BIOMARKER RESPONSES OF ESTUARINE MACROINVERTEBRATES OF LAGOS LAGOON EXPOSED TO DRILL CUTTINGS

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CERTIFICATION

This is to certify that the Thesis:

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CERTIFICATION

I certify that the work embodied in this thesis for degree of Doctor of Philosophy (Marine Biology) by **ANAGBOSO MARYKATE UKAMAKA** has been carried out under our supervision in the Aquatic Toxicology and Ecophysiology Laboratory Department of Marine Sciences, University of Lagos.

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DEDICATION

To the memory of my late grandmother, Mrs. Abigail Okafor.

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"Let love be sincere. Hate what is evil and hold to whatever is good"

Romans 12 vs 9

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ABSTRACT

The toxicity, accumulation of hydrocarbon and selected heavy metals, and biomarker responses were investigated in three macroinvertebrate species of Lagos lagoon exposed to drill cuttings. The test species, *Tympanotonus fuscatus*, *Pachymelania aurita* and *Sesarma huzardi* were handpicked from the Lagos lagoon and the surrounding mangrove swamp and were acclimatised for 7 days under laboratory conditions. On the basis of the $96hLC_{50}$, the relative sensitivity of the organisms to drill cuttings was found to be. *Tympanotonus fuscatus* > *P. aurita* > *S. huzardi*. Exposure of the test animals to sublethal concentrations of drill cuttings based on 0.01 and 0.1 of the $96hLC_{50}$ concentrations of the toxicant for a period of 32 days for the periwinkles and 16 days for the crabs revealed that there was no hydrocarbon accumulation ($P > 0.05$) in the digestive gland of all test species. The accumulations of heavy metals (Iron, Copper and Zinc) were also found to be neither time nor concentration dependent ($P > 0.05$) suggesting that the organisms have the capacity to regulate these essential metals over the period of observation. Levels of Cadmium and Silver in the digestive glands were found to be below the detection limit of 0.001. The sublethal studies carried out to identify early warning biological responses to hydrocarbon and heavy metal pollution involved the monitoring of the following biomarkers: metallothionein (MT) induction, lysosomal membrane stability, antioxidant enzymes (catalase (CAT), superoxide dismutase (SOD) - SOD was monitored only in the crabs -, a phase II biotransformation enzyme glutathione *S*-transferase (GST), and lipid peroxidation (LPO) activity. All biomarker analyses were performed using the digestive gland of each of the test species but for lysosomal membrane stability, which was determined using the haemolymph of the organisms. Measurements of biomarkers were

also performed using spectrophotometer method except for lysosomal membrane stability which was evaluated with the neutral red retention assay. MT synthesis was enhanced in *T. fuscatus* and *S. huzardi* on exposure to sublethal concentration of drill cuttings ($P < 0.05$). On the other hand, MT synthesis was depressed in *P. aurita* on exposure to sublethal concentration of drill cuttings ($P < 0.05$). Lysosomal membrane stability decreased with exposure time and concentration of the toxicant in all the species ($P < 0.05$). Antioxidant enzymes activities were similar in the two periwinkle species during the study. Antioxidant enzymes activities were also similar in both sexes of the crabs. CAT activity increased significantly ($P < 0.05$) above the background level of 6.7 CAT units/mg protein in the periwinkles and 1.0 CAT units/mg protein in the crabs by the end of 16 day exposure. The background value of SOD decreased with time in all treatment groups during the experimental period ($P < 0.05$). Increase in GST values observed in drill cuttings exposed periwinkles was not sustained to the end of the exposure period in the periwinkles while GST values remained significantly ($P < 0.05$) elevated in drill cuttings exposed crabs. In exposed periwinkles, lipid peroxidation initially rose significantly ($P < 0.05$) above background values, but subsequently fell, such that by the end of exposure period, its activity was below the observed background level. In the crabs, LPO activity value increased significantly ($P < 0.05$) in all drill cuttings exposed groups during the experiment as well as in the control groups. Lysosomal integrity tests integrated the results of the findings above and demonstrated that exposure to sublethal concentrations of drill cuttings has toxic effects on the test organisms. The implication of the findings of this study and the incorporation of biomarkers in environmental biomonitoring are discussed.

1.0 INTRODUCTION

1.1 BACKGROUND TO STUDY

Aquatic organisms are subject to various natural and anthropogenic-induced stressors, which vary both spatially and temporally. The impacts from these stressors can be detected at several levels of biological organization, from that of the subcellular and molecular to that of the ecosystem. Several tools are available for detection of effect of environmental stressors at every level of biological organisation (Depledge and Hopkin 1995). Population and community level effects are ecologically more relevant because they reflect changes in ecosystem structure and function but are relatively insensitive. Effects which can be measured at the lower levels are more sensitive, but need to be prognostic to be ecologically relevant (Köhler *et al.*, 2002).

Effects are through two basic pathways in animal populations; directly mainly through effects on metabolic pathways, and indirectly through effects on food chains and on the behaviour of the organism (Adams *et al.*, 1998). In both cases however, effects may result in changes in populations and communities, for instance when energy for growth is channeled for induction of metallothionein in response to heavy metal exposure (Calow, 1991), or when pollutants influence the quality and quantity of food available to consumers. Hence, effects which are not readily visible at the molecular and cellular levels may manifest at higher biological organisation, at which point damage may have already occurred. However, death is an extreme endpoint for ecological assessment, hence it is important that measures which are able to detect subtle effects at the molecular and cellular levels are put in place before adverse effects manifest in ecological change.

These measures need to be ecologically relevant and also able to minimise impact by indicating the health status with a prognostic capability.

Damiens *et al.* (2004) have pointed out that marine pollution has been traditionally documented in terms of chemical concentrations of contaminants; and that these measurements did not provide estimations of the deleterious effects on living organisms and are now complemented with biological criteria, especially with the measurement of biomarkers. Biomarkers are measurements of biological tissues, fluids or cells that can be used to determine if an organism has been exposed to a contaminant or if a contaminant has caused or is causing biological changes (Depledge and Hopkins, 1995). It is generally accepted by environmental scientists that, biomarker-based techniques do have a major role to play in the overall effort of environmental monitoring and protection (Moore *et al.*, 2004; Handy and Depledge, 1999; Handy *et al.*, 2003; Lam and Wu (2004); Depledge, 1994; Peakall, 1994; STAP 2004). This is because early detection of effects of pollutants on populations will enable protective measures to be put in place before large scale ecological problems occurs. Establishment of prognostic biomarkers will enable biomarkers to be incorporated in routine monitoring of chemical effects, which before now has relied heavily on measurement of chemical concentration in water, and in some cases in biota.

One of the main sources of environmental contamination of our coastal waters is the exploration and exploitation of petroleum and other hydrocarbon products. Hydrocarbon development and production activities produce environmental risks and impacts resulting

from not only accidental spills and blowouts, but also from permissible discharges of operational wastes such as produced water, drilling muds and drill cuttings. Produced water represents the largest volume discharge from offshore production platform, and is derived from the wells along with the hydrocarbons, and is also a major source of oil in the marine environment. It comprises of a mixture of "formation water" naturally present in the petroleum reservoir, and in some fields, "injected water" – seawater with chemical additives that is injected into the rock formations through additional wells to increase recovery of hydrocarbons (Somerville *et al.*, 1987). In all offshore fields, produced water is separated from the desired petroleum products on the production platforms and is then discharged overboard. The volume of discharge depends on the characteristics of the particular field and tends to increase during the life of each well. Though produced water is made up of mostly water, it is often at high temperature, and contains a wide variety of dissolved and suspended contaminants, including hydrocarbons and various process chemicals added to the stream on the platform (Somerville *et al.* 1987).

Drilling muds are essential component of the rotary system used to drill for oil and gas in both land and aquatic environments. They are complex mixtures of a very large number of substances with exact composition being varied to meet technical demands of the drilling. The functions as outlined by Norwegian Oil Industry Working Group (1996) include: assist in the removal of cuttings from the well bore; control formation pressure; maintain bore hole stability; protect producing formations by minimising formation/fluid interaction among other functions.

Drill cuttings are particles of crushed rock produced by the grinding action of the drill bit as it penetrates into the earth. They range in size from clay sized particles to coarse gravel and have angular configuration. Their chemistry and mineralogy reflect that of the geologic origin of the parent rock. Drill cuttings contain some amounts of the drill fluid used in drilling in addition to the formation solids. Although the drill cuttings are considered toxicologically inert, there is concern that any adhering fluid additive may be toxic particularly if the cuttings are produced during drilling with oil based or synthetic mud (Neff *et al.*, 2000).

Concern about the disposal of drill cuttings centres on the amount of drilling muds adhering to the cuttings. Bentonite and barite which form bulk of the mud are toxically inert, however these have been pinpointed as the source of trace metals present in the mud (Roddie *et al.*, 1999). Barite and bentonite are known to prevent plant growth when dumped on land (Murphy and Kehe, 1984). Cuttings discharge on land may alter soil pH and salinity, and increase soil metal concentration as well as cause poor microbial and plant growth (Kinigoma, 2001). In the marine environment, discharged cuttings may physically smother the benthic fauna, alter species structure and abundance, as well as cause increased sediment concentrations of base fluid and metals (Terrens *et al.*, 1998; Sayle *et al.*, 2002). Cutting piles may also form depending on the oceanographic condition (Roddie *at al.*, 1999). Piles represent long term sources of environmental contaminants and toxicity as components leach into the water column.

The need to reduce the impact associated with hydrocarbon exploitation is a matter of priority involving both industry and environmentalists. To this end, increased effort is being channeled towards the development of techniques that will reduce or eliminate pollution from drill cuttings especially in the developed world (Hird and Tibbetts, 1996). Reduction of impact from drilling operations can be achieved if information that will help to determine the oil content limitations for drilling discharges such that the cuttings will not cause a significant environmental impact on the substratum is readily available. Impact can also be minimised by the development of protocols for assessment of environmental impact of future drilling discharge. Information from toxicity testing, especially at sublethal effect is very valuable (Roddie *et al.*, 1999).

1.2 STATEMENT OF THE PROBLEM

The activities of hydrocarbon development and production generate large amounts of impacts and effects, arising not only from accidental blowouts but also from permissible discharges of such wastes as drill cuttings, which impact negatively on the aquatic biota. Protection and renewal of these vulnerable resources are of high priority and requires objective, credible and timely scientific information. It is important to understand what the potential effects are, and if and how they can be mitigated. This information needs to be incorporated into a continually evolving scientifically based regulatory framework for the hydrocarbon industry that protects valuable living resources and other aesthetic uses of water.

