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Haemoglobin Oxidation: A possible mechanism of crude oil toxicity in fish

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Abstract

Haemoglobin oxidation occurs in erythrocytes. It involves the loss of an electron from the ferrous (Fe^{2+}) ion to the ferric (Fe^{3+}) state in the presence of an oxidant. Crude oil, which contains polycyclic aromatic hydrocarbons (PAHs), has the capacity of serving as oxidants to fish haemoglobin to yield the methaemoglobin (Fe^{3+}) species which has lost the capacity to bind oxygen. This study reveals that various quantities (20, 60, 100 and 200 µl) of crude oil used to contaminate and incubate fish haemoglobin at 25 °C increased the methaemoglobin absorbance at 630 nm wavelength due to methaemoglobin (Fe^{3+}) while there was a decrease at 540 nm and 577 nm. Since polycyclic aromatic hydrocarbons (with alkylated side chains) are the only component in crude oil that is absorbed by fish, it is suggestive that these compounds may be responsible for haemoglobin oxidation in fish.

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Introduction

Haemoglobin is an iron-protein compound found in erythrocytes that binds and delivers oxygen to various tissues of an organism (Onwubiko *et al*., 1982; Boyiri *et al*., 1995). It has been identified as one of the most important haem-protein in nature (Hall and Gray, 1929). The delivery of oxygen to the various specialized cells of the organism regulates the production of most of the organism's energy in the form of adenosine triphosphate (ATP) because oxygen is the final electron acceptor in the electron transport chain (Onwubiko *et al*., 2000). Thus, the inability of an organism to capture and deliver oxygen to cells leads to the loss of the organism's capacity to obtain energy from consumed foods which can halt the metabolism and in severe cases, could lead to death of the organism (Onwubiko *et al*., 2000). Cell asphyxiation could occur when the capacity of the haemoglobin to deliver oxygen to cells is reduced via the process of oxidation of the haem-bound ferrous iron at the ligand binding site (Wallace *et al*., 1982).

Studies carried out on the mechanism of oxidation of haemoglobin show that there is a loss of an electron from ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron in the presence of an oxidant (Jaffe and Neumann, 1964; Misra and Fridovich, 1972; Rifkind, 1974; Brunori *et al*., 1975; Wallace *et al*., 1978; Jarolim *et al*., 1990; Reza *et al*., 2002). Crude oil, which contains polycyclic compounds has the capacity of serving as oxidants to fish haemoglobin to yield the methaemoglobin species. Haemoglobin oxidation could be one of the reasons why fishes and other marine organisms suffocate by thick sludge of oil on water surface from crude oil pollution. Frequent spillages of crude oil and its products in creeks and rivers as reported by Ekweozor (1989) have resulted in a marked reduction in the number of both freshwater and marine creatures.

In the present study, we have determined the toxic effect of crude oil on marine life with haemoglobin oxidation as a possible mechanism. This was done by the characterization of some freshwater fish haemoglobin contaminated with crude oil using UV-visible spectroscopy.

The results provide useful information on the toxicity of crude oil on marine organisms and the mechanism of its action.

Materials and methods

Study location

This research was conducted at the Department of Biochemistry postgraduate laboratory, University of Nigeria, Nsukka, Enugu State, Nigeria. Crude oil (Akwa-Ibolite) was procured from the *Ebok* field (Afren Nigeria) in Akwa Ibom State, Nigeria. Three freshwater fish species including Nile Tilapia (*Heterotis niloticus*) and two African Catfish species (*Clarias gariepinus* and *Chrysichthys nigrodigitatus*) were used for the experiment. *Clarias gariepinus* was sourced from Imo River at Oyibo, Rivers State while *Heterotis niloticus* and *Chrysichthys nigrodigitatus* were sourced from Anambra River at Otu-Ocha, Anambra State, Nigeria.

