Lipid Peroxidation and Antioxidant Biomarker Activities as Indicator of Pollution in Blue Crab *Callinectes amnicola* from Lagos lagoon

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ABSTRACT
Lipid peroxidation and antioxidants biomarker activities in blue crabs *Callinectes amnicola* sampled from Makoko (receives domestic waste), Ibese (receives textile effluent) and Ajah (control station) were investigated in Lagos Lagoon for 18 months between February 2010 to August 2011. The gills and muscle of blue crabs were used for the biomarker analysis. The activities of lipid peroxidation (MDA), superoxide dismutase (SOD) and reduced glutathione (GSH) were investigated in the tissues of blue crabs. There was an increase in lipid peroxidation activity in the tissues of *Callinectes amnicola* from the entire stations sampled. In contrast, the superoxide dismutase (SOD) and reduced glutathione (GSH) activities were insignificantly low ($p<0.05$) in gills of blue crabs sampled from Ibese. Oxidative stress was generated in tissues of the blue crabs in the entire station. However, the overall increase in lipid peroxidation can explain the oxidative stress and the equivalent oxidative damage observed in the muscle and gills of *C. amnicola*. The induced oxidative stress observed in muscle and gills of the blue crabs in this study confirmed the fact that those stations are highly polluted, in other words, Lagos lagoon is polluted. Hence, there is need for regular monitoring of pollution status in the Lagos lagoon.

Keywords: Reduced Glutathione, Oxidative damage, Superoxide dismutase (SOD), Gills, Muscle.

INTRODUCTION
Lagos lagoon is the largest of the eight lagoons that make up the lagoon systems of Nigeria and probably the most exposed to anthropogenic influence. The pollution status of the Lagos lagoon is generally attributed to the direct discharge of wastes (domestic and industrial etc) due to the high level of urbanization and industrialization of the city of Lagos and its environs. Since the later part of the 19th century, the Lagos lagoon has served as the ultimate sink for disposal of untreated domestic sewage (Ajao and Fagade, 1990).

Lagos lagoon has continued to be under pressure from pollution, such as from petrochemical materials (Ekundayo, 1977), Untreated sewage and sludge (Akpata and Ekundayo, 1978; Akpata, 1987 and Ajao, 1990), faecal pollution and discharge of bio-degradable wood wastes from sawmill located along the lagoon (Ekundayo, 1977 ; Nwankwo *et al.*, 1994; Nwankwo, 1998. Akpata, 2002), industrial effluent (Oyewo, 1998; Amund, 2000).

Living marine resources; including fish, shell fish, benthic organisms etc, have been affected by the state of pollution and they have been used immensely by ecotoxicologist for pollution assessment and monitoring in the aquatic environment. Shell fish, including crabs are utilized as food and are also a source of protein for people in the Nigerian coastal states thus, their states of health are important to public health. They have been used for monitoring pollutants in aquatic environments (Eickhoff *et al.*, 2003, Dugan *et al*., 2005, Koenig et al., 2008; Dissanayake *et al*., 2008; Morales-Caselles *et al*., 2008).

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Biomarkers are measurements in body fluids, cells or tissues indicating biochemical or cellular modifications due to the presence and magnitude of toxicants, or of host response (NRC, 1987). Examples include superoxide dismutase (SOD), catalase (CAT), glutathione transferase(GT), glutathione peroxidase(GPx), reduced glutathione(GSH) and lipid peroxidation(LPX) etc. They are used after exposure to environmental or anthropogenic sources of pollution, to elucidate cause-effect and dose-effect relationships in health risk assessment, in clinical diagnoses and for monitoring purposes. Generally, biomarker responses are considered to be intermediates between pollutant sources and higher-level effects (Suter, 1990). When these compensatory responses are activated, the survival potential of the organism may already have begun to decline because the ability of the organism to mount compensatory responses to new environmental challenges may have been compromised (Depledge and Fossi, 1994). The most compelling reason for using biomarkers is that they can give information on the biological effects of pollutants rather than a mere quantification of their environmental levels.