With respect to drill cuttings, the effects on marine environment are both physical and chemical. Physically, there is the severe damage to the benthic fauna due to smothering effect, which increases substantially when multiple wells are drilled. Chemically, the effects will depend on the type of mud used in drilling. Varying degree of toxicity is attributable to the base of the mud, though generally, water based muds (WBM) and synthetic based muds (SBM) are regarded as being less toxic than oil based muds (OBM), and most countries allow the discharge of these without treatment offshore. Productive systems such as estuaries and wetlands, which support a diverse fish community, which in turn can support a productive, sustainable fishery are extremely sensitive to pollution. Bioaccumulation of toxic components by edible species and possible trophic transfer to man is also a serious matter of concern.

In Nigeria, on site disposal in offshore or deep-waters of water based drill cuttings with an oil content that does not cause sheen on the receiving water is allowed by the regulatory authority, otherwise cuttings is to be transported onshore for land disposal/treatment e.g. incineration or desorption/oil recovery. Discharge of cuttings contaminated with water/oil based mud and/or esters is prohibited in inland and nearshore areas (Department of Petroleum Resources, DPR, 2002). Nevertheless both WBM and SBM contain varying amount of oil depending on their specification and will therefore constitute a hazard to the marine biota.

Presently in Nigeria, only WBM and SBM are used in drilling activities, though OBM have been used in the past. However, OBM may be used in difficult formation if

approved by the Director of Department of Petroleum Resources (DPR, 2002). With WBM the overall quantities discharged can be very high. The water component will naturally disperse into the ocean, but the other constituents represent substantial contamination, for instance heavy metals such as barium. Cr^{6+} associated with lignosulphonate, low molecular aromatic hydrocarbons, biocides, etc, and these are expected to have effects on marine biota if bioavailable.

OBM are regarded as being especially toxic because of their high oil content. Their discharges are allowed only after treatment to a level specified by regulatory authority. while some countries forbid the discharge of OBM coated cuttings. In Nigeria, The Department of Petroleum Resources stipulates that treatment should reduce oil content of cuttings contaminated with low toxic mineral OBMs to less than 10g oil per kg cuttings, i.e. <1 % oil on cuttings, less than 50g oil per kg of cuttings (i.e. <5 % oil on cuttings) for synthetic/pseudo oil based mud system containing linear alpha olefins (LAO), isomerised olefins (IO), n-paraffin polyalpha olefins (PAO), and 100g oil per kg cuttings (i.e. 10 % oil on cuttings) for cuttings coated with ester based muds before they can be discharged offshore (DPR, 2002).

The biological effects of drill cuttings discharges in the marine environment will be basically either through physical smothering of benthic species, or chemically through ingestion, or assimilation of food particles or water contaminated with drill cuttings. There is also the effect arising when cutting piles form in the marine environment. Piles

represent long term sources of contamination as contaminants leach from the piles into the surrounding water, mostly at chronic levels.

As pointed out by Roddie *et al.* (1999), most work to determine the toxicity of drill cuttings and mud had been restricted to acute toxicity tests which try to determine the LC_{50} values of the toxicants, with practically no chronic tests to study the sublethal effects of cuttings and muds on biota. Thus there is a huge gap in the understanding of the organisms' response when exposed to sublethal concentrations of chemicals from drilling operations. This study therefore proposes to use multiple biomarker approach to study the effects of drill cuttings on the selected estuarine organisms.

Biomarkers provide early warning signals of the impact of chemicals long before the effects result in changes in community composition/structure. They are sensitive molecular endpoints which are measures of sublethal impact by different types of contaminants and help to better understanding of pathways of contaminant metabolism, detoxification and toxic action (Correia *et al.*, 2002). For instance with metals, once their capability is exceeded, they become free to interact with a number of subcellular system and induce several manifestations of toxicity. The biomarker approach will enable remediation measures to be put in place long before serious damage is done to the ecosystem.

1.3 PURPOSE OF STUDY

1. To determine the heavy metals and the total hydrocarbon content of the drill cuttings used in this investigation.

2. To determine the acute toxicity and relative sensitivity of drill cuttings on the periwinkles and mangrove crab test species: *Pachymelania aurita*, *Tympanotonus fuscatus*, and *Sesarma huzardi* of the Lagos Lagoon.

3. To investigate the accumulation of hydrocarbons and heavy metals from the drill cuttings by the test species, *Pachymelania aurita*, *Tympanotonus fuscatus*, and *Sesarma huzardi* of the Lagos Lagoon.

4. Determine the biological responses such as lysosomal stability and metallothionein induction which are routine monitoring indicators of general stress and heavy metal contamination in these estuarine macroinvertebrates.

5. Determine the toxicological effects of drill cuttings on the estuarine macroinvertebrates using antioxidant enzymes, (catalase (CAT), superoxide dismutase (SOD)), a phase II transformation enzyme (glutathione *S*-transferase (GST) and lipid peroxidation as biomarkers of oxidative stress.

2.0 LITERATURE REVIEW

The concern about the disposal of drill cuttings into the aquatic environment is as a result of the amount of drilling mud that coats the cuttings, especially when oil based mud are used in drilling (Roddie *et al.*, 1999; DPR, 2002). Drilling muds are suspensions of solids and dissolved materials in base of water or oil that are used to maintain hydrostatic pressure control in the well, lubricate the drill bit, remove drill cuttings from the well and stabilise the walls of the well during drilling or workover operations (DPR, 2002).

2.1 CLASSIFICATION OF DRILLING MUDS

Drilling muds can be broadly divided into two categories: water based drilling muds (WBM); and non-aqueous based drilling muds (NADM) (Neff *et al.*, 2000).

2.1.1 Water Based Drilling Muds (WBM)

Water based muds typically consists of water (fresh, seawater) or brine e.g. KCl. The salinity ranges from 0 to about 900 kg/m^3 depending on the salt type and density of the desired mud (Norwegian Oil Industry Working Group, 1996). Depending on the needed characteristics of the mud, various additives may be added including barite, clay, caustic soda, lignite, lignosulfonates and/or water soluble polymers. WBM may also contain low concentrations of specialty chemicals added to solve some particular problems that affect mud properties, e.g. tributylphosphate to control foaming, ammonium bisulphite to remove oxygen, sodium bicarbonate to remove excess calcium ions. Diesel fuel, mineral oil or another insoluble organic liquid may be added to a WBM at a concentration of a few percentages to improve the lubricity of the mud in difficult formations (Neff *et al.*,

2000). The oil is dispersed in the water phase and the cuttings remain water wet. Additives of particular concern from pollution viewpoint according to DPR (2002) are ferrochrome lignosulphate and lead compounds.

2.1.2 Non-Aqueous Based Drilling Muds (NADM)

NADM has the continuous phase as a liquid hydrocarbon mixture or other insoluble organic chemical. NADMs also contain barite, clays, emulsifiers, water, calcium chloride, lignite and lime. Water or saline water, brine (usually containing $CaCl_2$) at a concentration of 10 – 50 % is dispersed into the hydrocarbon phase to form a water-in-organic phase emulsion with water droplets less than 1 μm in diameter. This emulsion is called an invert emulsion because water is dispersed in the organic phase, and the formation solids that come in contact with the NADM become oil-wet. The three types of NADMs based on the chemical composition of the base fluid used in the mud formation include oil based mud, enhanced mineral oil based muds and synthetic based muds.

2.1.2.1 Oil Based Muds/Fluids (OBM/OBF)

These contain diesel oil or conventional oil as the continuous phase and are considered the most toxic to environmental receptors. Mineral oils were developed as low toxicity replacements for diesel oil OBFs in an attempt to reduce the environmental impacts of discharged OBF contaminated drill cuttings.

2.1.2.2 Enhanced Mineral Oil Based Muds (EMOBM)

These contain an enhanced mineral oil as the continuous phase. Enhanced mineral oils are conventional mineral oils that have been hydro-treated or otherwise purified to

remove all aromatic hydrocarbons and are thus less toxic to environmental receptors than OBMs.

2.1.2.3 Synthetic Based Muds

These were developed to simultaneously improve drilling and environmental performance. The continuous phase is a water insoluble synthetic organic material. The US Environmental Protection Agency (EPA) (1996) defines a synthetic material as applied to synthetic based drilling muds as "A material produced by the reaction of a specific purified chemical feedstock, as opposed to the traditional base fluids such as diesel and mineral oil, which are derived from crude oil solely through physical separation processes". Physical separation processes include fractionation and distillation and/or minor chemical reactions such as cracking and hydroprocessing. Since they are synthesised by the reaction of purified compounds, synthetic materials suitable for use in drilling fluids are typically free of polycyclic aromatic hydrocarbons (PAHs), but laboratory findings have sometimes shown levels of PAHs up to 0.001 weight percent PAH expressed as phenathrene. On the other hand, the Norwegian Regulatory Authority (Norway, 1997) defines SBM as "A drilling fluid where the base fluid consists of non-water soluble organic compounds and where neither the base fluid nor the additives are of petroleum origin".

Neff *et al.* (2000) further divided synthetic base into four general categories:

I. Synthetic Hydrocarbons: These include polymerised olefins such as linear alpha olefins (LAO) formed by the polymerisation of ethylene; poly alpha olefins

(PAOs) produced by oligomerisation of LAOs of desired length chain; and internal olefins (IOs) formed by isomerisation of LAOs in the presence of heat and a suitable catalyst. Linear alkyl benzene (LAB) was included in this category. They however contain a benzene molecule, and have been reclassified as environmentally unacceptable and are no longer used. Linear paraffins (LP) also called n-paraffins are sometimes included in this class; however they are usually prepared from a petroleum feedstock. Synthetic LPs are available commercially for use in SBMs, but are not widely used because of their cost.

II. Ethers: These are saturated hydrocarbons with an oxygen atom in the center. They are more stable both chemically and biologically than esters or acetals, with high hydrolytic stability and low biodegradability following disposal at sea.

III. Acetals: They are formed by an acid-characterised reaction of an aldehyde with an alcohol or carbonyl compound. They are relatively stable under neutral and basic condition but may revert back to the aldehyde and alcohol under acidic conditions.

IV. Esters: They are formed by the reaction of a carboxylic acid with an alcohol under acidic conditions. They can also be made by oxidation of the terminal bond of LAOs. Esters are somewhat polar and more water soluble than would be expected from their molecular weight (typically 396.4 with chemical formula C26H52O2). Chain length and branching of the fatty acid and alcohol are modified to optimise viscosity, pour point and hydrolytic stability. Esters may be mixed with synthetic hydrocarbons (LAO, IO or PAO) in SBM to attain some particular drilling

performance characteristics. They are relatively stable under neutral conditions but may revert back to their constituents under acidic or basic conditions.

2.2 DRILLING WASTE GENERATION

According to Cline and Piper (1994), drilling mud is pumped into the well for a variety of reasons including the removal of drilled solids. At the surface, an attempt is made to remove dried solids from the drilling mud, a process called solids control. However, not all the solids are removed from the mud, and the amount left is considered contamination. Various amounts of contamination by drilled solids can be tolerated, but if the contamination level begins to affect the mud properties, then whole mud must be discarded and replaced by clean mud, a process known as dumping and diluting. However in cases where SBM are used, the mud is usually recycled and reused. An attempt however is also made to remove drilling mud from the cuttings, but this is incomplete as some amount of mud still adheres to the cuttings though the amount is variable depending on the grain size of the cuttings particle, type of mud, efficiency of the cuttings processing equipment, and geologic characteristics of the formation being drilled (Annis, 1997). As a general rule, if comparable cuttings cleaning equipment is being used, the amount of mud adhering to cuttings increases as particle size decreases and mud viscosity decreases (Neff *et al.*, 2000).