Isolation of fish haemoglobin

The isolation of fish haemoglobin was carried out following the method of Onwubiko *et al*. (2000). Blood samples were drawn from the heart region of the fish species using syringe with needle and preserved in vials containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The blood cells were then washed with normal saline solution three times, and thereafter centrifuged at 4,000 rpm for 10 minutes. The fish red blood cells (pellets) were then collected and lysed with distilled water for 30 minutes before centrifuging at 4,000 rpm for another 30 minutes. The supernatant, containing the haemoglobin, was collected and further purified by gel chromatograghy technique with Sephadex G-25 gel which had been equilibrated with 10 mM potassium phosphate buffer (pH 7.8). The pellets which contained membrane structures were then discarded.

Crude oil contamination

The purified fish haemoglobin samples of the species were put in vials containing EDTA. Each haemoglobin sample was divided into five portions where the various levels of crude oil contamination (the control, i.e., no contamination, 20 µl crude oil, 60 µl crude oil, 100 µl crude oil, and 200 µl crude oil) were applied.

Spectroscopy readings

The absorbance of purified haemoglobin was read using JENWAY 6405 UV/visible spectrophotometer after calibrating the instrument with phosphate buffer of pH 7.8. The absorbance of the contaminated haemoglobin samples with varying concentrations of crude oil was taken after calibrating the instrument with the same phosphate buffered solution.

Results

The effects of the various concentrations of crude oil on the haemoglobin of African catfish species (*Chrysichthys nigrodigitatus*, *Clarias gariepenus*) and Nile tilapia, *Heterotis niloticus* are shown in the figures 1 to 9 below. For *Chrysichthys nigrodigitatus* (Figure 1), when 20 µl crude oil was used to contaminate the haemoglobin, the concentration of haemoglobin at 540 nm and 577 nm bands decreased from 0.037 to 0.033 mM and 0.033 to 0.030 mM, respectively, while the absorbance at 630 nm band which characterizes the methaemoglobin (Fe^{3+}) species, increased from 0.006 to 0.015 mM. When contaminated with 60 µl crude oil (as shown in Figure 2), the concentration at 540 nm, 577 nm and 630 nm bands increased from 0.037 to 0.040 mM, 0.033 to 0.036 mM, and 0.006 to 0.010 mM, respectively. For 100 and 200 µl crude oil contaminations, the same pattern as shown in Figure 2 was observed. Regardless of the increase in the oxyhaemoglobin species, the difference in concentration was not substantial compared to that of the methaemoglobin species. This clearly shows haemoglobin oxidation.

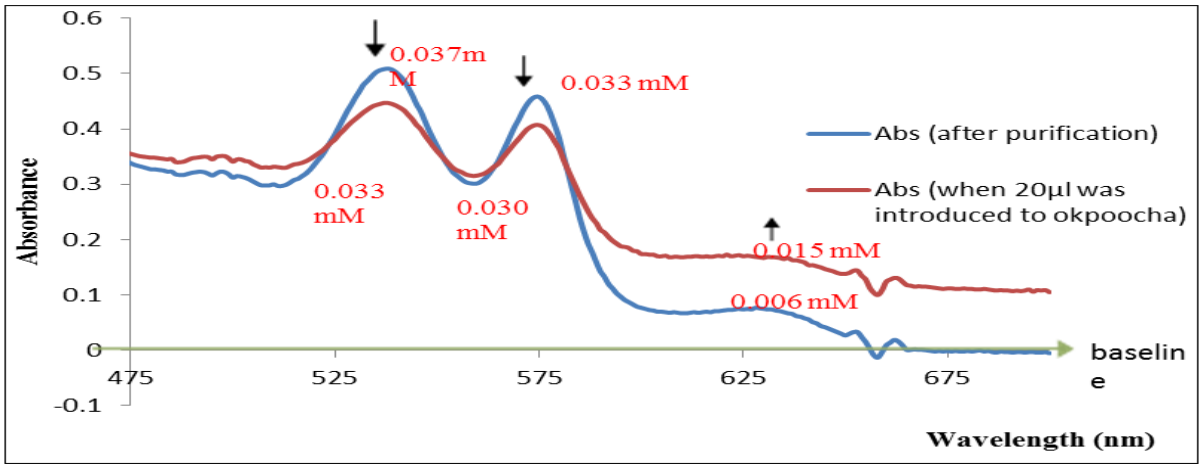

Fig. 1. Effect of 20 µl crude oil on the haemoglobin of *Chrysichthys nigrodigitatus* at concentration 0.037 mM in phosphate buffer of pH 7.8 with baseline at zero.