Lipid peroxidation reflects oxidative damage to lipid-rich components such as cell membranes that occurs as a result of increased OH• radicals, especially in the presence of elevated levels of redox reactive metals such as copper, zinc, iron, chromium etc. Moreover, the free radical induce damage and propagates additional cytotoxic products that can damage DNA and enzymes (Kehrer, 1993; Yu, 1994). Increased lipid peroxidation has been demonstrated in response to contaminant exposures in fish and bivalves (Di Giulio et al. 1989; Viarengo et al., 1990; Ringwood et al., 1998b; Livingstone, 2001).

Superoxide dismutase (SOD) constitutes the first most important defense line. These virtually exist in all organisms, and its major function is to scavenge superoxide anion. SOD catalyses the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide (McCord and Fridovich, 1969).

$2O_2 - + 2H+ \rightarrow H_2O_2 + O_2$

The SODs are a group of metalloenzymes that catalyse the conversion of reactive superoxide anions (O_2^-) to yield hydrogen peroxide H_2O_2, which in itself is an important ROS as well. H_2O_2 is subsequently detoxified by two types of enzymes: CATs and glutathione dependent peroxidases (GPOXs).

Glutathione (GSH) is an abundant tripeptide that is regarded as one of the most important "first-line" defense mechanisms of cells. Glutathione is the most abundant intracellular thiol and an important antioxidant detoxification mechanism. Potential oxidative damage associated with metal exposures can be ameliorated when GSH binds metals and effectively sequester them from interaction with other cellular components, thereby potentially reducing adverse effects. Animal cells may respond to contaminants by increasing GSH levels as well as other detoxification mechanisms such as metallothioneins in an effort to reduce adverse effects. However, if the detoxification mechanisms are overwhelmed, the GSH production and recycling may be impaired, leading to decreased or depleted GSH levels. Therefore elevated GSH levels can indicate that organisms are exposed to a pollutant, but ultimately adverse effects are associated with GSH depletion as the detoxification mechanisms are overwhelmed. Glutathione depletion has been observed in mammalian systems as well as marine organisms, and it has been hypothesized that GSH depletion is both a signal of stress and a predisposing factor for increased adverse effects (Meister and Anderson, 1983; Viarengo et al., 1990; Regoli and Principato, 1995). Eventually these cellular stress effects would be reflected in more general physiological effects such as decreased growth and reproduction, and ultimately cause significant effects on the sustainability of organisms' populations. The purpose of this study was to determine the biomarker activities in tissues of blue crabs *Callinectes amnicola* to assess the potential biological effects of heavy metal and organic pollution in the Lagos lagoon system.

For this assessment, crabs were sampled from Makoko and Ibese which receives domestic waste and textiles effluents respectively and Ajah as control site, from February 2010 to August, 2011 in order to evaluate the effects on the accumulation of the pollutants in tissues, cellular biomarker responses, and overall physiological condition.

MATERIALS AND METHODOLOGY

Blue crabs *Callinectes amnicola* were sampled monthly for 18 months between February 2010 to August 2011 from 3 stations on Lagos lagoon (Makoko, Ibese and Ajah as shown in figure 1) in Lagos, Nigeria. Makoko receives domestic waste, Ibese receives industrial effluents, but Ajah is devoid or with little waste. They were transported to the laboratory where the mophometric and meristic features were measured and dissected after identification with FAO identification guide. The gills and muscle of the *C. amnicola* sample were taken and preserved at -20^{0}C for further analysis.

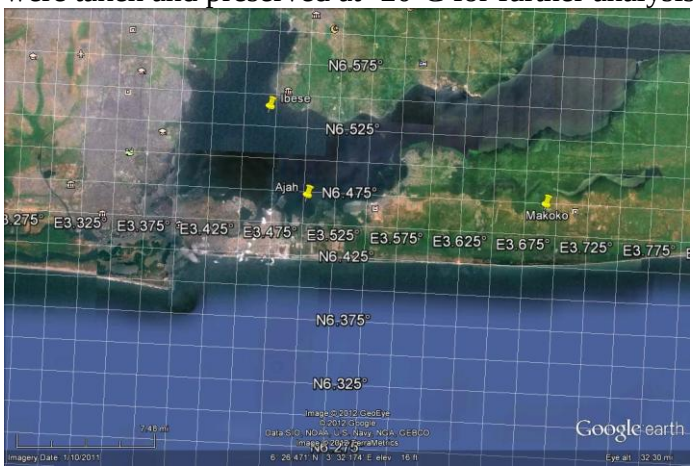

Fig I shows the map of Lagos lagoon showing the sampling stations

Biomarker analysis: The oxidative stress and damage was investigated in the gills and muscles of *Callinectes amnicola* by the measurement of the activity of the selected antioxidant biomarkers; Lipid Peroxidation (MDA), Reduced Glutathione (GSH) and Superoxide dismutase (SOD).

