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Comparative acute toxicity and oxidative stress responses in tadpoles of *Amietophrynus regularis* exposed to refined petroleum products, unused and spent engine oils

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The relative acute toxicity of refined petroleum (diesel, kerosene and petrol), unused and spent engine oils as well as their abilities to alter the activities of superoxide dismutase (SOD) and cause lipid peroxidation in tadpoles of the common African toad, *Amietophrynus regularis* were evaluated. After 48 h of exposures, kerosene was found to be the most toxic (LC_{50}= 4930 mg/L) while the least toxic was unused engine oil (LC_{50} = 7777 mg/L). However, by 96 h of exposure, spent engine oil was found to be the most toxic (LC_{50} = 2915 mg/L) while unused engine oil remained the least toxic (LC_{50}= 7353 mg/L). Further, assessment of oxidative stress markers was conducted using sub lethal concentrations of the test compounds (1/100th 96 h LC_{50}). There was significant inhibition of SOD in exposed tadpoles compared to the control ($P<0.05$) with the least activity recorded in tadpoles exposed to petrol, while unused engine oil recorded the highest. The results of the lipid peroxidation assay, determined by measuring the levels of malondialdehyde (MDA) indicated significantly higher levels in the exposed individuals compared to the control. Unused engine oil caused the highest level of MDA production while diesel induced the least level. Tadpoles exposed to diesel, kerosene, petrol and spent engine oil exhibited consistent responses among the three test parameters, however inconsistent responses were observed in tadpoles exposed to unused engine oils. The relevance of the comparisons in biomarker selection and ecotoxicology were discussed.

Key words: Petroleum products, toxicity indices, tadpoles, oxidative stress.

INTRODUCTION

Crude oil refining, transportation and use according to Pacheco and Santos (2001), is associated with spillage of petroleum products which is one of the most important pollutant of concern in aquatic ecotoxicology. Their

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toxicological effects have been evaluated in a number of fish species (Sunmonu and Oloyede, 2007; Simonato et al., 2008; Jahanbakhshi and Hedayati, 2012) given the inevitability of contact and possible uptake once they enter aquatic ecosystems.

In Nigeria, petroleum product spills apart from those occurring during refining of crude oil may also result during transfers at the jetty, accidents involving tankers, dispensing of products to vehicles as well as vehicular and generator repairs. Since, the advent of crude oil exploration in the country in 1956 (Akpofure et al., 2000) spillages of crude and petroleum products have been commonplace, raising concerns regarding their polluting effects in aquatic ecosystems (Kadafa, 2012). Petroleum products are a mixture of hydrocarbons and additives which could produce free radicals (Achuba and Osakwe, 2003). Free radicals are one of the major precursors to oxidative stress, binding with the unsatu-rated fatty acids of the phospholipids of cell membranes, resulting in lipid peroxidation damage (Timbrell, 2000).

Exposure of animals to pollutants such as hydro-carbons in their natural environment and laboratory conditions has been reported to result in oxidative stress (Esiegbe et al., 2012). Oxidative stress is a state in which the balance between the production of reactive oxygen species (ROS) and their removal by antioxidant defences before they can cause damage is upset (Collins, 2009). Although biological systems, are constantly exposed to free radicals and ROS, there exist a repertoire of anti-oxidative stress enzymes which naturally serves to minimize oxidative damage to cells (Azqueta et al., 2009).

High concentrations of toxicants or chronic expo-sures may overwhelm the anti-oxidative stress mecha-nisms resulting in oxidative stress (Reznick et al., 1998). Oxidative damage to cell membranes leads to the release of by-products such as alkanes, ketones and aldehydes including 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal and malondialdehyde (MDA) (Zielinski and Portner, 2000). The presence and activities of enzymes such as superoxide dismutase, catalase and glutathione consti-tutes a formidable defence system against oxidative stress (Brucka-Jastrzębska, 2010). Superoxide dismu-tase (SOD) catalytically breaks down super oxide radicals generated in peroxisomes and mitochondria into oxygen and hydrogen peroxides (Li et al., 1995) making them less lipid soluble and more liable to biochemical action.