2.3 DISTRIBUTION OF DRILL CUTTINGS IN THE MARINE ENVIRONMENT

Groenewoud *et al.* (1999) has pointed out that the spatial pattern of initial deposition of discharged cuttings material depends on the manner in which the cuttings are discharged and on the tidal current velocity. When cuttings are discharged into the sea, the coarser particles tend to settle quickly to the seabed, especially when SBMs and OBMs are used in drilling, leading to formation of cutting piles. Flocculation of drilling wastes particles also encourage settling of the particles to the floor (Milligan and Hill, 1998). However, local environmental variables such as depth, current and wave regimes, and water stratification will affect the settling behaviour of wastes. Hurley and Ellis (2004), however, pointed out that parameters related to settling rates of different size particles under various conditions are not known, and can lead to error in prediction. In addition, regional and temporal variations in physical oceanographic processes that determine the degree of dilution and waste suspension, dispersion and drift in the benthic boundary layer, have a large influence on the potential zone of influence of discharged drilling wastes. The appearance and size of piles once formed will be affected by such processes as seafloor transport and erosion, either through bottom currents or by wave action that take place, trawl fishing may play also a significant role in the existence and redistribution of piles (Groenewoud *et al.*, 1999).

Dulfer (1999) defined a cutting pile as a deposition of 10 mm of cutting materials. According to Groenewoud *et al.* (1999) modeling exercise has revealed that a short term deposition of more than 10 mm materials depending on the local current condition is to

be expected within 50 m of the discharged points on the Dutch Continental Shelf. However, cutting piles were not noted to exist for long in the shallow waters of the Dutch Continental Shelf, but are quickly eroded due to the relatively high current velocities near the seabed. On the other hand, the bulk of drilling mud discharges settle rapidly and can accumulate on the seabed on the energetic offshore banks of Canadian east coast. (Muschenheim and Milligan, 1996), as a result of flocculation to the order of 0.5 - 1.5 mm of drilling waste particles in seawater (Milligan and Hill, 1998). Roddie *et al.* (1999) have also pointed out that localised cutting piles are a feature of drilling activities in the North Sea. Neff *et al.* (2000) reported that SBF cuttings discharges in the waters of North Sea, Gulf of Mexico, offshore Australia and Ireland accumulate in a very irregular pattern in sediments around a drilling rig. Cuttings coated with NAM are more likely to form piles than cuttings coated with WBM because of the greater adhesion of the latter which encourages them to settle quickly to the bottom.

2.4 ENVIRONMENTAL ISSUES ASSOCIATED WITH DRILL CUTTINGS DISCHARGES

The immediate effect of drilling waste discharge would probably be smothering of the benthic community and the development of cutting piles depending on the oceanographic characteristics of the water (Roddie *et al.*, 1999). Other impacts will be affected by fate, persistence and biodegradability of cutting components, which include changes in sediment grain size and composition (physical alteration of habitat); organic enrichment and sediment anoxia caused by the decomposition of the base fluid; toxicity to aquatic biota; bioaccumulation/bioconcentration by aquatic biota; leaching of non-water based

drilling fluids into water column; and tainting of commercially exploited demersal fish stock.

2.4.1 Fate

When drill waste is discharged into the sea, the water components are likely to disperse into the water column especially when WBM is used in drilling. OBMs have high settling velocity, and thus will be quickly removed from the water column and may form piles. Modeling of oil based mud cuttings in the North Sea by Maynard (1996) showed that in general treatment to reduce oil concentrations on cuttings is likely to increase oil concentration in water column from zero to values that exceed background concentration, though at a concentration that may not be toxic to marine life. Treatment also means that a larger area of seabed receives OBM cuttings leading to reductions in the amount of oil and cuttings deposited per unit area, a situation that favours seabed recovery following cessation of discharges. Untreated oily cuttings on the other hand are quickly removed from the water column settling in thick patches covering small areas, with longer impacts on marine life and sediment.

2.4.2 Persistence

After deposition of the cuttings on the bottom, the physical persistence of the cuttings will depend on the natural energy of re-suspension and transport of the seafloor as well as on the rate of biodegradation of the mud coating the cuttings. In shallow waters, bottom currents may act more to remove and redistribute cuttings than in deep waters, leading to more rapid disappearance of zones of high concentrations. Lower temperature and slower

currents will lead to longer persistence if other factors are constant. Also, the tendency of cuttings containing non aqueous mud to adhere together, resuspension of NAM cuttings requires higher current velocities than those required for WBMs.

2.4.3 Biodegradation

Biodegradation processes may reduce pile size and possible toxicity (Kjeilen *et al.*, 1999). The rates will be affected by factors relating to seafloor conditions including temperature, oxygen availability, sediment type, mud concentration in sediments and mud type. Aerobic degradation is more important on the pile surface than in deeper areas, where anaerobic process predominates.

2.4.4 Toxicity to biota

The primary concern about the discharge of drill cuttings is the level of the base drilling fluids adhering to the cuttings discharged into the environment, and the potential sources of toxicity of drill cuttings can be indicated by the assessment of its chemical constituent. Roddie *et al.* (1999) however reported that there is at present no sound scientific basis for estimating the toxicity of whole cuttings from the knowledge of its composition. Complexation and speciation of individual components will influence their availability and the physical formulation of the original drilling mud will influence interaction between components. These authors however believe that toxicity tests can provide useful tools to investigate and understand components interaction, but this approach according to them has rarely been used in this context in relation to oil industry activities. In the North Sea however, drilling mud chemicals must be pre-approved by determining

their toxicity to three aquatic species representing the aquatic food chain, namely an algae e.g. *Skeletonema costatum;* a herbivore e.g. *Acartia tonsa*; and a sediment reworker such as amphipods according to OSPAR (1995) as quoted by Mairs *et al.* (1999). Furthermore in Nigeria, the DPR requires a 96hr LC_{50} toxicity to be conducted on all mud systems and base oils for OBM using two local species prior to usage (DPR 2002). In some cases discharge approval is given based on the compliance with effluent toxicity limits at the point of discharge. For instance the Department of Petroleum Resources in Nigeria requires that discharged drilling fluids meet both a daily minimum and a monthly average minimum 96hr LC_{50} of at least 30000 ppm in a 9:1 seawater to drilling fluid suspended particulate phase (SPP) volumetric ratio using *Palaemonetes africanus*, a marine/brackish shrimp species, and *Desmocaris tripinosa* a fresh water species, while in Gulf of Mexico the test is carried out using *Mysidopsis bahia* (Mairs *et al.*, 1999).

2.4.5 Bioaccumulation

Issues concerning bioaccumulation relates to significant concentration of heavy metals from the cuttings in the tissues of organisms, and is affected by the bioavailability of the drilling mud/cutting components to the aquatic biota. Most metals in the cuttings are associated with the barite matrix. However barite is stable in seawater under oxidising condition (Neff, 2005). Some metals may be released when barites dissolve under anoxic condition which regularly occurs near installation platforms (Schaanning *et al.*, 2002). Experiment performed with the muscles and liver of dab fish *Limanda limanda* exposed to treated cuttings showed no difference in metal accumulation between control and exposed fish (Stagg and McIntosh, 1996). Reviewing several literatures on

bioaccumulation of metals from drilling mud/cutting, Neff (2005) concluded that these metals have a low bioavailability, and are not biomagnified. When accumulated in the tissue of benthic organisms, they remain in the tissue as insoluble, inert concretions, probably of the original barite particles.

Polyaromatic hydrocarbons (PAHs) are other components of the drill cuttings that have raised environmental concern, and were thought to be the cause of toxicity in earlier drilling. Generally, synthetic drilling fluids have a high n-octanol/water partition coefficient (Log P_{ow}) and are not expected to bioaccumulate in aquatic life (APPEA, 1998). However, vertebrates are also able to metabolise aromatics (Stegman, 1981). Experiments by Stagg and McIntosh (1996) have revealed that uptake of hydrocarbons from oil coated cuttings in the dab fish *L. limanda* was initially rapid but declined with time of exposure possibly due to metabolism of hydrocarbon and excretion associated with exposure.

2.4.6 Leaching of non-water based drilling fluids into water column

The toxicity of cuttings is as a result of the original mud formulation used in drilling. While much is known concerning the base oils, very little is known about the mud additives. The latter is an important issue concerning long term (chronic) effects of cuttings discharged into marine environment as a process such as leaching of the pile constituents will ensure their presence at sublethal concentrations. Factors such as the type of drilling fluid, its persistence and its solubility, manner of burial and prevailing oceanographic conditions will affect the rate of leaching (APPEA, 1998). Additives such

as defoamers, descalers, thinners, viscosifiers, lubricants, stabilizers, surfactants and corrosion inhibitors have been implicated as having effects on marine organisms ranging from minor physiological changes to reduced fertility, lower feeding rates and higher mortality depending on concentrations (Roddie *et al.*, 1999). Hydrocarbons in the cutting piles do not leach out in any substantial amounts over time, but will stay bound to the sediment particles trapped within pore water and degrade slowly. Delvigne (1996), and Stagg and McIntosh (1996) have shown that exposure of fish to low tox oil based fluids, both treated and untreated, resulted in limited leaching of hydrocarbons, and no leaching of metals. Biodegradation may lead to the release of smaller amounts of water soluble metabolites. Other desorption processes such as, presence of surface active compounds, diffusion of components between oil and water, weathering characteristics of base oils and production of surfactants may cause the release of chemicals from particles. These processes however occur slowly, and leaching is thus not considered important for impacts in the undisturbed pile (Kjeilen *et al.*, 1999). However, if the pile structures are disturbed, accumulated solubilised materials trapped in stagnant pore waters may be released to the water column, with the potential acute effects on organisms living in water masses or on the seabed surfaces. Dissolved material will however be rapidly diluted in the water, showing acute effects only over a very limited period of time.

2.4.7 Exposure of sediment to hydrocarbon and production of anoxic sediment

Elevated sediment concentration of base oil which can persist for long periods are issues of concern when drilling with OBMs and SBMs, as deposition of cutting materials will lead to exposure of the sediment to oil. In the North Sea for instance, it is believed that oil

on drill cuttings probably represents the major source of oil discharged offshore (Stagg and McIntosh, 1996). However, sediments near platforms where only WBM cuttings were discharged usually do not contain high concentrations of total hydrocarbons (THC) and PAH (Neff, 2005). Furthermore, processes which cause re-suspension and subsequent deposition of cutting materials will further increase the area of sediments contaminated with oil (Roddie *et al.*, 1999), with the greatest effect to be felt on sediments along current direction (Terrence *et al.*, 1998; Groenewoud *et al.*, 1999). Several post drilling investigation have revealed persistent elevated sediment concentration of hydrocarbons in sediments few months to several years after the cessation of drilling activity (Candler *et al.*, 1995; Olsgard and Gray, 1995; Daan *et al.*, 1996; Terrens *et al.*, 1998; Groenewoud *et al.*, 1999; Sayle *et al.*, 2002; Daan *et al.*, 2006).