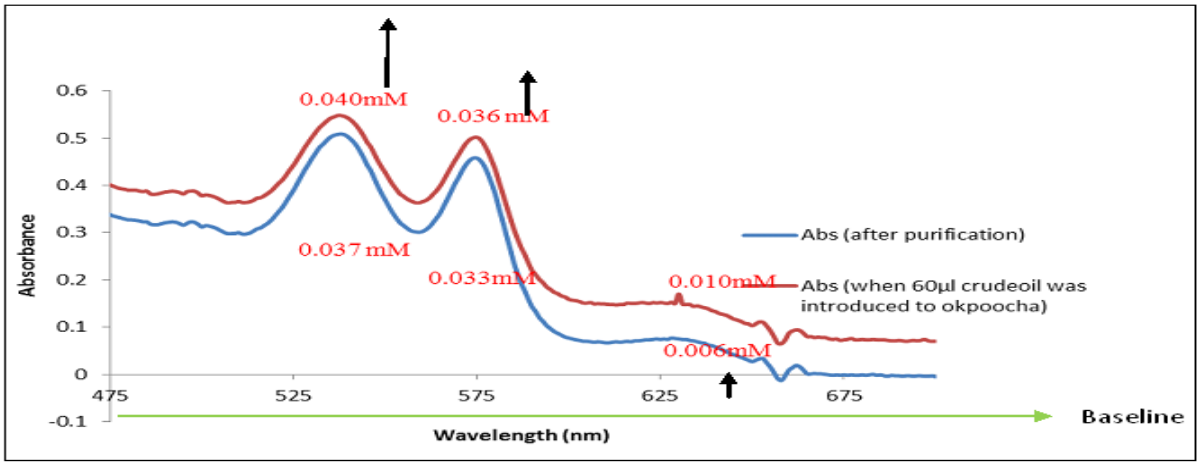

Fig. 2. Effect of 60 µl crude oil on the haemoglobin of *Chrysichthys nigrodigitatus* at concentration 0.006 mM in phosphate buffer of pH 7.8 with baseline at zero.

Figure 3 shows the effect of 20 µl crude oil on the haemoglobin of *Clarias gariepenus*. A decrease in the 540 and 577 nm bands with no difference in the 630 nm band was observed. For the 60 µl crude oil contaminated haemoglobin, a decrease from 0.064 mM to 0.062 mM for the 540 nm band and 0.059 mM to 0.058 mM for the 577 nm band was observed. There was also an increase from 0.001 mM to 0.007 mM in the 630 nm band (Figure 4).