The consistency in reports regarding the link between exposures to pollutants and subsequent lipid peroxidation damage (Sreejai and Jaya, 2010; Brucka-Jastrzębska, 2010; Esiegbe et al., 2012) implies that they could be suitable candidates for use as biomarkers. Given the simplistic nature of overall measured responses in making toxicological deductions, the use of biomarkers has become commonplace.

This study investigates the acute toxicity and level of oxidative damage in tadpoles of the African common toad, *Amietophrynus regularis* (Reuss, 1833) following exposures to acute and sub-lethal concentrations of petroleum products (diesel, kerosene and petrol) and engine oils (spent and unused) to explain the possible mechanism of toxic action as a continuation of the discourse on potential biomarkers for environmental pollution monitoring. *A. regularis* is a common tadpole in the rainforests and mangrove swamps of southern Nigeria (Onadeko, and Rodel, 2009). Urbanization and forest clearance destroys their natural habitats and brings them closer to sites of human activities which further threaten their survival. Amphibians worldwide are reported to be on the decline and the drivers have been reported to include global warming (Houlahan, 2000), disease (Kiesecker, 2001) and in some cases aquatic pollution (Ezemonye and Tongo, 2010). It is com-monplace to sight their tadpoles in ponds and open gutters which are receptacles and easy dumping sites for spilt petroleum products and spent engine oils. This therefore justifies a study evaluating the toxicities of these products to the tadpoles as well as the indicators of oxidative stress so as to ascertain the extent to which they pose threats to their survival. The enzyme, SOD together with levels of some metabolites, such as MDA are used as biomarkers of oxidative stress (Idowu et al., 2014). Thus, their levels in fishes exposed to petroleum products and engine oils would give an indication of the stress levels in the exposed toads.

MATERIALS AND METHODS

Collection and acclimatization of tadpoles

Tadpoles of African common toad, *A. regularis* (Approximate Average length =0.80±0.15 cm), which are commonly found in ponds and gutters were collected from an undisturbed pond (N 6° 31' 1.5960", E 3° 23' 59.7840") at the University of Lagos campus, Lagos, Nigeria, during the breeding season (July 2013), 1 to 2 days after hatching. Hand nets were used in the collection of the tadpoles and care was taken not to agitate them during the process. The tadpoles were transferred into plastic cans containing their habitat water collected from the same pond before transporting to the Ecotoxicology laboratory about 50 m away. The natural pond water was also used during the acclimatization of tadpoles in the laboratory. They were kept in large plastic tanks (l x w x h = 60 cm x 35 cm x 30 cm), half-filled with water and aerated with a 220 v air pump so as to maintain dissolved oxygen levels in the tank. They were left to acclimatize to laboratory conditions (temperature, 26 to 28°C; humidity, 65 to 75%; Light: dark, 8:14 h) for a minimum of 72 h before using them in bioassays. Only tadpoles in tanks having mortality of less than 1% were employed for the study.

Test compounds

Refined petroleum products (Diesel (Automotive Gas Oil- AGO), kerosene (Dual Purpose Kerosene- DPK), petrol (Premium Motor Spirit- PMS) approved for use in automobiles in Nigeria by the Department of Petroleum Resources (DPR) and engine oils (Motor

Oils- SAE 40: unused and spent) were used for this study. The petroleum products and unused engine oil were of the global grade imported for use in Nigeria. Their specific gravities were diesel: 990 mg, kerosene: 1000 mg, petrol: 990 mg, unused engine oil: 1010 mg and for spent engine oil, 1100 mg. They were purchased in plastic kegs from a filing station at Bariga, near the University of Lagos campus. The spent engine (fuel) oil was collected in 1 L plastic keg from an Auto mechanic workshop also at Bariga. The collected petroleum products and engine oils were stored in the laboratory at room temperature of 26 - 28°C prior to use.

