Bilirubin; Biomarker, Pollution level, Crude oil injection.

Introduction

The indiscriminate dumping of refuse materials, industrial wastes and sewage into water bodies causes water pollution. Pollutants that are dumped into water not only destroy aquatic life but constitute poisons to humans, especially when man consumes such seafoods as fish, crayfish and shrimp. Crude oil spills from oil rigs and tankers also pollute water and destroy aquatic life. Ekwu (2000) reported that some toxic substances in an oil spill might evaporate rapidly. Therefore, catfish exposure to crude oil compound is reduced with time and this reduction is usually limited to the initial spill area.

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Although some organisms may be seriously killed soon after they come in contact with oil in a spill, non-lethal effects can be subtler and long lasting. For example, aquatic life on reefs and shorelines are at risk of being smoothered by oil that washes along the shore. Sometimes, aquatic biota are slowly poisoned by their long term exposure to oil trapped in shallow waters. Lee (1976) stated that the harm done to an aquatic environment often leads to harm for one or more species in a food chain, which ultimately leads to damage of other species further up the chain.

The potential utility of biomarkers for monitoring both environmental quality and the health of organisms inhabiting polluted ecosystem has recieved increasing attention during recent years (Lopes *et al*, 2001; Samecka-Cymerman and Kempers, 2003; Gauthier *et al*, 2004). Many enzymatic markers have been applied to determine the degree of exposure of animals to pollutants. Several specific enzymes have been proposed for monitoring purpose of water pollution (Agradi *et al.*, 2000). Research has shown that Glutathione S-transferase activity involved in defense against oxidation stress could reveal effective biochemical markers of toxic effect (Petrivalsky *et al.*, 1997). Esterases can be used as a biomarker for the random use of insecticides in an aquatic system especially when the risk of contamination of non-target organism is involved (Ozmen *et al.*, 1999., Brewer *et al.*,2001). Other biochemical parameters such as carboxyl esterase, lactate dehydrogenase, alkaline and aspartate aminotransferase as well as alkaline and acid phosphatase are considered useful biomarkers to determine pollution levels(Asztalos *et al.*, 1990; Raberg and Lipsky, 1997; Baron *et al.*, 1999., Basagalia, 2000). Some of these enzymes are perceived good bioindicators for animals chronically exposed to ocntaminants such as crude oil and heavy metals (Almeida *et al.*, 2001; Mazorra *et al.*, 2002).

There is dearth of published information in Nigeria on the effect of crude oil pollution on enzyme activities in *Heterobranchus bidorsalis*. This study, therefore was designed to investigate the effect of exposing *H. bidorsalis* adults to different concentrations of Bonny-light crude oil on bilirubin enzyme concentrations in the fish. The essence was to assess the response of this enzyme to varying concentrations of the oil pollutant and as an index to liver function. The significance of the possible use of bilirubin enzyme as a biomarker to determine oil pollution levels in Nigeria is discussed.

Materials and Methods

Eighteen (18) aerator –equipped, transparent plastic aquaria (55 x 30x 30cm^3) were randomly stocked with 360 adults of *Heterobranchus bidorsalis {* mean weight ± standard error of mean (s.e.m.) , 130.80± 0.36g} at 20 fish per aquarium. The experiment was designed to have 15 aquaria (5x3) with 25cm^3 dechlorinated tap water and with fish injected with doses of Bonny-light crude oil (BLCO) at 10.00, 20.00, 30.00, 40.00 and 50.00µl.g^{-1} i.e. T_1, T_2, T_3, T_4, and T_5 respectively. Three (3) aquaria had fish not injected with BLCO and served as the controls (0.00 µl.g^{-1})(T_5). The injection of fish with graded concentrations of BLCO was carried out with the aid of 2.50ml disposable hypodermic syringes, just below the dorsal fin.