Treatment of cutting materials to reduce the oil content will probably reduce the amount of oil deposited on the sediment, increase oil concentration in water 1000 m from the discharge point since the settling velocity of the cuttings is reduced so that oil remains in the water column although at non toxic value, increase the area of seabed that receives oil coated cuttings leading to reductions in the amount of oil and cuttings deposited per unit area, a situation that favours seabed recovery following cessation of discharges (Maynard, 1996). For instance, Stagg and McIntosh, (1996) were also able to demonstrate using artificial sediment that treatment of cuttings to decrease the oil content reduced concentrations of hydrocarbons in the sediment, as much as 40 fold for aliphatics and 10-20 fold for aromatic hydrocarbons.

Degradation of hydrocarbon is possible through aerobic and anaerobic means, but will lead to depletion of oxygen species and the production of anoxic sediment (Sayle *et al.*, 2002). Sanders and Tibbetts (1987) had pointed out that microbiological hydrocarbon oxidation is almost exclusively an aerobic phenomenon, mediated mainly by aerobic bacteria, though yeasts and fungi may be involved in co-oxidation. Bioremediation techniques that increase levels of electron acceptors within pile materials have, therefore, been proposed to aid biodegradation of cutting materials (Backwell, 2000).

2.4.8 Biological impacts of discharged drill cuttings in the marine environment

The immediate biological impact of discharged drill cuttings on the sea bed is associated with the marine benthos. This could result from physical smothering, alteration of sediment properties, and also in the case of non aqueous mud from a decrease in oxygen level resulting from biodegradation of the mud coated cuttings, leading to reduced species richness and macrofaunal density (Daan *et al.*, 1996), and this may last for many years, especially if biodegradation of the mud component is slow or limited, such that sediment concentration of base oil remains significantly high.

Berge (1996) designed two types of experiments to investigate biological effect of treated oily cuttings on benthic community; the community structure experiment intended to test effects on an established community (mainly as mortality); and the settling experiment to test the settling behaviours (avoidance, attraction) of larvae to investigate the effect of treated oily cuttings on benthic recruitment. Both experiments ranked the effects of the

different cuttings treatments similarly, though the species composition was different. Most severe effects were caused by cuttings with high base oil content (15-20 %). Cuttings with base oil content of 2-3 % also caused significant but less severe effects, while cuttings with oil base content less than 2 % caused no significant effect. In the established community experiments, 3 mm layer of cuttings were added on a natural benthic community. Sampling of the area some months later showed a changed community structure with *Capitella capitata* dominating, and 7 months after, *Capitella* was still high, but there was also massive settlement of the polychaete *Polydora* species in all plots reducing the dominance of *Capitella*. On the other hand the sample collected in the settling experiment showed the crustacean *Upogebia deltaura* as the dominant species. The author calculated the threshold for effects on gross community structure at oil concentration of approximately 1000 ppm, and showed that the no-effect concentration at the species level based on oil content was somewhere in the range 150-990 ppm.

In their analysis of the long term biological effect of oily mud on the Dutch Continental Shelf, Groenewoud *et al.* (1999) used the sea urchin, *Echinocardium cordatum* as an indicator species. The authors reported that *E. cordatum* densities have substantially recovered in 90 to 99 % of the seabed initially affected 6 to 8 years after drilling. However, strong effect and less than 50 % recovery can still be expected within a distance of 50 to 200 m from the discharge point. Sampled data of the species showed that of the 15 OBM wells locations investigated, there was a reduction in abundance of either adult or juveniles at 12 of the locations 8-13 years after cessation of drilling within

100 m compared to remote stations. The authors further divided the affected area of each well into three sub areas based on the degree of impact on the abundance of *E. cordatum*, namely, 'strong effect distance' with abundance reduction of 50 % or more; 'minimal effect distance' with abundance reduction greater than 5 %, and maximal effect distance, where reduction in abundance is greater than 1 %. In general, the area affected as measured by the abundance of *E. cordatum* was greatest in the sedimentation area of the DCS, followed by the transition area and least in the erosion, and corresponds to 6.2 km^2, 2.5 km^2 and 2.3 km^2 respectively, i.e. 12 km^2 of the total DCS.

Groenewoud *et al.* (1999) also reported that reduction in densities of *E. cordatum* seems to have also occurred outside the areas where oil concentrations were expected. This according to the authors may be explained by the following two hypotheses. One is that the reduced population density in this outer area may be the consequence of a short term effect due to the fact that recovery of initially affected populations of adults may take several years, and recruitment failure resulting from natural causes may retard such recovery. The second hypothesis is that the distribution of oil in sediments is extremely patchy and is conceivable that at longer distances from well sites such patches do not always turn up in the samples, erroneously suggesting that the sediment of the entire area may not be contaminated. However, mobile animals creeping around the sediments may periodically come in contact with these oil patches. Moreover, even if the concentrations that the animals encounter are below the lethal level, the chronic exposure may impair their tolerance to natural stress and, thus lead, to mortality.

In investigating fields offshore Brunei in South China Sea, drilled with different mud types Sayle *et al.* (2002) reported that WBM tend to disperse more widely than ESBM (up to 4000 m versus 200 m), however the benthic community recovers more quickly from WBM disposal. The data showed reduction in abundance of animals at well sites Egret-9 and Selangir-1 drilled with OBM to be 92 % and 77 % respectively; reduction in species abundance at well site for Iron Duke cluster and Meragi-1 which are ESBM wells are 99 % and 95 % respectively, while that of the WBM, Iron Duke-15 was only 16 %. Reduction in diversity at well site for Egret-9 and Selangir-1 82 % and 66 % respectively, 77 % and 75 % respectively for Iron Duke cluster and Meragi-1; and 27 % for Iron Duke 15. It is also instructive to note that while the species composition of the OBM wells was dominated by the opportunistic polychaete *Capitella*, the species composition of the ESBM well, Meragi-1 was dominated by the Polychaete *Sternapsida*, while that of the WBM well, Iron Duke 15 was dominated by Isopoda.

An initial low level impact, which was limited primarily at the class level of taxonomic grouping and mainly as reduction in abundance of nematodes and crustaceans, the effects of which disappeared four months after cessation of drilling with ester SBM was observed by Terrens *et al.* (1998) on monitoring of the Fortescue field in Gippsland Basin, eastern Bass Strait. The authors reported that significant effect was confined to sampling station FTA1, 100 m from the platform, which was consistent with the observed ester distribution within the sediments.

2.5 THE ROLE OF BIOMARKERS IN ENVIRONMENTAL ASSESSMENT

The effects of marine pollution on marine organisms are immediate as well as long term due to the elimination of sensitive species and reduction in overall abundances of food in the benthic communities, thereby adversely affecting the fishery industry, and decreasing biodiversity. Suter *et al.* (2002) had pointed out that the greatest limitations of many coastal indicators are the lack of linkage with the cause or causes for change. Thus sensitive pollution indicators that will provide early warning of problems before extensive damaged is caused are needed to aid the management of the coastal environment, monitor the impact of industry, and encourage ecologically sustainable industrial development.

A biomarker is a biological measure of an organism's response to a contaminant, defined by Peakall and Walker (1994) as "A biological response to a chemical or chemicals that gives a measure of exposure, and sometimes, also of toxic effect". Biomarkers are first and foremost measures of individual health (Mineau, 1998), and integrate the temporal exposure of organism to contaminants and the multiply pathway of exposure by measuring an actual exposure or biological response to a contaminant. According to De Coen and Janseen (2003), biomarkers can generally be considered measures of the initial changes caused by toxicological interactions between the chemical and the (biological) receptor site. This interaction the authors continue "Induces a cascade of events starting at the subcellular level (e.g., disturbance of gene transcription and interference with metabolic pathways) and ultimately leads to adverse effects at higher levels of biological organization. The effects, which are normally studied in conventional toxicity tests

(impaired growth, reproduction, or survival), can thus be considered the final result of accumulating damage at the subcellular level".

Typical specific molecular biomarkers used for environmental monitoring include vitellogenin, which is induced by xenoestrogens, cytochrome P450 1A, induced by polycyclic aromatic hydrocarbons and dioxins, and metallothionein, induced by heavy metals. These inductions may be detected by increases in mRNA or protein levels, or in the case of cytochrome P450, by enzyme activity. However, organisms are exposed to mixture of chemicals in their environment, and there are also other effects due to environmental changes including changes in temperature and diet, the age of the organisms, and stage of the breeding cycle. Therefore individual biomarkers may provide misleading results, consequently, the use of suites of biomarkers will offer more advantage since no single biomarker can fully give the true picture of the extent of deterioration, or improvement during remedial action (Galloway *et al.*, 2002; Lam and Wu, 2004, Brown *et al.*, 2004; Handy *et al.*, 2003). Exceptional cases however are known where a single biomarker may be able to show that a specific chemical exposure has caused a specific effect. For instance, exposure to tributyl tin (TBT) produces specific degrees of imposex in the dogwhelk *Nucella lapillus* (Evans *et al*, 2000).

Biomarker responses are affected by environmental factors (English and Storey, 2003) Time taken to induce the biomarker as well as the persistence of the biomarker is issue to consider in incorporating biomarkers in biomonitoring studies (Howard *et al.*, 2002). As pointed out by Leung and Furness (2001), if a biomarker occurred but then receded

despite continued exposure or adverse effect, it may be misinterpreted and lead to wrong conclusion that the population is unaffected.

2.5.1 Classification of Biomarkers

Biomarkers can be classified into markers of exposure, effect and susceptibility.

1. **A biological marker of exposure** is an exogenous substance or its metabolite or the product of a xenobiotic agent on some target molecule or cell that is measured in a compartment within an organism. They are responses that indicate that exposure to a chemical or a class of chemical has occurred, but do not provide knowledge of adverse effects at the level of the organism, though they may have harmful effects at the level of population (Walker, 1998).
2. **A biological marker of effect** is a measure of molecular, biochemical or physiological change or other alteration within an organism that can be recognized as an established or potential health impairment or disease. They are therefore responses that indicate that both exposure and adverse effects have occurred. Issues to be addressed include exposure time, time taken to induce the biomarker, persistence of the biomarker during exposure and the sensitivity of the biomarker to sudden short term changes in exposure. Handy *et al.* (2003) have suggested that biomarkers which do not reflect short-term responses to pollution effect, but deteriorating health and biological function associated with low environmental quality to be most useful in measuring chronic effect in organisms.
3. **A biomarker of susceptibility** is an indicator of an inherited or acquired limitation of an organism's ability to respond to the challenge of exposure to a

xenobiotic substance (National Research Council, 1989). They are responses that are used to assess an organism's inherent or acquired limitation to cope with a chemical exposure.