Homogenization: 0.5g of the muscle and gills of *Callinectes amnicola* were homogenized with 5ml of 0.4 M Phosphate buffer using pestle and mortal. The organism homogenate was centrifuged at 3000 r.p.m for 15minutes and the supernatant samples were stored at -20^{0}C for biochemical analysis. This is the first step in biomarker analysis and it was repeated in all the sample of blue crabs used for this study.

Reagent Preparation and Procedure

Lipid peroxidation **(LPX)**

The lipid peroxidation level was measured in malondialdehyde (MDA) concentration in tissues of *C. amnicola* by the method of Niehaus and Samuelsson, (1968). 0.1ml of tissue homogenate (Tris-Hcl buffer, pH 7.5) was treated with 2ml of (1:1:1 ratio) TBA-TCA-HCl reagent and place on water bath for 15min, cool and centrifuge at room temperature for 10min at 3,000 rpm. The clear supernatant was transfer into 1.5ml cuvette and the absorbance was measured against reference blank at 535nm using spectrophotometer.

Reduced Glutathione **(GSH)**

Reduced glutathione (GSH) activity was determined by the method of Ellman, (1959). 3ml of the 10% TCA was added to 3ml of homogenate and centrifuge at 3000rpm for 10min. Then, 1.0 ml of supernatant was treated with 0.5ml of Ellmans reagent and 3.0ml of phosphate buffer(0.2M, pH 8.0), before the absorbance was read at 412nm using spectrophotometer.

Superoxide Dismutase (SOD)

Superoxide dismutase activity was determined by measuring the inhibition of auto-oxidation of epinephrine at PH 10.2 at 30^{0}C as described by McCord and Fridovish, (1989). 3.0ml of Na_2CO_3 buffer was added to 0.02ml of tissue homogenate (Tris-Hcl buffer, pH 7.5) and treated with 0.03ml epinephrine reagent and centrifuge at room temperature for 10min at 3,000 rpm. The clear supernatant was transfer into 1.5ml cuvette and the absorbance was measured against reference blank at 480nm using spectrophotometer.

Total Protein

Total protein was measured by the method of Lowry et al., (1951). The diluted biuret reagent was added to 0.02ml of the samples**,** while blank reagent was used for the preparation of the protein standard and left at room temperature for 10 minutes. The clear supernatant was transferred into 1.5ml cuvette and the absorbance was measured against reference blank at 546 nm using spectrophotometer.

Data Analysis

The System Analysis System 9 (SAS) statistical package was used for the analysis. The data were analysed using T-test to ascertain if there is significant difference and relationship between the activity of the selected antioxidant biomarkers (SOD, GSH, and MDA) in *Callinectes amnicol*a from polluted sites and control site. Also, to investigate the oxidative stress generated in the gill and muscle of *Callinectes amnicola* at $p< 0.05$.

RESULT

Laboratory Analysis

Superoxide Dismutase (SOD)

Mean activities of antioxidant biomarkers are shown in Tables 1 and 2. The mean SOD activities at Makoko were found to be insignificantly higher ($P< 0.05$) in muscle and gill of the crabs when compared to mean activities in crabs from the control site (Ajah site) Fig 1. However, there was a insignificant difference $p<0.05$ in gills of crabs sampled from Ibese when compared to mean activities in crabs from Ajah (fig 2), though the SOD activities was higher in Ajah than ibese in gills and muscle. The activity of SOD was generally low in all the tissues; gills and muscle. ***Reduced Glutathione (GSH)***

The activity of GSH was observed to be insignificantly low ($p<0.05$) in all the tissues of Crabs collected from the sample sites; Makoko, Ibese and Ajah (Table1 and 2). The activities of GSH in muscle of crabs collected from both Makoko and Ibese is insignificantly higher ($p<0.05$) compared to Ajah, however, in gills reverse is the case.