Acute toxicity bioassay

Preliminary tests were carried out to determine suitable range of bioassay concentrations for the study in an initial test which lasted for 96 h. The range of bioassay concentrations selected for the definitive tests were as follows: diesel: 2970, 4950, 7425, 9900 mg/L and untreated control; kerosene: 1500, 3000, 5000, 6000, 7000 mg/L and untreated control; petrol: 2970, 4950, 6930, 9900 mg/L and untreated control; unused engine oil: 7070, 7575, 8080, 10100 mg/L and untreated control; used engine oil: 1100, 3300, 5500, 7700, 8800 mg/L and untreated control. Four active tadpoles (7 to 12 days old) were randomly selected into an experimental tank (L x W x H = 13.5 x 11 x 7 cm) containing 500 ml of their natural habitat water contaminated with the respective concentrations of toxicants. Each experiment was replicated twice to make a total of 16 tadpoles per concentration. Mortality assessments was carried out every 24 h over a 96 h period and tadpoles were considered dead if there were no body movements or they become turned upside down and did not respond to repeated gentle prodding with the blunt end of forceps. Bioassay conditions were same as for acclimatization.

Assessment of sub lethal effect

The tadpoles were further exposed to concentrations equivalent to 1/100th of the calculated LC_{50} for 28 days. Given the limitation of size, whole tadpoles were used for the determination of the activities of SOD and levels of MDA. The whole body was homogenised (9% w/v) in 100% methanol and centrifuged at 10,000 rpm for 15 min at 4°C using the technique of Hermes-Lima et al. (1995) as described in King et al. (2012) and the supernatant was used for the assays.

Measurement of superoxide dismutase activity

The SOD enzyme activity was measured by its ability to inhibit the antioxidation of epinephrine (that is, determining the difference in the level of superoxide anion production and decomposition) at an absorbance of 450 nm, using the method of Sun and Zigma (1978). The concentrations so determined were expressed as unit/mg protein, of which one unit is defined as the amount of enzyme needed to inhibit 50% epinephrine reduction per minute and per mg of protein at 25°C and pH 7.8.

Lipid peroxidation assay

The thiobabituric acid reaction (TBARS) assay was used to determine the level of lipid peroxidation in the supernatant of the tissue homogenates. Specifically, MDA, the measure of lipid peroxidation damage was determined by measuring absorbance at 535 nm in a spectrophotomer (Yagi, 1998).

Statistical analysis

The dose-response data of quantal responses (mortality) of the tadpoles to the petroleum products were analysed by probit analysis after Finney (1971). The indices calculated from the probit analysis includes: LC_5: sub-lethal concentration that causes 5% response (mortality) of exposed tadpoles at 95% confidence interval; LC_{50}: lethal concentration that causes 50% response (mortality) of exposed tadpoles at 95% confidence interval; LC_{95}; lethal concentration that causes 95% response (mortality) of exposed tadpoles at 95% confidence interval and toxicity factor:

$$TF = \frac{\text{LC50 of the other Chemical}}{\text{LC50 0f the most toxic chemical}}$$

The analysis of variance (ANOVA) of the SOD and MDA values were carried out at 5% ($P<0.05$) level of significance using SPSS version 16.

Toxicity ranking

The respective acute toxicity, induced SOD activities and MDA levels in the tadpoles were ranked for the five test substances on a scale of 1 to 5 (1= highest, while 5= least) in order to design a uniform semi qualitative assessment to determine the extent of consistency of the measured responses.