2.5.2 Types of Biomarkers

Different biomarkers have been developed and validated. These are grouped into different types including molecular, biochemical (including metabolic products and enzymes), physiological, histopathological, morphological, reproductive and behavioural However for the purpose of environmental pollution monitoring, the use of a combination of more than one biomarker is advocated.

2.5.3 Application of biomarkers

The aim of using biomarkers is to relate toxic chemical presence in the environment to effects on living organisms as the net result of exposure and toxicity is an effect, which is measurable in the case of a biomarker (Lam and Wu, 2004). The application of biomarker in environmental monitoring will necessitate the understanding of the molecular basis of protection of the cells from the effects of xenobiotics (Moore *et al.*, 2004), and will include characterising the membrane protein pumps that directly remove xenobiotics from cells, as well as biotransformation processes by which the enzymes of cells either detoxify pollutants to harmless excretable products, or activate them to more toxic forms, thus building a weight of evidence case that pollution is affecting the health of natural populations and that particular classes of pollutants are responsible (Handy *et al.*, 2003).

The following areas of applications have been identified according to Handy *et al.* (2003).

1. **Environmental Assessment:** To identify locations and circumstances in which environmental chemicals may have biological effect.
2. **Long-term Monitoring:** To establish baseline (normal ranges and variability) for a particular species or area. Good for identifying deterioration and /or improvements.
3. Field Studies.
4. Identification of organisms/population at risks.
5. Identification of chemical modes of action.

2.5.4 Biomarker responses

Exposure of organisms to contaminants may induce measurable responses in the organisms, which could be implored in monitoring programmes. Some of the most commonly measured biomarkers in routine environmental monitoring are lysosomal stability, a general indicator of stress, metallothionein, commonly associated with heavy metal pollution, ethoxyresorufin O-deethylase (EROD) activity, which commonly indicates oxidative stress.

2.5.4.1 Metallothionein (MT)

Metallothioneins are cysteine rich, low molecular weight proteins of about 7 kDa and apparent molecular weight of 13 kDa. MT pool is made up of different isoforms with certain amino acids substitutions but for the highly conserved cysteine residues (George

and Olsson, 1994). The isoforms have different physiological roles and different ways of induction, depending on the cell types and tissues.

The primary role of MT remains known, but the protein is widely believed to be involved in homeostasis regulation and compartmentalisation of essential metals such as zinc and copper (Roesijadi 1994). MT can donate Zn or Cu to metalloproteins, such as carbonic anhydrase, pyridoxal kinase and haemocyanin (Petering *et al.*, 1991; Churchich *et al.*, 1989); while thionein, the apoprotein form can remove metals from Zn-finger proteins (Zeng *et al.*, 1991; Brouwer and Brouwer-Hoexum, 1992) thus possibly being involved in gene expression by controlling the availability of Zn to regulatory factors (Klaassen *et al.*, 1999). MT may, however, be influenced by various biotic and abiotic factors (English and Storey, 2003; Leung *et al.*, 2001).

Another important function that has been attributed to this protein is the sequestration and detoxification of harmful metals such as Cadmium and Mercury (Coyle *et al.*, 2002) Additional metallothionein synthesis may represent a specific response of the organisms from polluted populations or following exposure to metals such as cadmium, Copper, Zinc and mercury (Hamer, 1986), MT induction has been reported to begin in 24 hours and to reach its maximum levels by 48 hour during a 96 hour exposure to heavy metals in the grass shrimp *Palaemonetes pugio* (Howard *et al.*, 2002). Cellular interception of metals by metallothionein possibly occurs either directly by binding of metal ions that are initially taken up by the cell or by removal of metals from non-thionein ligands that include cellular targets of toxicity. Cd-induction of MT results in relative concentration

of metallothionein-bound metals in the organism (Coucelo *et al.*, 2000; Howard *et al.* 2002,) and thus confers enhanced metal tolerance to the organism (Klaassen *et al.*, 1999; Hogstrand *et al.*., 1996). This concentration of metals especially in species that serve as food source to human has raised concern over trophic transfer of metals (Wang, 2002). Butler and Roesijadi (2001) were able to demonstrate protective function of MT against Cadmium toxicity by showing that disruption of MT expression results in increased Cadmium toxicity in the haemocytes of the oyster *Crassostrea virginica*. It is suggested that the capacity to induce MT is greatest in tissues and organs that are involved in metal uptake, storage and excretion, such as the gill, digestive organs and the kidney. The accumulation of MT has therefore been proposed as a biomarker of exposure to heavy metals. Production of MT also functions to protect the cell from oxidative stress caused by Cadmium exposure (Viarengo *et al.*, 1999).

It has been suggested that inclusion of measurement of MT in biomonitoring will of necessity include the quantification of the different isoforms and their induction patterns (Lacorn *et al.*, 2001), major site of induction and the determination of the half-life/persistent (Leung and Furness, 2001; Howard *et al.*, 2002). The half ($t_{0.5}$) is the time required for pollutant concentration to be reduced by half due to physical and chemical processes, including transformation to its degradation products.

Brown *et al.* (2004) showed that induction of MT in *Carcinus maenas* was dose dependent on exposure Copper, and that overexpression of MT in organisms exposed to 68.1 µg Cu/l was able to protect the organism against deleterious effects at higher

biological level. However, no induction of MT was observed in *Patella vulgata* treated to 6.1 µg Cu/l. This observation the authors believed was consistent with protein catabolism witnessed in this organism, and the reason the organism was very susceptible to Copper toxicity. Brown *et al.* (2004) also reported that *Mytilus edulis* was relatively insensitive to Copper over 7 days of exposure to 68.1 µg Cu/l, probably as a result of reduced Copper uptake due to reduced feeding. Aside reduction in NRR time, which may be attributable to indirect effect of Copper due to fasting, the organism was not affected by Copper over 7 days of exposure. The authors concluded that the exposure Copper concentration was too low to have induced physiological changes in this species over the experimental 7 days period.

Domouhtsidou *et al.* (2004) reported higher metallothionein values in the digestive cells of *Mytilus galloprovincialis* than recorded in the gills and mantle/gonad complex of the same species, which the authors attributed to the high Cu and Zn values found in the digestive gland of the animal. Also, the MT content of the gill and the mantle/gonad complex significantly correlated negatively with the lysosomal stability values. In addition, the authors were able to recognise that the synthesis of MT in this species reflected the pollution gradient of the sampling stations, with the least values being recorded in the reference site.

Coucelo *et al.* (2000) reported significant production of metallothionein in the hepatic tissue of a marine teleost *Halobatrachus didactylus* 24 hours and 7 days after intraperitoneal injection of 1 µg/g Cd^{2+} ($CdCl_2$). Liver concentration of Cd also increased

confirming that MT mops up free metals in the cell thereby protecting the cell from metal toxicity.

Leung and Furness (1999) investigated the production of MT in different tissues of the dogwhelk *Nucella lapillus* on exposure to 0.5 mg Cd/l for 60 days and depuration for another 110 days. The authors found a positive correlation between whole body Cadmium concentrations and MT levels during exposure period. No significant difference was found in the concentration of Cu and Fe between control and Cd-treated animals, but the concentration of Zn significantly increased in treated animals suggesting that that Cd-induce MT may bind to cytosolic Zn. The half life of MT in this species was calculated to be approximately 40 days, while Cadmium concentration increased in the treatment and leveled off only after 120 days indicating that *N. lapillus* could not regulate Cd. The authors also reported the induction of MT in the different tissues of this species, and at the end of the depuration period ranked the order of MT levels as Leiblein gland > gonad > kidney > digestive gland > gills > other tissues; and the order of Cd concentration as Leiblein gland > digestive gland > gonad > kidney > gills >other tissues. The authors therefore suggested that the pattern of induction of MT indicate that the protein may be involved in the detoxification of Cd in *N. lapillus*. Also following the high levels of MT induction in the Leiblein gland and its persistence even after prolonged depuration, the authors advocated the inclusion of the measurement of MT in this tissue in biomonitoring programmes.

Antioxidant role of MT in mussels has been demonstrated. This role is achieved by oxyradical scavenging activity and binding of Fenton activity metals (Cu, Fe), by MT and is effective in protecting cells and the entire organisms from oxidative stress (Viarengo *et al.*, 1999). Pitt *et al.* (1997) also reported protection of MT against oxidative stress in sheep pulmonary artery endothelial cells (SPALC). However, though MT can protect against oxidative stress, the protein itself is poorly inducible by oxidants at least in invertebrates (Viarengo *et al.*, 2000a).

In the study carried out by Viarengo *et al* (1999), exposure of mussels to Cadmium and/or Iron resulted in significant increase in metallothionein induction. MT induction in Cadmium preexposed mussel remained nearly unaltered after treatment to Iron, a prooxidant transition metal, and was responsible for preventing prooxidant processes due to Fe treatment, thus increasing the survival rate of pretreated Cadmium mussels. Pitt *et al.* (1997) isolated and cultured endothelial cells from the proximal pulmonary artery of sheep (SPALC). The cells (n=10) were treated to Cadmium concentrations up to 1.0 mM for 24 hours. The authors noted concentration and time dependent increases in sheep cells MT mRNA and protein levels, and demonstrated that preexposure of SPALC to Cadmium resulted in cells with increased levels of MT and that are also resistant to oxidative stress. They also provided evidence via direct gene transfer that MT was participating in the antioxidant defenses of pulmonary endothelium to tert-butyl hydroperoxide (*t*-BOOH a), hyperoxia and 2,4-dimethylvaleronitrile (AMVN), a peroxyl radical generator, though antioxidant protection of MT in SPALC may be phospholipid selective.

MT protection against nonmetallic chemicals such as carbon tetrachloride (CCl_4) has been demonstrated by Klaassen and Liu, (1998), who showed that MT induction by Zn in the hepatic cells of mice were able to bide ^{14}C from ^{14}C-CCl_4 in the MT-induced animals than in controls, with a concomitant reduction of covalent binding of ^{14}C-CCl_4 to cellular protein and lipid.

Induction of MT however is not restricted to heavy metals alone. For instance, Ghoshal *et al* (1998) have demonstrated MT induction in response to restraint stress. Also, MTs are also known to have been induced in response to environmental stress in the marine periwinkle *Littorina littorea* (English and Storey, 2003). The authors reported that exposure to anoxic and freezing conditions in this gastropod resulted in 3-6 fold increase in MT mRNA gene levels and that induction response was essentially the same in both cases, rising quickly and remaining high for at least 12 h of stress exposure; oxygen deficiency being the trigger for transcription of MT gene. The upregulation of the MT gene is probably part of the antioxidant defense system, and is aimed at combating oxidative damage from reactive oxygen species on reintroduction of oxygen.