Lipid peroxidation (MDA)

Levels of MDA in blue crabs tissues are shown in table 1 and 2 below. The mean values of MDA in gills and muscle of crabs were insignificantly higher ($p<0.05$) in all the sample sites. However, the level of MDA in muscle of blue crabs was higher when compared to gills. The MDA level in gills of blue crabs sampled from Makoko and Ibese were found to be higher when compared with the respective values from Ajah.

Table 1: Lipid peroxidation and antioxidant activity in the Muscle of *Callinectes amnicola* (Blue crab) collected from Makoko, Ibese and Ajah in Lagos lagoon.

Parameters	**Makoko**	**Ibese**	**Ajah**
SOD(Umol/mgprotein)	307.57±431.96	243.76±256.88	275.85±249.24
GSH(µmol/ml)	0.63±0.42	0.55±0.35	0.54±0.28
MDA(Umol/ml)	26.01±12.87	29.92±17.22	27.18±17.26
* = significant difference			

Table 2: Lipid peroxidation and antioxidant activity in the Gills of *Callinectes amnicola* (Blue crab) sampled from Makoko, Ibese and Ajah in Lagos lagoon.

Parameters	**Makoko**	**Ibese**	**Ajah**
SOD(Umol/mgprotein)	381.23±552.39	197.47±243.6	237.56±220.16*
GSH(µmol/ml)	0.41±0.23	0.41±0.2	0.42±0.19*
MDA(Umol/ml)	27.80±21.15	25.64±13.19	24.58±13.64
* = significant difference			

DISCUSSION

The activities of antioxidant biomarkers; SOD and GSH in tissues of *C. amnicola* investigated in this study was generally low in all the polluted sites, hence the reason for higher concentration or activities of lipid peroxidation in tissues of *C. amnicola* in all sites, this was also parallel with the studies of Sole et al. (1996) who have demonstrated the reduction of antioxidants level in the mussel M. edulis exposed to the Aegean sea oil spill.

In tables 1 and 2, Superoxide dismutase (SOD) concentration in gills and muscle of *C. amnicola* were very low, this was also reported by Neves et al., (2000), Vijayavel et al., (2004).

SOD is the first enzyme to deal with oxyradicals (Kappus, 1987; Viarengo, 1989 and Vijayavel, 2004) by accelerating the dismutation of superoxide (O_2^-) to H_2O_2 which damages the membrane and biological structures. Thus it is understood that increased intracellular ROS activities (due to heavy metal contamination and organic pollution) leads to decreased concentration of SOD observed in this study. However, the significant decrease of SOD showed in gills of *C.amnicola* from Ijora-iddo axis in this study, decrease in antioxidant enzymes in gill of fish sampled from Lagos lagoon was also reported by Doherty et al., (2010). This explained the function of gills in this study; they are more exposed to contaminated water and as such metal can penetrate through their thin epithelial cells (Nwaedozie, 1998). Gill a first point of contact with environmental xenobiotics, indicates gill is a sensitive biochemical indicator of environmental pollution in fishes as reported by Kono and Fridorich, (1982). However, decrease in antioxidant biomarker found in the gills of *C. amnicola* in this study could account for the marked lipid peroxidation observed.

Reduced glutathione (GSH) is considered one of the most important antioxidant agents involved in protection of cell membranes from lipid peroxidation by scavenging oxygen radicals(yielding glutathione disulfide, GSSG) (Meister,1983). Moreover, glutathione is the cofactor of many enzymes catalyzing the detoxification and excretion of several toxic compounds. From the result, low GSH concentration or activities observed in tissues (gill and muscle) suggest the toxicity encountered in the blue crab from the sites due to metal contamination and organic pollution as it was also reported by Vijayavel et al., (2004) who demonstrated sub-lethal effect of Naphthalene on lipid peroxidation and antioxidant status in the edible crab *Scylla serrata* and Suhel et al., (2006) who worked on biomarkers of oxidative stress in *Wallago attu* during and after a fish-kill episode at Panipat, India. Low significant difference ($p<0.05$) of GSH concentration observed in gills of *C. amnicola* from okobaba (due to heavy metal contamination) may be due to the enhanced oxidative damage due to free radicals, which agrees with the findings of Doyotte et al., (1997) in aquatic invertebrates exposed to trace metals.