RESULTS

Relative acute toxicity of the petroleum products and engine oils to *A. regularis*

On the basis of the 48 h LC_{50}, kerosene was found to be the most toxic (LC_{50}= 4930 mg/L) having a toxicity factor (TF) value, 1.6 times higher than the least toxic test substance, unused engine oil. The toxicity ranking at 48 h therefore was as follows: kerosene (most toxic) followed by petrol, spent engine oil, diesel, and unused engine oil (least toxic) (Table 1). By the 96th h following exposures, spent engine oil was found to be the most toxic (LC_{50}= 2915 mg/L), being 2.5 times more toxic than unused engine oil which remained the least toxic. The order of toxicity also included spent engine oil (most toxic), followed by kerosene, spent engine oil, petrol, diesel and unused engine oil (least toxic) (Table 2).

Lipid peroxidation

The levels of MDA was significantly lower ($P<0.05$) in the control tadpoles than in those exposed to the petroleum products (Figure 1). MDA levels were highest in those exposed to unused engine oil (Table 3). The next highest MDA level was recorded in tadpoles exposed to kerosene, followed by spent engine oil, while the least level was measured in those exposed to diesel.

Superoxide dismutase activity

The results show that the SOD activity was significantly

Table 1. Relative 48 h acute toxicity (mg/L) of kerosene, diesel, petrol, spent engine oil, and engine oil acting singly against tadpole (*Amietophrynus regularis*).

Test compounds (ml/L)	LC_5 (confidence interval)	LC_{50} (confidence interval)	LC_{95} (confidence interval)	SE	DF	Probit line equation	TF
Kerosene	2850 (1030-3720)	4930 (3830-5960)	8530 (6750-20230)	2.20	2	y=1.59+3.01x	1
Diesel	1881 (0-3445)	7286 (4455-129650)	29957.4 (13186-1156062)	1.28	2	y=1.03+2.25x	1.5
Petrol	2693 (426-3990)	6702 (4059-10949)	16682 (10474-163657)	1.46	2	y=1.17+2.95x	1.4
Unused engine oil	5555 (1071-6626)	7777 (6252-8878)	10898 (9282-46904)	4.70	2	y=4.23+2.36x	1.6
Spent engine oil	2761 (22-4301)	7524 (5478-24673)	20515 (11506-19283242)	1.65	2	y=1.28+2.46x	1.4

SE = Standard error; DF = degree of freedom, TF= toxicity factor.

Table 2. Relative 96 h acute toxicity (mg/L) of kerosene, diesel, petrol, spent engine oil, and engine oil acting singly against tadpole (*Amietophrynus regularis*).

Test compounds (mL/L)	LC_5 (Confidence interval)	LC_{50} (Confidence interval)	LC_{95} (Confidence interval)	SE	DF	Probit line equation	TF
Kerosene	1120 (70-1990)	3880 (2400-6610)	13480 (7430-258180)	1.08	2	y=0.67+2.69x	1.3
Diesel	2456 (653-3495)	5207 (3703-5801)	11207 (8088-34046)	1.51	2	y=1.17+3.07x	1.8
Petrol	1436 (39.6-2564)	3871 (1535-5267)	10454 (7019-100713)	1.45	2	y=1.05+2.16x	1.3
Unused Engine oil	6020 (111-6777)	7353 (4050-7929)	8969 (8161-171983)	9.16	2	y=8.03+2.04x	2.5
Spent engine oil	1023 (198-1727)	2915 (1727-4202)	8294 (5401-27973)	1.03	2	y=0.58+2.62x	1.0

SE = Standard error; DF = degree of freedom; TF= toxicity factor.