2.5.4.2 Lysosomal Stability

Lysosomes are membrane bound organelles that contain hydrolytic enzymes for breaking substances within a cell (autophagy) or substances that have been taken in from outside the cell (heterophagy). Accumulation of toxic substances such as heavy metals, PAHs, PCBs, oil derived hydrocarbons could cause alteration in the lysosomes leading to a

reduction in the stability of the lysosomal membranes and an increase in lysosomal enzyme activities (Lowe *et al.*, 1992). Lysosomal enlargement in response to contaminants has been observed in laboratory studies using both bivalve (Porte *et al.*, 1998) and fish cells (Lowe *et al.*, 1992; Roméo *et al.*, 2000). Harmful algal blooms have also been recorded to reduce lysosomal membrane stability in oysters as reported by Keppler *et al.* (2005). The authors reported that laboratory exposures to cultured alga *Heterosigma akashiwo* cultures, and natural water samples collected during a *H. akashiwo*-dominated bloom resulted in significant lysosomal membrane destabilization rates (>39 %) in the digestive gland cells of oysters *Crassostrea virginica* compared to control, indicating pronounced malfunctioning of the hepatopancreas. No mortality was however recorded after 4 days of exposure to the alga in the laboratory, and destabilisation rate however continued to increase 7 days after recovery period.

Nolde *et al*, (2006) suggested that serious damage of cells and organism-level effects could appear when lysosomal membrane stability is reduced by more than 40 % using the neutral red retention protocol or 30 % if the lysosomal latency assay is used.

Significantly lower lysosomal membrane labialisation period in Copper treated digestive gland cells of *Mytilus galloprovincialis* compared to control cells have also been reported in an inter-calibration exercise involving different laboratories (Viarengo *et al.*, 2000b). The authors reported that each laboratory was able to distinguish between control and Cu-exposed mussels, and no significant differences existed among the laboratories. The labialisation induction factor, i.e. the ratio between treated and control samples were very

similar for all laboratories. The authors concluded that inter-calibration exercise is necessary for the quality control of biological data collected in large biomonitoring programmes as in the case of MED POL programme for the Mediterranean Sea. Moore *et al.* (1999) also reported a strong correlation of lysosomal integrity with pollution gradients in a large scale biomonitoring program for the Black sea.

Lysosomal damage can thus be employed as a prognostic biomarker for pathology and reduced fitness and may have the utility to predict population and community status (Ringwood *et al.*, 2004; Köhler *et al.*, 2002). Factors such as season and reproductive state of the organism may also confound results (Taleb *et al.*, 2007). Previous history of exposure is also a factor to consider as tolerance of organisms to chronic exposure of substances in their environment and/or non availability of pollutants may give higher NRR time than may be expected (Nolde *et al.*, 2006).

2.5.4.3 Biomarkers of Oxidative Stress

Oxidative stress is a state of unbalanced tissue oxidation, involving enhanced intra- and extracellular reactive oxygen species (ROS) production, peroxidation of lipids, proteins, and DNA, and often causes a general disturbance of the cellular redox balance i.e. the ratio of reduced to oxidized glutathione (GSH/GSSH) and the NADH/NAD (Abele and Puntarulo, 2004). Oxidative stress is a product of one of three factors listed by Dorval *et al.* (2005), namely, increase in reactive ROS, an impairment of antioxidant defense systems, or an insufficient capacity to repair oxidative damage.

Reactive oxygen species (ROS) refers to oxygen free radicals, i.e. partially reduced intermediates of the 4 electron reduction of oxygen to water: superoxide anion ($O_2^{\cdot-}$) and hydroxyl radicals ($^{\cdot}OH$), as well as the non-radical active species such as hydrogen peroxide (H_2O_2) (Abele and Puntarulo, 2004). Abele (2002) listed their effects within the cell to include genetic degeneration and physiological dysfunction leading to cell death, and progressive ageing of the organism. ROS are unstable presenting one or more lone electrons, and will yield or tear off electrons from molecules in order to achieve stability (Manduzio *et al.*, 2005). The superoxide radical is a poor oxidant and will not result in oxidative damage to the organism, but in the presence of Iron will undergo the Haber-Weiss reaction, to form molecular oxygen and the extremely reactive hydroxyl radical $^{\cdot}OH$. The hydroxyl radical can also be produced by hemolytic fission of hydrogen peroxide in the presence of transition metals (Iron, Copper) through Fenton's reaction. Other ROS include the hydroperoxyl $ROO^{\cdot}$ and alcoxyl $RO^{\cdot}$ radicals that arise from lipid peroxidation.

Nitric oxide $NO^{\cdot}$ is regarded as a reactive nitrogenised species (RNS) and can play a role both in the production and destruction of radicals. It can be formed from endogenous or exogenous NO donors or from L-arginine by the activity of the enzyme nitric oxide synthase (NOS). It plays a role in the neurotransmission and cellular immune defence. High concentration of NO impedes cellular functions such as DNA synthesis and mitochondrial respiration.

The antioxidant defense systems function by either repairing the oxidative damage or by directly scavenging oxygen radicals. The systems include the antioxidant enzymes (AOX) of the cell namely; superoxide dismutase (SOD) that metabolises superoxide radical to H_2O_2, catalase (CAT) that converts H_2O_2 to water and oxygen, and glutathione peroxidase (GPx) that detoxifies hydroperoxides. GPx uses reduced glutathione (GSH) as electron donor and generates oxidised glutathione (GSSG). Glutathione reductase (GRx) may also be included as a member of the antioxidant enzymes because it reduces the oxidised glutathione according to a NADPH-dependent process, and is thus at the base of regeneration of reduced glutathione (GSH) necessary for the activity of GPxs and other enzymes in the cell. The second component of the antioxidant defense systems is the free radical scavengers such as glutathione, vitamins E, A and C, urate, biliverdin Metallothionein has also been implicated in protection of the cell against oxidative damage (Viarengo *et al.*, 1999; Klaassen *et al.*, 1999; Coucelo *et al.*, 2000; English and Storey, 2003), mainly due to its sulfhydryl nucleophilicity and also metal complexation (Viarengo *et al.*, 2000a).

Glutathione S-transferases (GST): GSTs are important catalysts for the conjugation reactions involving glutathione and various electrophilic substances during phase II metabolism. The enzymes exist in different forms and their induction has been reported in response to different contaminants. Substrates for GST share three basic features: they must be hydrophobic to some degree, they must contain an electrophilic carbon atom and they must react nonenzymatically with glutathione at some measurable rate (Sipes and Gandolfi, 1991). However, Hyne and Maher (2003) have pointed out that their inclusion

as biomarker requires more study because of their lack of specificity and qualitative response of the enzymes to contaminants.

The antioxidant systems are sensitive indicators of exposure to xenobiotics and may thus be employed as biomarkers of oxidative stress in exposed organisms. Substances such as Polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and metals are capable of exerting oxidative stress in invertebrate (Viarengo *et al.*, 1999). Effects of pesticides in bivalve larvae have also been reported to include oxidative stress indicated by measured thiobarbituric acid (TBARS) levels (Damiens *et al.*, 2004). Copper has been shown to increase the rate of lipid peroxidation measured by the level of malondialdehyde (MDA) by 365 % at 200 µM with no significant effect on the activity of catalase when added in vitro to kidney extracts of the sea bass *D. labrax* after 20 mins, where as Cadmium at 500 µM raised the MDA only by 20 %, while at the same suppressed catalase production in the liver extract (Römeo *et al.*, 2000). Organisms exposed to severe environmental stress such as freezing and hypoxia may experience elevated production of ROS on reintroduction of oxygen and would therefore build up defense mechanisms during the state of hypoxia (English and Storey, 2003). Increased ROS may be experienced directly during the hypoxic state (Chandel and Schumacker, 2000). Warming has been reported to increase mitochondria production of ROS and oxidative stress in the clam *Mya arenaria* (Abele *et al.*, 2002), and in the marine polychaete lugworm *Arenicola marina*, which was associated with higher activities of the antioxidant enzymes catalase and SOD, as well as lower mitochondria densities (Keller *et al.*, 2004).

Downs *et al.* (2002) investigated the correlation between levels of oxidative damage products, antioxidant enzymes and specific components of cellular structural integrity with coral bleaching, seasonal and increased sea surface temperature (SST) and water depth in a bid to establish that coral bleaching is related to specific molecular mechanism to prevent the production of ROS or to protect against the damaging effect of ROS. Temperature were monitored at depths 3 m, 9 m and 18 m, and temperature values at all sites exceeded 28^{O}C from June to September with a peak in July when temperature at the 18 and 9 m site were above 30^{O}C. By September when bleaching event occurred in response to July temperature, bleaching was positively correlated to depth, but negatively correlated to temperature. Corals at the 18 m depth were completely bleached, partial bleaching was observed at the 9 m depth and no bleaching at 3 m water depth. Recovery was observed in October when temperature decreased to below 25^{O}C in the 3-9 m depth and below 27^{O}C at the 18 m depth. Bleaching was associated with oxidative damage as was indicated by the levels of protein carbonyl and lipid peroxidation which were positively correlated with ocean temperature. Significant negative correlation was calculated between carbonyl and chlorophyll a content. The authors also reported that the measured antioxidant enzymes, (glutathione, SOD) and heat shock proteins Hsp60 and Hsp70 showed these to be negatively correlated with correlated with levels of oxidative damage. In summary the work shows that photosynthesis II was the source of ROS responsible for oxidative stress in the coral. Severe oxidative damage products correlated with, and preceded coral bleaching, and also affected cellular integrity. There was an

increased energy demand to maintain homeostasis and the antioxidant defense and stress proteins were involved in protecting the cells.

On measuring antioxidant responses in three tissues of the fish *Halobatrachus didactylus* exposed to sublethal concentration of cadmium, Coucelo *et al.* (2000) reported significant increase in the activity of SOD and CAT in the mitochondrial fraction of the liver and kidney of the fish 24 hours after exposure to cadmium. On the other hand, cytosolic SOD activity showed significant increase 7 days after exposure to Cadmium in the hepatic and cardiac tissues. Kidney CAT and SOD activities showed a decrease 24 hours after exposure to cadmium, and increased significantly between 24 hours and 7 days of exposure in the mitochondrial fraction, where as no difference was observed in the cytosolic fraction. The authors related the early induction of mitochondrial antioxidant enzymes to the activation of defensive mechanism against Cadmium toxicity by the cell since Cd is known to inhibit oxidative phosphorylation process, as well as interfere with mitochondrial oxidative mechanism. The increased mobilization of the antioxidant defense is also responsible for protecting the cell against oxidative damage as no significant change in lipid peroxidation products was observed.