However, the higher biomarker activities that was observed in muscle compared to gill, may be due to the rate of metabolism in crab or attributed to the rate of bioconcentration of the heavy metals in the muscle of the crab, crab do not metabolise and excrete xenobiotics from tissue as rapid as fish. They are closely in contact with sediments, assuming that concentrations of contaminants in crabs generally reflect the sediment is reasonable. Hence, they are important benthic species for pollution assessment.

The low concentration of the antioxidant biomarker (SOD and GSH) observed in gills and muscle of blue crab in this study might be the reason for elevated concentration of lipid peroxidation found in the tissues of blue crab. Several studies have shown enhanced lipid peroxidation in aquatic organisms exposed to high concentrations of pollutants (Thomas and Wofford, 1984; Gabryelak and Klekot, 1985; Viarengo et al., 1989; Ribera et al., 1991) and of pollutants in contaminated sediments (DiGiulio etal., 1993; Livingstone, 1993; Sole et al., 1996). Elevated lipid peroxidation was observed by Wilhelm Filho et al. (2001) in cichlid fish

taken from polluted sites, compared to clean sites. Oakes and Van Der Kraak (2003) and Oakes et al., (2004) have demonstrated increase in lipid peroxidation in gonads of white suckers exposed to pulp and paper mill effluents as well as municipal sewage treatment plant effluents. Increase in lipid peroxidation was observed in naphthalene exposed marine crab Scylla serrata by Sole et al. (1996).

Lipid peroxidation expresses the oxidative damage in a biological system. Oxidative damage set in when there is no equilibrium between the reactive oxygen species (ROS) generated as a result of bioaccumulation of contaminant and the antioxidant biomarker response. Alternatively, the ROS overwhelm the production of antioxidant biomarkers. The elevated lipid peroxidation concentration observed in this study due to pollutants exposure might be due to the microsomal metabolism of xenobiotics and microsome mediated redox cycling which gives rise to oxyradicals capable of oxidizing membrane lipids. This indicates that some cell damage may be occurring since the increase is insignificant. However, oxidative stress was observed in tissues (gill and muscle) of blue crab *C. amnicola* in this study.

Oxidative stress has been implicated in several pathologic conditions in mammals (e.g. mutagenesis, atherosclerosis, ischaemia-reperfusion, inflammation, etc.) (Davies 1994) and in molluscs and fish (Dio Giulio et al. 1989, Bainy et al. 1996), there is an interest in the antioxidant systems in crustaceans. Various stress responses have been observed in crustaceans. These include black gill syndrome, molt retardation, and disoriented behavior as a consequence of aquatic pollution (Sindermann 1996). These effects can render animals more susceptible to parasitic infections that may affect the health of whole population. Oxidative stress may also result when oxygen availability is low (Storey 1996), and in response to various chemical compounds (xenobiotics) (Videla et al. 1995, Bainy et al. 1996).

In conclusion, the present study confirmed the use of biomarkers activities in assessment of the potential biological effects of heavy metal and organic pollution in tissues (gills and muscle) of blue crab *Callinectes amnicola*. The oxidative stress generated in the tissues due to exposure to pollutants or other xenobiotics from Makoko and Ibese which causes the production of potent oxidants and free radicals capable of damaging important cell components such as proteins, lipids and DNA. The induced oxidative stress observed in muscle and gills of the blue crabs in this study established the fact that those station are highly polluted, in other words, Lagos lagoon is polluted. Hence the result obtained from this research can be use as a base line data for the usage of crab as a sentinel for pollution assessment in Lagos lagoon and also as guide for biomonitoring to assess toxicity level in aquatic environment and crab state of health for consumption along the food chain. Measurement of antioxidants biomarkers such as SOD, GSH activities and lipid peroxidation may be promising indicators of an oxidant impact on aquatic organisms. Biomarkers have the advantage of providing a quantitative response as well as valuable information of ecological relevance on the chronic adverse effects caused by water pollution. The high level in MDA of the crabs sampled from the polluted sites compared with the relatively control sites also confirmed that the Lagos lagoon is polluted.

Lastly, links between oxidative stress responses and damage at higher levels of biological organization would be valuable for water quality protection efforts, as aquatic biomonitoring is aimed at protecting populations, communities and ecosystems, not only individual health.

It is however recommended that antioxidant biomarkers should be used for environmental monitoring in aquatic environment, since it gives an early warning signal of effect of xenobiotics on aquatic organisms at molecular level which help to prevent the effect at organismal level.

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