The study was categorized into two sections namely: the oil exposure (toxicity) period and the post oil-exposure (recovery) period. The toxicity period lasted for 4 days, while the recovery period lasted for 42 days and was monitored fortnightly. At the end of the toxicity period, the surviving fish and plastic aquaria were washed and replenished with dechlorinated tap water. A 38% crude protein diet (Table 1a) was fed to the fish at 3% body weight per day (bwd^{-1}) during the toxicity period and at 5% bwd^{-1} during the recovery period. The proximate composition of the test diet (Table 1b) was carried out as described by Windham (1996). The filteration system of the aquarium aided in the collection of faeces and other residues. Records of the water temperature (26±0.60^{0}C) and pH(6.60±0.40) were taken with the aid of a maximum and minimum mercury-in-glass thermometer and a pH meter (Model Ph-l-20-l) respectively. The percent mortality (PM) and the percent survival (PS) of the fish were taken during the toxicity and recovery periods of the study.

Liver samples of fish from each triplicate treatment of BLCO and the control were dissected with sharp surgical blades and scissors and washed in distilled water to remove traces of blood. The liver samples were macerated and homogenized as described by Devi *et al* (1993) and placed in ice-cold 0.25M sucrose (Oluah *et al.*, 2005). The liver homogenate was centrifuged at 5000rpm for 15 minutes at 4^{0}C and the supernatant transferred to clean micrafuge tubes. The samples were stored at –80^{0}C until enzymatic assays were carried out(Ozmen *et al.*, 2005).

Serum bilirubin enzyme estimation, which served as the liver function test (LFT) , was carried out at the Cynbald Diagnostic Laboratory, Abakpa Nike-Enugu Nigeria. The serum estimation method was based on the principle that bilirubin enzyme reacts with diazol reagent in the presence of methanol to form a purple coloured compound of azobilirubin. The intensity of the colour was estimated colorimetrically and was directly proportional to the bilirubin enzymes present. Hence, at the Cynbald Laboratory, the stored liver supernatant was thoroughly mixed with diazol reagent, methanol and water in three clean test tubes and allowed to stand for 30 minutes. This mixture was later read in the colorimeter at 540nm. The values obtained were averaged. A blank estimation was also carried out with the liver supernatant, distilled water, diazo blank and methanol as the reagents. Calculations for the bilirubin enzyme concentration in each samples was expressed as:

$$\text{mg bilirubin/100ml} = \frac{Total\ Value}{S\tan dard\ value} \times \frac{4}{1},$$ where the total value of bilirubin enzyme concentration was obtained when the liver supernatant, diazo reagent, methanol and water were used for the test while the standard value was when distilled water was used as the blank.

The data obtained were subjected to analysis of variance (ANOVA)(Steel and Torrie, 1990) to determine statistical difference between treatment means. Simple percentages were used where appropriate to explain the analyzed data.

Results

The gross and proximate compositions of the diet fed to *H. bidorsalis* during the experimental period are shown on Tables 1a and 1b respectively. Table 2 shows the bilirubin enzyme concentrations (BEC)(mg/100ml) of the fish injected with 10.00-50.00 µl.g^{-1} BLCO and the control (0.00 µl.g^{-1}) during the toxicity and recovery periods. Table 3 shows the percent mortality and survival of the fish. The control fish recorded significant ($P < 0.01$) higher values of BEC in the livers than those injected with the various concentrations of BLCO (Table 2). This situation was prevalent both at the toxicity and recovery periods of the study. The BEC values in the control fish livers were relatively of the same magnitude; ranging between 0.94 to 0.97mg/100ml within both study periods (Table 2).

The BEC values in the livers of fishes injected with BLCO concentrations (10.00-50.00 µl.g^{-1}) decreased with increasing concentrations of oil injection (Table 2). Both at the toxicity and recovery periods of the study, 10.00 µl.g^{-1}BLCO concentration was associated with the highest bilirubin enzyme concentration in the fish liver when compared to the values recorded with the other BLCO concentrations (20.00-50.00 µl.g^{-1}) (Table 2). However, there were significant differences in the values of BEC in the fish livers as a result of fish injection with different concentrations of BLCO and the control ($P < 0.05$; $P < 0.01$) (Table 2).