Dorval *et al.* (2005) revealed altered levels of the antioxidants (CAT, GPx and GSH), lipid peroxidation, and plasma cortisol in the liver and adrenal tissues of the white sucker fish (*Catostomus commersoni*) sampled in water impacted by agricultural chemicals. According to the findings of these authors, the activities of CAT and GPx were significantly higher in both tissues of fish from reference site compared to those of

contaminated sites, and no significant difference was observed between the three contaminated sites. Higher activities were also found in the liver than in the adrenal in both reference and contaminated fish. Higher GST activity was found in the liver than in adrenal of the fish though GST levels was not significantly different in fish from contaminated and reference sites, possibly because GST uses GSH for activity, and the former has been depleted in contaminated sites. It was also observed that the level of lipid peroxidation was significantly higher in tissues of fish sampled in contaminated sites than in those from reference sites, adrenal levels being higher than the levels found in the hepatic tissue. In conclusion, chronic exposure to agrochemicals was associated to an impairment of antioxidant enzymes and increased lipid peroxidation, potentially compromising adrenal and hepatic cell function and leading to reductions of hormone levels and glycogen.

In a bid to investigate the subunit expressions of GST activities in a salmonid species, Pérez-López *et al.* (2002) injected sample of the rainbow trout intraperitoneally with 3 doses (5, 20, 70) μg/g body weight of a commercial PCB mixture (Arochlor-1254) in 0.5 ml corn oil, and a reference group that received only 0.5 ml corn oil. All the three tissues, namely the liver, kidney and gill examined showed no specific significant GST activities compared to reference group. Total GST activity was however significant in the hepatic fraction, but the kidney and gill fractions showed significant total GST activity different from the control group of the fish only when determined against ethacrynic acid (ETHA). Moreover, 5 isoforms of the GST were identified, of which only forms 1, 2 and 3 expressed significantly different profile pattern in the liver, and only form 1

(corresponding to π-class) showed a significantly different profile pattern in the kidney and gill at the end of the 9 week study. The authors discussed the importance of characterisation of the different GST isoforms in order to establish the existence of an enzymatic induction since the inhibition or induction of a specific GST isoenzyme cannot be revealed by measurement of GST activity alone. This characterisation is important for the validation of the use of trout GST as biomarkers of environmental contamination, more so as π-related subunits can be directly associated to PCB-mediated enzymatic induction in salmonid fish. The increase in general GST activity observed in the reference was attributed to the corn oil which was administered to this group. Corn oil contains a high proportion of polyunsaturated fatty acids, which activate the peroximal fatty acid oxidation system leading to oxidative stress.

2.5.4.4 Other Biomarkers

There are several other biomarkers which may be used to investigate effects of chemicals on individuals and many of these have successfully been employed in biomonitoring programmes. These include **molecular biomarkers (genotoxicity)** that measures responses in nucleic acids of natural biota, which may manifest either as damage to the genome or some adaptive change in gene expression (Lam and Wu, 2004). As proposed by Anderson *et al.* (1994), the concept of genetic ecotoxicology included measurement of direct DNA damage, epigenetic effects and changes in gene pools attributable to toxicant exposure. However, seasonal variations in environmental conditions, diet, hormonal status may have a marked effect on the enzymes that activate and detoxify genotoxicants (Mitchelmore and Chipman, 1998). For instance the work of Everaarts *et al.* (1994) on

DNA integrity and EROD induction in the flatfish *Limanda limanda* and the asteroid echinoderm *Asterias rubens* sampled from a transect of the North Sea revealed strand breaks which did not correspond with contamination gradients of PCBs and PAHs concentrations, and responses that may be indicative of environmental ambient conditions and not necessarily due to xenobiotics.

Changes in genetic materials in lower vertebrates and invertebrates are associated with various diseases including impairment of enzyme function, general metabolism and immune response, enhanced protein turnover, production of initiators of cytotoxic injuries, inhibited growth, decreased scope for growth, decreased fecundity, and faster aging (Depledge, 1994). Population level effect of genotoxicants are however regarded as having more of ecological importance than the individual effect because it is at this level that evolutionary processes manifest (Anderson *et al.*, 1994). Anderson *et al.* (1994) advocated interdisciplinary collaboration including the area of molecular biology, ecotoxicology, population genetics and community ecology in order to forge comprehensive studies of genetic alterations in natural populations.

Physiological Markers: Pollutant effect on organisms may include the disturbance of the physiological process including respiratory, cardiovascular, osmoregulatory, neurological, and endocrine impairments. Physiological markers integrate subcellular and cellular processes, and may be indicative of the overall fitness of the organism (Hontela, 1997). Campbell *et al.* (2003) reported that field collected fish exposed to heavy metal contamination exhibited endocrine impairment which was absent in fish from reference stations, and that the impairment was associated with a reduced capacity to increase

plasma cortisol levels in response to metal stress. The authors also reported metabolic impairment in fish collected in metal impacted lakes. Increase in standard metabolic rate has also been associated with exposure to heavy metals in crayfish *Procambarus acutus* (Rowe *et al.*, 2001). Brown *et al.* (2004) recorded a decrease in heart rate in the common limpet *Patella vulgata* exposed to Copper. Handy and Depledge (1999) have pointed out that the use of physiological assays requires the establishment of the normal resting response of the organism in defined environmental conditions.

Mixed Function Oxidases: The enzymes of the cytochrome P450 family are involved in the biotransformation of organic xenobiotics. Their activity can be measured using test such as the induction of ethoxyresorufin O-deethylase (EROD). EROD activity is a well-established *in vivo* biomarker of exposure to several planar/halogenated aromatic hydrocarbons (PHH/PAH) and many structurally related compounds (Lam and Wu, 2004). CYP1A a member of the cytochrome P450 (CYP450) enzymes is induced by several groups of polyaromatic compounds, planar chlorinated biphenyls and polychlorinated dibenzo-p-dioxins and dibenzofurans, (Tom *et al.*,2003). The Ah receptor is the major pathway for the induction of P450, and Hahn *et al.* (1992) has shown this receptor to be present in fish, but not in invertebrates. Increase in CYP1A levels in the carp *Cyprinus carpio* on exposure to Ah receptor agonists has been demonstrated, however, EROD activities decreased with increasing concentration after reaching a maximum level (Smeets *et al.*, 1999). Fish CYP1A is evaluated at the transcript, protein and catalytic activity levels. Reverse transcription-competitive polymerase chain reaction (RT-cPCR) for the quantitative assessment of fish transcript

CYP1A level, and the enzyme-linked immunosorbent assay (ELISA) for quantitatively assess protein level have also been reported in the literature (Tom *et al.*, 2003). The use of this enzymes as biomarkers of exposure in invertebrates is however limited because of the low rate of induction in invertebrate species.

Acetylcholinesterase Activity (AChE): Acetylcholinesterase is essential for the functioning of the nervous system, and uses acetylcholine as the transmitter substances for transmission of impulse at neuromuscular junctions and at synaptic knobs (Schmidt-Nielsen, 1990). Hydrolysis of this transmitter substance will yield choline and acetate as metabolites which is then recycled (Nwosu *et al.*, 1992). The inhibition of this enzyme is specifically employed as a marker of organophosphorus and carbamate pesticide exposure, and the effect will exist long after the organism may have metabolised or eliminated the pesticide from its body (Hyne and Maher, 2003). However, inhibition of this enzyme by exposure to Copper in the shore crab *Carcinus maenas* has been documented (Brown *et al.*, 2004). Sibley *et al.* (2000) demonstrated that AChE activity can be used as indicator of exposure and mortality in zooplankton, and that the enzyme has the capacity to predict population level effects (Hyne and Maher, 2003). However, in imploring the use of this enzyme in field studies of population density, choice of species and its AChE activity must be considered.

Stress Proteins: They are produced by organisms in response to stressed environmental condition, as found in the mollusk, *Mytilus edulis* which produces these proteins in response to both heat shock and Cadmium (Sanders, 1993). They are produced during stress to restore and protect normal cell function by refolding damaged proteins.

resolubilising protein aggregates, and helping to protect protein synthesis (Sander *et al.*, 1994). Stress 70 and chaperon 60 (cpn60), are the most studied stress proteins, which have been used as biomarkers in a range of test organisms (Lewis, et al., 1999).

Histo-cytopathological Biomarkers: Histo-cytopathological biomarkers are lesions that signal effects resulting from prior or on-going exposure to one or more toxic agents, and one of the most rapid methods for detecting effects in organisms from the field. Histopathologic biomarkers are higher level responses – reflect prior alteration in physiological and/or biochemical function, with lesions often persisting after exposure has ended. Their use presumes the normal microscopic anatomy to be known and the consideration of the full range of natural variability. The summary below is mostly adapted from the review of Au (2004).

Most histopathological symptoms are responsive to a variety of pollutants (at least more than one) and are therefore only indicative of the general quality of the environment rather than specific types of pollutants. Further, since many pathological incidences are species-specific, the restriction in natural distribution of many species may make monitoring results not directly comparable between regions/locations. Except for a few symptoms (e.g. liver tumors, lysosome integrity, lipopigment content, peroxisome proliferation), the cause–effect relationships and detailed mechanisms leading to the development of most pathological symptoms are not clear. Further laboratory and field studies are thus required to understand the mechanisms involved in order to provide a sound scientific basis for monitoring programs.

1. Necrosis as in fin erosion (Lindesjöö and Thulin, 1990). Fin erosion may be caused by effects of chemicals on mucus and/or skin epithelium, though fish stressed by chronic exposure to contaminants may be more susceptible to microbial infections. and factors such as fungal/bacterial pathogens and hereditary defects are associated with the disease development. Although induction of fin erosion in fish is not as specific as other toxicopathic lesions, the use of fin erosion as a biomarker have many advantages in environmental monitoring, which include, easy visual recognition of the symptoms visually, rapid diagnosis cost-effectiveness. As a consequence, this fish disease has been frequently employed as an indicator for environmental quality in various national monitoring programmes.
2. Hyperplasia /papilloma. Recognised as small, rounded, half-transparent, creamy white patches with a smooth surface measuring 2-10 mm in diameter. Its induction in fish is a sensitive, non-specific response to a wide range of chemical contaminants and physical stress (e.g. hypoxia), and a good dose-response relationship has generally been demonstrated.
3. Hypertrophy. Excessive growth, increase in size of individual cells
4. Neoplasm. Relentlessly growing mass of abnormal cells – tumors
5. Histopathology of liver. Different lesions have been identified in different fish species, including foci of cellular alteration (FCA), megalocytic heptosis (MH). hepatocellular nuclear pleomorphism (NP) and hydropic vacuolation (HV). Factors such as season, age, sex may confound results obtained. The use of liver histopathology as a biomarker of chronic exposure may be limited by the long latent period for tumor development.

6. Histopathology of gill: Epithelial hyperplasia with lamellar fusion, epithelial hypertrophy, telangiectasia, shortened gill filament, edema with epithelial lifting, and epithelial desquamation are examples of gills' lesion in response to a wide range of contaminants. Exposure of the goldlined seabream *Rhabdosargus sarba* to sub-bloom and bloom levels of *Chattonella marina*, a red tide causative species, elicited chloride cell proliferation on gill epithelial within hours, and this resulted to fish death shortly afterwards (Tang and Au, 2004). Response of fish gill to stress/pollutant exposure seems not to be affected by the biology of the fish (sex, age) or seasonal factors.