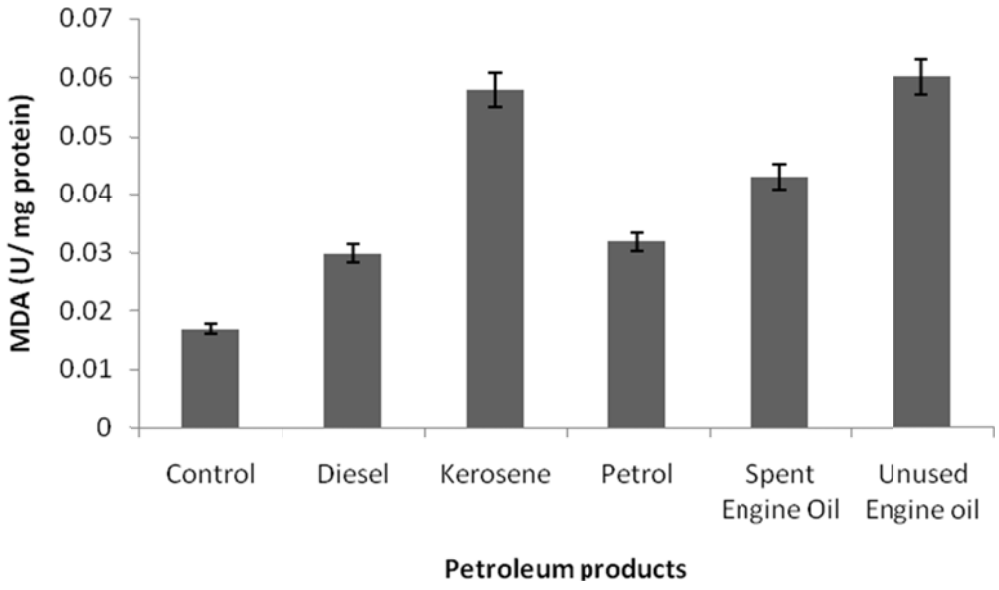

Figure 1. Comparison of the level of lipid peroxidation (MDA) in *Amietophrynus regularis* exposed to 1/100th of the respective LC_{50} of the petroleum products and engine oils.

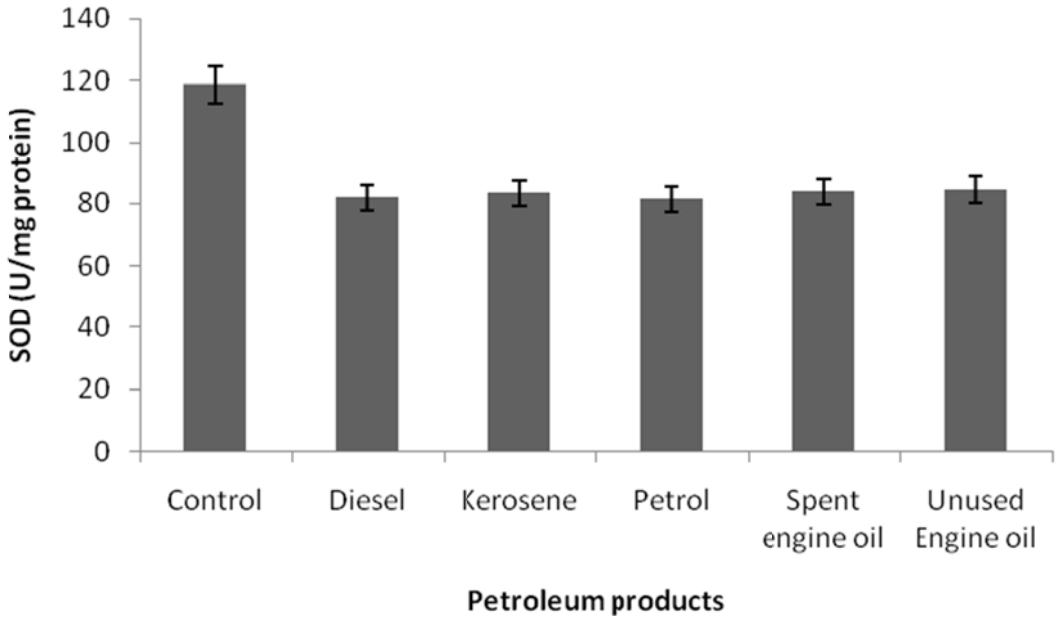

Figure 2. Comparison of the superoxide dismutase (SOD) activity in *Amietophrynus regularis* exposed to 1/100th of the respective LC_{50} of the petroleum products and engine oils.