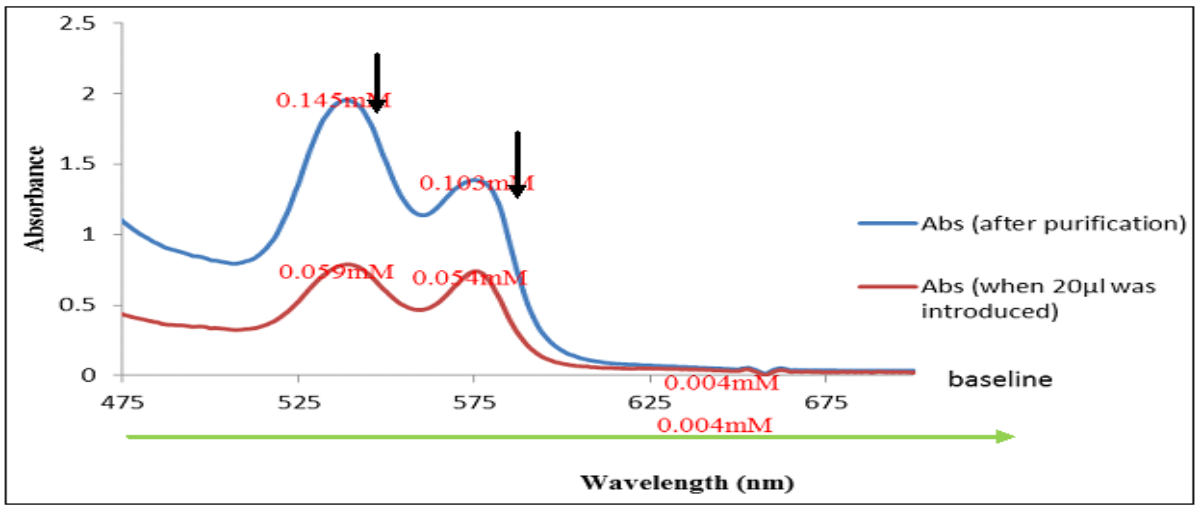

Fig. 3. Effect of 20 µl of crude oil on the haemoglobin of *Clarias gariepenus* at concentration of 0.145 mM in phosphate buffer of pH 7.8 with baseline at zero.

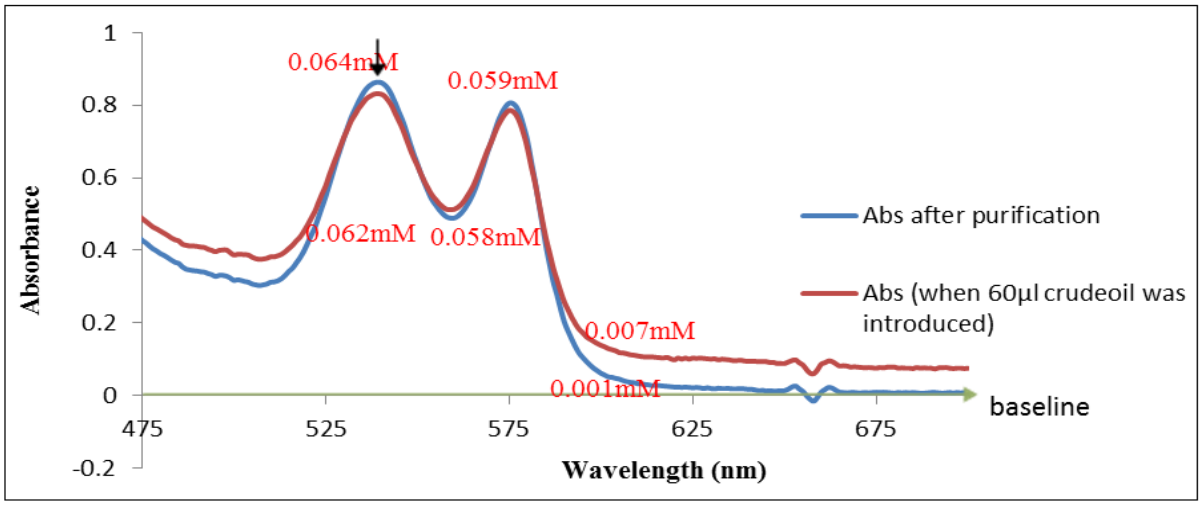

Fig. 4. Effect of 60µl crude oil on the haemoglobin of *Clarias gariepenus* at concentration of 0.001 mM in phosphate buffer of pH 7.8 with baseline at zero.

The same graph pattern in Figure 4 was observed when 100 µl crude oil was used for the contamination. For the 200 µl crude oil contaminated haemoglobin, a decrease from 0.145 mM to 0.072 mM for the 540 nm band and 0.103 mM to 0.066 mM for the 577 nm band was observed. There was also an increase from 0.010 mM to 0.011 mM for the 630 nm band (Figure 5). Figure 6 shows the effect of 20 µl crude oil on the haemoglobin of *Heterotis niloticus*. An increase in all bands was observed. For 60 µl crude oil haemoglobin contamination, a prominent decrease from 0.042 mM to 0.026 mM for the 540 nm band and 0.041 mM to 0.025 mM for the 577 nm band was observed. The 630 nm band increased from 0.002 mM to 007 mM (Figure 7). For the haemglobin contaminated with 100 µl crude oil,

the same graph pattern as shown in Figure 6 was observed, whereas the haemoglobin contaminated with 200 µl crude oil had the same graph pattern as shown in Figure 7. Due to the inconsistencies in the pattern of graph exhibited by some of the crude oil contaminated haemoglobin, further studies was carried out to check the effect of time on the crude oil contaminated haemoglobin. For *Clarias gariepenus*, after 48 hr incubation, the effect of 20 µl crude oil on the haemoglobin was prominent. A decrease from 0.064 mM to 0.057 mM for the 540 nm band and 0.059 mM to 0.048 mM for the 577 nm band was observed.