Increases in the BEC values of fish livers were obtained from day 14 of the recovery period: irrespective of the BLCO doses to which the fishes were previously injected (Table 2). Significantly, BEC values were increased by 23% at day 14, 15% at day 28 and 5% at day 42. Despite these improvements in the concentrations of the bilirubin enzymes as the recovery period extended up to day 42, the highest BEC value recorded with the fish injected with 10.00 µl.g^{-1} BLCO(0.71±0.06mg/100ml) did not approximate the BEC value of the control fish on day 42 (0.94±0.05mg/100ml(Table 2).

The percent mortality (PM) and the percent survival (PS) of the fish, both at the toxicity and the recovery periods (Table 3) indicated that the fish injected between 40.00 µl.g^{-1} and 50 µl.g^{-1} BLCO concentrations recorded highest mortality and least survivals. Comparatively the least fish mortality was recorded with fish injected with 10. µl.g^{-1} BLCO (Table 3).

Table 1a. Gross Composition of Experiment Diet

Ingredients	%Composition
Yellow maize	9.29
Soybean meal	54.84
Fish meal	16.65
Blood meal	10.97
Palm oil	5.00
Salt	0.25
Vitamin mix [1]	0.60
Mineral mix [2]	2.40
Total	100.00

[1]Vitamin mix provided the following constituents diluted in cellulose (mg/kg of diet): Thiamin, 10; riboflavin, 20; pyridoxin, 10; folacin, 5; pantothenic acid, 40; chorine chloride, 3000; niacin, 150; vitamin B12, 0.06; retinyl acetate (500,000 lu/g),6; menadione-Na-bisulphate, 80; inositol, 400; biotin,2; vitamin C, 200; alpha tocopheral, 50; cholecalipherol (1,000,000 lu/g).

[2]contained as g/kg of premix: $FeSO_4.7H_2O$,5; $MgSO_4$. $7H_2O$, 132; K_2SO_4, 329.90; KI, 0.15; $MnSO_4$. H_2O, 0.7; and cellulose, 380.97

Table 1b. Proximate Composition of Experimental Diet

Nutrient	%Composition
Crude protein	37.58
Ether extract	5.18
Ash	10.48
Dry matter	11.80
Nitrogen free extract	34.46
Total	100.00

Discussion

Various enzymes have been used as stress indicators or general biomarkers of stress to fish in polluted waters. Foremost among these enzymes are: lactate dehydrogenase(LDH), aspartate amino-transferase (AST), carboxyl esterase(CE) and acid phosphastase (ACP). These enzymes have been employed for diagnosing liver, muscles and gill damages caused to fish by pollutants (Neff, 1985). Reports showed that the activities of these enzymes decreased with increasing concentrations of metal such as cadmium, copper and lead in water (Ozmen *et al.*, 2005). The concentration of bilirubin enzymes (BEC) in *H. bidorsalis* adults of this study decreased with increasing concentrations of BLCO (Table 2) and agrees with Ozmen *et al.* (2005) report for other pollutants. Oluah and Njoku (2001) observed a linear relationship between tissue glucose levels in *Clarias gariepinus* and paraquat (a herbicide) concentration in water. Simon *et al.* (1983) had earlier obtained similar results when *Cyprinus carpio* was exposed to paraquat. All these results point to the fact that enzymatic response to the concentrations of pollutants in water is dose-dependent.