Table 3. Assessment of relationship between toxicity of the petroleum products, superoxide dismusae (SOD) activity and lipid lipid peroxidation product (MDA- Malondialdehyde) in *Amietophrynus regularis*.

Petroleum products	96 h LC_{50}^{ab}	SOD activity^{ac}	MDA^{bc}	Remark
Diesel	4	4	5 (lowest)	Very consistent
Kerosene	2	3	2	Very consistent
Petrol	3	5 (lowest)	4	Consistent
Spent engine oil	1 (most toxic)	2	3	Consistent
Unused engine oil	5 (least toxic)	1 (highest)	1 (highest)	Inconsistent

N.B, Similar alphabets implies no significant relationship (P>0.05).

inhibited (P<0.05) in the tadpoles exposed to sub lethal concentrations (1/100th LC_{50}) of the petroleum products and engine oils compared to the control after the 28 days period (Figure 2). Among those exposed to the petroleum products, petrol recorded the least activity, followed by diesel, kerosene and spent engine oil while unused engine oil had the highest activity. There was no significant difference (P>0.05) in SOD activity among the exposed tadpoles. Overall, the assessment of the relationship between the toxicity of the petroleum proucts and engine oils with their respective SOD activities as well as MDA levels by way of ranking (1-5; 1= highest, 5= least/lowest) indicated that there were no significant difference (P>0.05) between all three parameters. Summarily, the results indicated that diesel and kerosene showed very consistent relationships, petrol and kerosene showed consistent relationship while unused engine oil which had the least toxicity but the highest levels of SOD acivity and MDA was designated inconsitent (Table 3). The overall comparison of the ranks for the 96 h LC_{50} with the respective antioxidative stress markers (that is, SOD and MDA) using independent sample t test indicated that there was no significant difference (P>0.05) between each pair. However, there was a strong positive correlation (r=0.8) between the SOD and MDA ranks for the test groups.

DISCUSSION

Continued reliance of motor vehicles, generating sets, and other equipments fuelled by petroleum products make the potential for spills a continued environmental risk. There is, therefore a need for constant investigation

of their toxic effects on sensitive wildlife species such as amphibians. This study shows that petroleum products (diesel, kerosene, petrol), unused and spent engine oil are acutely toxic to the tadpoles of the common African toad (*Amietophrynus regularis*). With respect to the relative 96 h LC_{50}, the spent engine (fuel) oil was the most toxic and this could be related to the fact that being a waste product it may contain all sorts of toxic compounds/chemicals emanating from additives and heavy metals from worn engine parts. The differential toxicity of the petroleum products and engine oils to the tadpoles can be linked to their respective physical characteristics. Refined petroleum products are more volatile than the engine oils and therefore would not be retained for long in the bioassay medium. This may account for their lower acute toxicity compared to the used and unused engine oils. However, their relative toxicity should not override the fact that they all constitute environmental hazards being rich in hydrocarbons. This raises important ecotoxicological concerns given the ubiquity of petrol filling stations and auto mechanic workshops in major cities and highways in Nigeria. These facilities often leave little consideration to waste management in their design. Surrounding drainages and ponds becomes recipients of their wastes either by deliberate introduction or when they are washed off as run off after rainfall. Dumping of spent engine oils in gutters and drains is commonplace in auto mechanic workshops in Nigeria. There are no measures put in place for collection and management of spent oils and petroleum products from these workshops which are distributed across streets corners and major roads of the country. Thus, resulting in pollution concerns to animals inhabiting urban ecosystems.