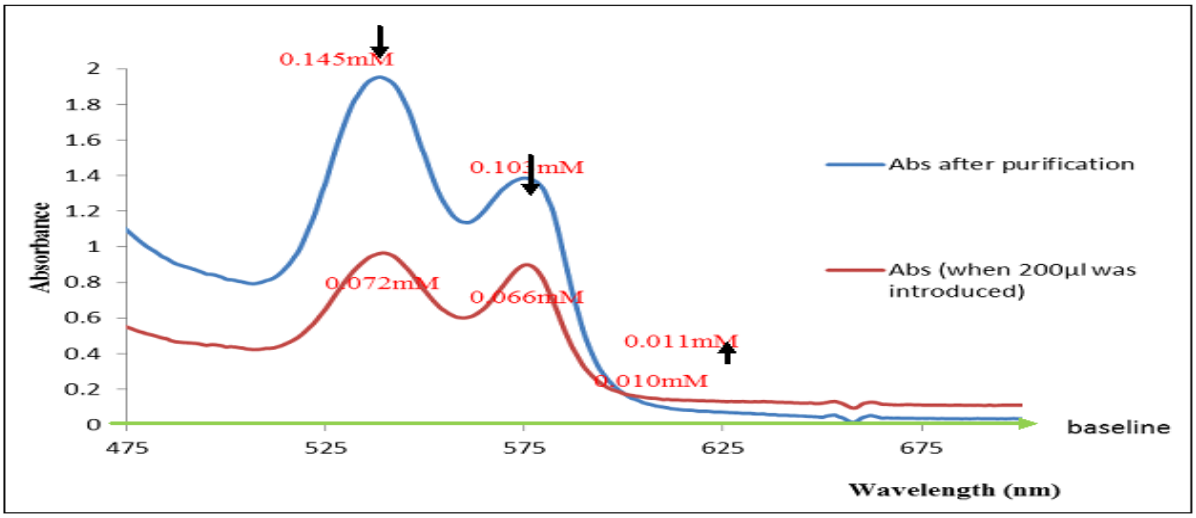

Fig. 5. Effect of 200 µl of crude oil on the haemoglobin of *Clarias gariepenus* at concentration of 0.145 mM in phosphate buffer of pH 7.8 with baseline at zero.