Table 2. Bilirubin Enzyme Concentration (mg/100ml) in *H. bidorsalis* Adults Injected with Different Concentrations of Bonny-light Crude Oil

Study Period	Overall Mean $\bar{x}$	Duration (Days)	Crude oil type	BLCO Concentration(µ/g^{-1}) 10.00	20.00	30.00	40.00	50.00	0.00
Toxicity period	0.34±0.03	4	BLCO	0.48^{a}±0.03	0.29^{b}±0.01	0.17^{c}±0.01	0.10^{c}±0.01	0.06^{d}±0.03	0.96^{e}±0.06
Recovery period	0.39±0.04	14	BLCO	0.59^{a}±0.04	0.36^{b}±0.02	0.21^{c}±0.02	0.12^{d}±0.01	0.08^{e}±0.03	0.95^{f}±0.07
	0.42±0.03	28	BLCO	0.68^{a}±0.04	0.41^{b}±0.02	0.24^{c}±0.01	0.14^{d}±0.02	0.09^{e}±0.04	0.97^{f}±0.06
		42	BLCO	0.71^{a}±0.06	0.43^{b}±0.02	0.25^{c}±0.03	0.15^{d}±0.02	0.10^{d}±0.01	0.94^{e}±0.05	0.43±0.03

[1]Bonny-light crude oil; Numbers in the same row with similar superscripts are not significantly different (P>0.05); Numbers in the same row with different superscripts differ significantly (P< 0.05,P<0.01).

Table 3: Percent Mortality and Survival of *H. bidorsalis* Adults Injected with Different Concentrations Bonny-light Crude Oil within 4 Days (Toxicity) and 42 Days (Recovery) Periods .

Study Period	Duration (Days)	%Mortality **BLCO1 Concentrations (mlL^{-1})** →					Control		% Survival **BLCO Concentration (mlL^{-1})** →						Control
		0.050	1.00	2.00	4.00	8.00	0.00		0.50	1.00	2.00	4.00	8.00	0.00	
Toxicity period		4	2.00	5.00	5.00	40.00	50.00	0.00		98.00	95.00	95.00	60.00	50.00	100.00
Recovery Period		14	2.00	3.00	4.00	32.00	40.00	0.00		98.00	97.00	96.00	68.00	60.00	100.00
	28	1.00	2.00	2.00	24.00	36.00	0.00		99.00	98.00	98.00	76.00	64.00	100.00	
	42	0.00	1.00	1.00	16.00	26.00	0.00		100.00	99.00	99.00	84.00	74.00	100.00	

[1]Bonny-light crude oil.

This assertion was exemplified in this study by the significant decreases (P <.0.05; P <0.01)(Table 2) in the BEC values of fish livers as the dose concentrations of BLCO increased from 10.00 µl.g^{-1} to 50.00 µl. g^{-1}. Increases in BEC values during the recovery periods at day (14) (23%), day 28(15%), and day 42(5%) (Table 2) implies that the previous injections of the fish with BLCO had tremendous effects on the activities of bilirubin enzymes within the liver. This result agrees with the suggestion proferred by Ozmen *et al.*(2005) on the necessity for a comparative monitoring of biomarkers on pathological changes in liver tissues in order to use good enzymatic markers as indicators of organ dysfunction. Although this study recorded percent increases in the values of BEC as the recovery period progressed from day 14 to day 42 (Table 2), such increases were also influenced by the concentrations of BLCO previously injected into the fish. This results is therefore consistent with Ozmen *et al.* (2005) which stated that cholinesterase (ACHE) activities in *Cyprinus carpio* during recovery from pesticide exposure may be influenced by the concentration of previous exposure to the pollutant. Sancho *et al. (*2000), nonetheless, reported that in animals previously exposed to pesticides in their environment and later transferred to clean water, ACHE concentration was found to be reduced.

Ozmen *et al.* (2005) stated that although some biomarkers such as CE, LDH, ACP, AST, and ACHE are being used world wide in several pollution monitoring-programmes, some enzymes' activities still require further research before they can be used routinely in pollution biomonitoring. The bilirubin enzyme is one such enzyme that needs further research because of its activities in the fish liver.

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