Previous investigations have evaluated the toxicity of crude oil and petroleum products on frogs (Udofia et al., 2013) and guppies (Simonato et al., 2008) linking them with acute toxicity as well as a number of sub lethal effects following long period of exposure to minute concentrations. This study confirms the toxicity of petroleum products to tadpoles, specifically of the common African toad. The toxicity of petroleum products to the tadpoles were found to increase with time of exposure, consistent with the findings of King et al. (2012) who suggested that the reason for this trend in catfishes and hermit crabs could be due to a number of factors including permeability of the skin. The LC_{50} values obtained from this study for petrol, diesel and kerosene were lower than those reported by King et al. (2012) against early life stages of catfishes and hermit crabs. Amphibians typically have a characteristic permeable skin adapted for cutaneous respiration (Hickman et al., 2008). Lipohilic pollutants such as petroleum hydrocarbons may easily diffuse through their skin, resulting in toxic effects. This together with other physiological and morphological differences may account for the increased toxicity to the tadpoles reported in this study. Ayoola and Akaeze (2012) however reported 96 h LC_{50} value of 562 ml/L in catfishes exposed to spent oil, a value which is over 200 times less than that observed for tadpoles in this study. Besides differences in species susceptibility, this may be due to the wide variation in the constituents of the spent engine oils and other practices in the automobile workshops from where they were collected. Thus, the difficulty in comparing responses between species as well as used/spent engine oils is hereby noted.

The assessment of MDA, the by-product of oxidative damage to the phospholipids of cell membranes indicated significant harm to cells in tadpoles exposed to the petroleum products relative to the control individuals. Lipid peroxidation damage is one of the first indicators of damage to cells by toxicants and represents a key biomarker of oxidative stress (Cini et al., 1994). Much of the work on lipid peroxidation resulting from petroleum products and their components in Nigeria have been focused on fishes (Achuba and Osakwe, 2003; Avci et al., 2005; Doherty, 2014). Avci et al. (2005) have earlier reported lipid peroxidation in the muscles and liver of fishes obtained from a river contaminated petroleum products from a nearby refiner. This study therefore provides an opportunity to extend the knowledge of the oxidative stress impacts of petroleum products on tadpoles of the common African toad.

The results from the biochemical assays indicated that there was inhibition of SOD activities in the exposed tadpoles relative to the control. Inhibition of SOD activities have been reported in the African sharp tooth catfish (*Clarias gariepinus*) exposed to polycyclic aromatic hydrocarbons (Otitoloju and Olagoke, 2011). This gives credence to the possibility of oxidative stress resulting from the hydrocarbon fractions of the petroleum products and confirms results from the lipid peroxidation assay in this study. Specific petroleum hydrocarbons such as benzene, ethylbenzene, toluene and xylene have been also found to induce oxidative stress at sub lethal concentrations, in *Clarias gariepinus* (Doherty, 2014). SOD, though involved in the protection of biological systems from the actions of free radicals and may be overwhelmed in the event of excessive toxic onslaught, resulting in oxidative stress, a condition that may be characterized by its eventual inhibition. This therefore justifies its use as a biomarker for assessing the toxic effects and responses to toxicants in this study.

Conclusion

The findings from this study points to a largely consistent relationship between the toxicity of petroleum products and spent engine oils and their respective SOD activity and MDA levels. This conclusion is based on the fact that

rank differences between the three parameters did not exceed 1 (one) for all toxicants except for unused engine oil. The relatively consistent relationship between SOD and MDA reported in this study was also consistent with the findings of Brucka-Jastrzębska (2010) who reported inhibition of SOD which was simultaneously associated with increase in MDA in catfishes exposed to heavy metals, lead and cadmium. The importance of antioxidative enzymes as sensitive biomarkers in monitoring environmental pollution therefore cannot be downplayed owing to the large number of investigators who have demonstrated this in a variety of animal groups as documented by Otitoloju and Olagoke (2011).

This study therefore justifies the use of MDA levels and SOD activity as suitable compliments for monitoring oxidative stress resulting from exposure to petroleum products. The consistent relationship between these biomarkers and 96 h LC_{50} values for some of the tested products is noteworthy and presents an opportunity for more investigative studies so as to understanding the mechanisms of action and make a case for their use in routine assessments of impacts of such spills in the environment.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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