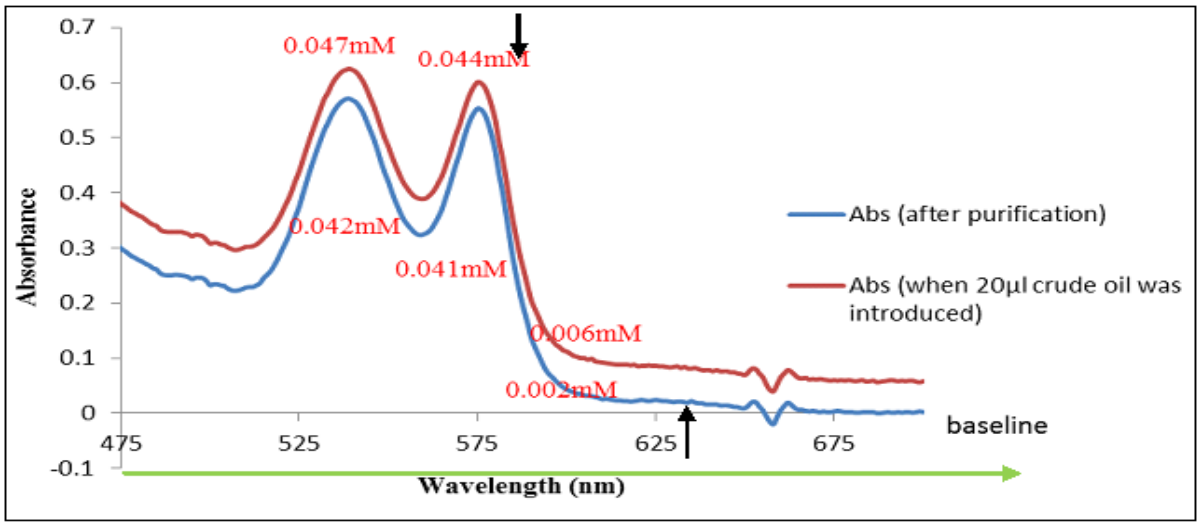

Fig. 6. Effect of 20 µl crude oil on the haemoglobin of *Heterotis niloticus* at concentration of 0.002 mM in phosphate buffer of pH 7.8 with baseline at zero.

There was also a prominent increase in the 630 nm band from 0.01 to 0.020 mM (Figure 8). Same graph pattern as shown in Figure 8 was observed in the 60, 100 and 200 µl crude oil contaminated haemoglobin. For *Heterotis niloticus* after 24 hr incubation, the effect of 20 µl crude oil on the haemoglobin was prominent. There was a decrease in the 540 and 577 nm bands from 0.042 to 0.039 mM, and from 0.041 to 0.031 mM, respectively. The 630 nm band increased from 0.002 to 0.019 mM (Figure 9). Same graph pattern as shown in Figure 9 was observed in the 60, 100 and 200 µl crude oil contaminated haemoglobin. From these results,

it took 48 hr incubation for the bands to be prominent in the haemoglobin of *Clarias gariepenus* while the haemoglobin of *Heterotis niloticus* was incubated for 24 hr for the bands to be prominent. This suggests that some fish species are more prone to toxicity than others.

Discussion

Haemoglobin A is known to have a maximum absorption when oxygen is bound with ferrous (Fe^{2+}) iron at 540 and 577 nm while the loss of oxygen and an electron to produce ferric (Fe^{3+}) methaemoglobin results in an increase in absorption of the 630 nm band of the visible region (Onwubiko *et al.*, 2000).

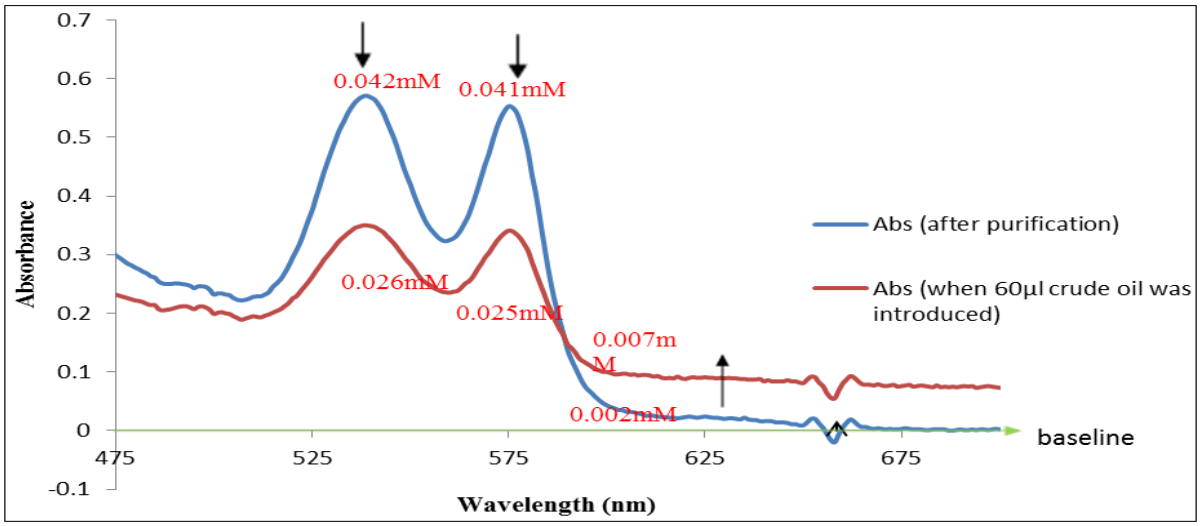

Fig. 7. Effect of 60 µl of crude oil on the haemoglobin of *Heterotis niloticus* at concentration of 0.042 mM in phosphate buffer of pH 7.8 with baseline at zero.

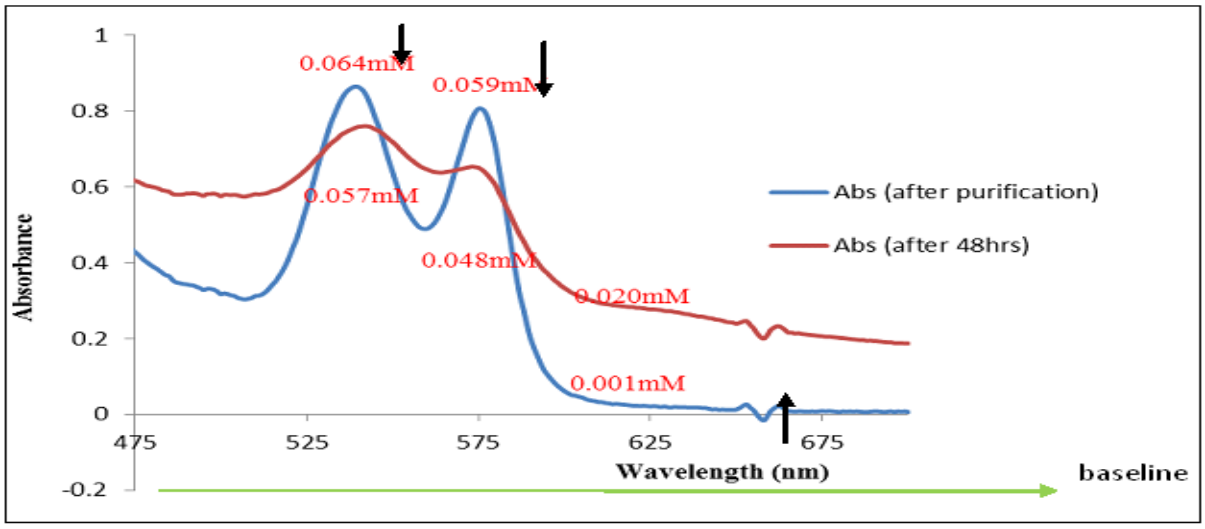

Fig. 8. Effect of 20 µl crude oil on the haemoglobin of *Clarias gariepenus* after 48hr incubation at concentration of 0.064 mM in phosphate buffer of pH 7.8 with baseline at zero.

The mechanism of haemoglobin oxidation is clearly shown in this study, from the spectra data given when various amounts of crude oil were used to contaminate the haemoglobin of the fish species. It showed an increase in the absorbance at 630 nm which could be attributed to the accumulation of oxidized (Fe^{3+}) methaemoglobin species.

African catfish species had more resistance to crude oil contamination unlike the Nile tilapia that was more prone to crude oil contamination based on the results obtained when the effect of time was studied. The loss of oxygen and an electron is due to the presence of an oxidant which acts as an electron donor to

oxyhaemoglobin oxidizing it from its ferrous to ferric (methaemoglobin) states (Jaffe and Neumann, 1964; Brunori *et al*., 1975; Reza *et al*., 2002).

$$Hb(Fe^{2+})O_2 + X \longrightarrow met\ Hb(Fe^{3+})X + O_2$$

With more than 17,000 compounds in crude oil (Marshall and Rodgers, 2004), it has the capacity of serving as oxidants to fish haemoglobin to yield the methaemoglobin species. Among the constituents of crude oil, only the aromatic hydrocarbons (PAHs) are absorbed by fishes because of their low molecular weight and their inability to dissolve in water due to their aromaticity.

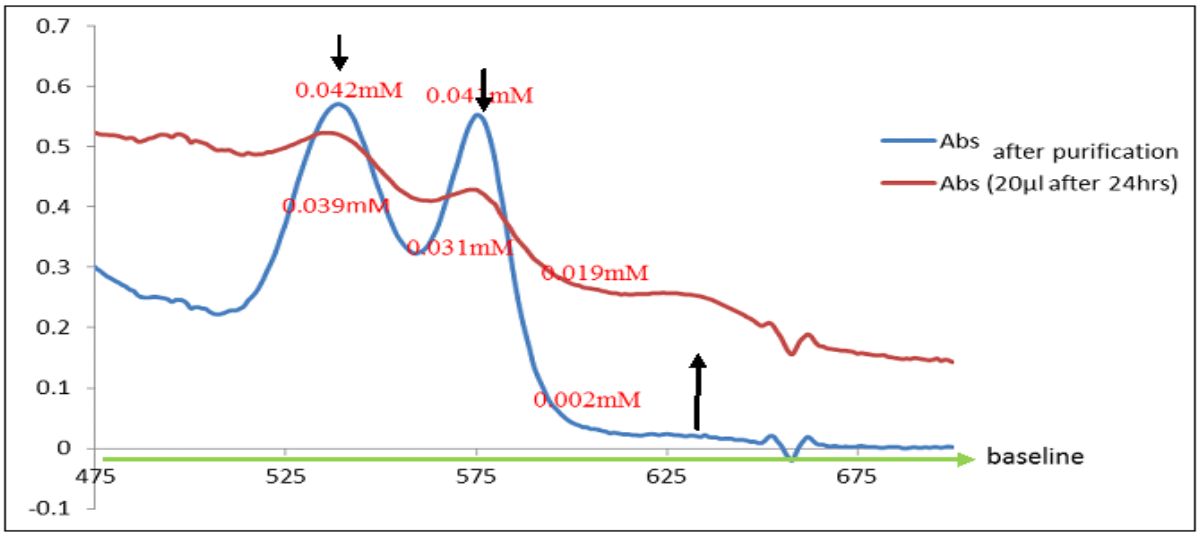

Fig. 9. Effect of 20 µl crude oil on the haemoglobin of *Heterotis niloticus* after 24 hr incubation at concentration of 0.042 mM in phosphate buffer of pH 7.8 with baseline at zero.

The alkylated PAHs, although less abundant in crude oil, have been reported to be more toxic than their unsubstituted congeners (Billiard *et al*., 1999; Rhodes *et al*., 2005; Carles *et al*., 2008). The mechanism of toxicity due to haemoglobin oxidants in mammalian erythrocytes shows that haemoglobin oxidation is followed by reversible hemichrome formation, protein denaturation and Heinz body formation (Jacob and Winterhalter, 1970). This would represent the initial reaction that is followed by reactions destructive not only to haemoglobin, but also to other components of the erythrocyte and in severe cases, could lead to death. Haemoglobin oxidation is achieved by breaking down the metabolism of the organism via the inability to obtain energy in the form of ATP from consumed foods. Since there are some structural (but no major functional) differences between fish haemoglobin and other haemoglobins, as reported by Perutz (1979) and Onwubiko *et al*. (2000), the same reaction is expected to take place.

Fish haemoglobin may even be more liable to oxidative attacks because of its enhanced Bohr effect and a number of factors such as temperature, as reported by Zavodnik *et al*. (1992); which tend to make fish haemoglobin unstable (Onwubiko *et al*., 2000).

Conclusion

The discernable decrease in oxyhaemoglobin followed by a resultant increase in methaemoglobin as observed in the present study is suggestive that the crude oil used to contaminate the fish haemoglobin contains an agent which could induce the oxidation of ferrous oxyhaemoglobin to ferric methaemoglobin. Since polycyclic aromatic hydrocarbons are the only group in crude oil that are absorbed by fish, and that out of the 16 PAHs present in 48 crude oils analyzed from around the globe, naphthalene, fluorine, phenanthrene and chrysene are more abundant with naphthalene having the highest concentration.

This suggests that one or all of these abundant compounds, or one or all of the less abundant compounds with high molecular weight, may be responsible for oxidizing the fish haemoglobin.

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