7. Histopathology of kidney: Non-specific kidney histopathological lesions (e.g. degenerative changes in tubular epithelium, dilation of tubular lumina, proteinaceous or cellular casts within tubular lumina, tubular necrosis and/or epithelial desquamation, and necrosis of interstitial hematopoietic tissues) have been observed in fish exposed to organochlorines, petroleum compounds, organophosphate, herbicides and heavy metals.

8. Macrophage aggregates (MA): Macrophages are large irregularly shaped cells present in liver, spleen and kidney, where they engulf cellular debris and microbes inside the organ. MA is a sensitive but non-specific indicator of exposure to environmental contaminants and has been reported in a variety of fish species (e.g Winter flounder, English sole, Rainbow trout, common jollytail (*Tasmanian blennies*) inhabiting degraded environments. Confounding biological factors such as starvation, bacterial infections, parasitic infections, heat stress may increase MA in fish.

9. Oocyte atresia: This is characterised by degeneration and necrosis of developing ova, and occurs in the ovaries of all fish species but can become pathologic following exposure to xenobiotic compounds, such as sublethal exposure to aromatic and chlorinated organic compounds, heavy metals, fuel oil and naphthalene. There may be species-specific differences, behavioural stage of sexual development are some of the compounding factors that affect interpretation, especially in field collected samples.

10. Embryonic defects: Embryonic defects may be indicated by morphological defects on non-synchronous cleavage, formation of loose cell aggregates during gastrulation, failure of blastospore closure and gross abnormalities in late embryos. Embryonic defects tend to be non-specific but sensitive to contaminants exposure Assessment however is restricted by the spawning season, which is short for most species.

11. Lipopigment content: Lipopigments tend to accumulate in the lysosomal compartments as residual bodies, which may be related to disturbance of lysosomal system functioning. In European flounder, decreased lysosome membrane stability coincided with increased size of lysosomes and increased lipid content (Köhler *et al.*, 2002). Standardisation of method for lipopigment determination is necessary for its worldwide application in pollution monitoring.

12. Peroxisome proliferation: This is defined as an increase in either peroxisomal volume or numbers, which may not always be accompanied by an induction of peroxisomal enzymes (Reddy and Mannaerts, 1994). Peroxisomes are single membrane-limited organelles that are involved in metabolic functions e.g. fatty acid

ß oxidation, and cellular respiration involving the metabolism of hydrogen peroxide (e.g. production of oxidases and catalase), (Fahimi *et al.*, 1993). Peroxisomes proliferations have been observed in response to xenobiotics both in laboratory and field studies. Factors such as season, site and sexual maturity could affect peroxisomal enzyme activities in mussel.

2.6 THE ECOLOGY AND BIOLOGY OF ESTUARINE FAUNA OF THE LAGOS LAGOON

Lagos lagoon is the largest of the four major lagoon systems that are found on the Gulf of Guinea in West Africa. It is a shallow expanse of water connected to the open sea via the Lagos Harbour (Hill and Webb, 1958) and thus is open to tidal influences from the sea resulting in diurnal variations in salinity. It also receives influx of fresh water from connected rivers and creeks including Majidun, Ogun, Ona, Shasha and Oshun which drain about 103,637 km^2 area (Oyewo, 1998), hence can also be regarded as an estuary (Chukwu, personal communication). Seasonal and diurnal variations in salinity are key factors that govern distribution of organisms within the lagoon system hence organisms inhabiting the lagoon must have a high tolerance for fluctuations in salinity. Seasonal environmental factors in the lagoon are attributable to rainfall distributive pattern. Lagos lagoon has a narrow intertidal zone that ranges from mangrove swamps to muddy and sandy shore. It also has a low benthic faunal composition and low diversity (Brown, 1991). Some of the organisms found in the lagoon include *Tympanotonus fuscatus*, *Pachymelania aurita*, *Clibanarius africanus*, *Sesarma huzardi*, *Neritina glabrata*, *Anadara selinus*, *Aloidis trigona*, *Polydora* sp, *Nereis succinea*, *Capitella capitata*,

Nerita senegalensis, various fish such as *Mugil* sp., *Tilapia* sp, , *Bathygobius soporator, Elops lacerta, Pomadasys jubelini, Caranx hippos* and many planktonic species. Recent studies however have shown increased diversity in fish species in the Lagos lagoon complex, possibly due to increased salt water intrusion (Emmanuel, 2009). Brown (1991) has pointed out that the faunal composition of the Lagos lagoon suggested an unstable environment

Owing to the location of the Lagos cosmopolitan city, Lagos lagoon serves as sink for various increasing array of waste types, including sewage, wood waste, municipal and industrial effluents among others (Akpata *et al.*, 1993; Nwankwo 1998; Chukwu and Nwankwo, 2004). Consequently, different investigators have studied the occurrence and distribution of industrial and domestic pollutants as well as microbial load in the lagoon system (Oyewo, 1998; Enajekpo, 2000; Ajayi and Akonai, 2003; 2005). These studies have revealed the degradation of the lagoon over time (Nwankwo, 2004), and the need to develop appropriate protocols that is aimed in safeguarding and encouraging the sustainable use of the Lagos lagoon system. Issues relating to bioaccumulation of pollutants and possible trophic transfer especially in species that serve as food to man are pertinent. For instance, Otitoloju and Don-Pedro (2004) demonstrated that the edible periwinkle *Tympanotonus fuscatus* is able to concentrate heavy metals in its soft tissues from binary mixtures of heavy metals in both field and laboratory experiment.

Tympanotonus fuscatus: These gastropods are commonly found in the brackish waters of West Africa. Two subspecies are found in the Lagos lagoon complex, *T. fuscatus fuscatus* (Linne) with tubercular spines and *T. fuscatus* (var *radula*) with smooth or

granular shell ornamentations. Both types are found within the inter-tidal zone: usually one variety predominates in a particular creek or shore. However there is increasing evidence to suggest that both varieties could be ecotypes of the same species (Oronsaye, 2002). *T. fuscatus* is edible and very rich in protein (Egonmwan, 1980, Adebayo-Tayo *et al.*, 2006), and thus serve as important source of protein to local inhabitants, but their high microorganisms content is a health issue which raises concern over the consumption of improperly prepared periwinkles (Adebayo-Tayo *et al.*, 2006).

Pachymelania aurita: This is another edible gastropod species that is found in sandy sediments of Lagos lagoon. Their high microbial content has also raised health issues (Adebayo-Tayo *et al.*, 2006). *P. aurita* is regarded as a dominant member of the faunal community of the Lagos lagoon with a mean annual production rate that varied from - 1.59 g and 0.79 g/0.5 m^2/yr and a production: biomass ratio that varies between 0.02 and 0.12 (Brown, 1991).

Sesarma huzardi: S. *huzardi* is a voracious and common crab species in tidal mangrove swamps, and is distributed up to the tidal limit in Lagos lagoon. The crab serves as an important source of protein to local inhabitants as well as being an important member of the mangrove community, where the adults feed on mangrove grasses and leaves. The adults also feed on other crabs and are considered as opportunity feeders. In water, the crabs are known to feed on algae, while the young crabs serve as food source to fishes. Therefore, the crab plays an important role in the food web of the mangrove community.

3.0 MATERIALS AND METHODS

3.1 TEST ANIMALS

The animal species used in the study were adult stages of the following:

1. *Tympanotonus fuscatus* var *radula L* (periwinkle) (Mollusca, Gastropoda, Megagastropoda, Melanidae),
2. *Pachymelania aurita* (periwinkle) (Mollusca, Gastropoda, Megagastropoda, Potamidae), and
3. *Sesarma huzardi* (mangrove crab) (Arthropoda, Crustacean, Decapoda, Grapsidae)

Periwinkles

P. aurita were collected from field by hand picking into a holding bucket from the edge of the Lagos Lagoon at low tide. *T. fuscatus* on the other hand were collected by hand from the mangrove swamp adjacent the Lagoon Front section of Lagos lagoon into a holding bucket. The collected specimens were transported to the laboratory for acclimatisation to the ambient laboratory environment. Lagoon water and sediments were also collected for use in the bioassay procedure.

Mangrove crabs

These were also handpicked from the mangrove swamp adjacent the Lagoon Front section of the Lagos lagoon in the evening and taken to the laboratory for acclimatisation before being used in the bioassay. Also mud sediments were collected from the same area

of collection of the crabs. Water samples were taken from the lagoon and also taken to the laboratory for experiments.

3.1.1 Laboratory Animal Acclimatisation and Selection

Periwinkles

T. fuscatus of similar shell length range (32-35 mm) and *P. aurita* shell length range (35-40 mm) were selected for all bioassays. The selected animals with unbroken shells were left in holding tanks with a thin layer of sediment serving as substrate and food source to organism, to allow them to acclimatise to laboratory conditions after the method of Tokolo (1988).

Mangrove crabs

Only adult crabs were used in this study. The crabs were sexed and only sexually immature male and female crabs were selected to minimise sexually related and reproductive effect differences. These were then left in holding tanks containing sediments from the original area of collection. Crabs were not fed and were acclimatised for 3 days before the start of the bioassay.

3.2 TEST CHEMICALS

The toxicant used for the bioassays is drill cuttings which were collected from Shell Petroleum Development Company of Nigeria Limited (SPDC) Western Division, Nigeria. The cuttings were coated with the pseudo-oil based mud used during the drilling process.

3.3 GENERAL BIOASSAY PROCEDURE

3.3.1 Bioassay Containers

For acute toxicity tests, bioassays were carried out in glass tanks measuring 46 cm X 30 cm X 30 cm for all animal species. This part of the tests lasted for 96 hours and provided the data for determination of the LC_{50} concentrations, which were used to determine the concentrations for sublethal tests.

For sublethal toxicity tests, plastic tanks were used for all animal species. The tanks used for the periwinkles, i.e. *T. fuscatus* and *P. aurita* measured 30 cm X 25 cm X 16 cm, while the tanks used for *S. huzardi* measured 45 cm X 30 cm X 20 cm.

3.3.2 Substrate Preparation

Lagoon sediments were used in tests involving the periwinkles, while mangrove sediments were used in the tests involving the mangrove crab. The sediments were sun dried to standardise the moisture content, and thereafter passed through a sieve (0.25mm) to obtain uniform substrate particles.

For acute toxicity test, a 200 g portion of the prepared sediment was used as a substrate in each tank of the bioassay. For sublethal tests, the mass of sediment used was 100 g for *T. fuscatus* and *P. aurita* because the tanks used were smaller in size. Whilst 200 g sediment was also used in cases of the sublethal tests involving the mangrove crabs *S. huzardi*. The

sediment was spread out to form a uniform bottom layer in each bioassay container at the commencement of bioassay.

3.3.3 Preparation of test media

Lagoon water was used as the diluents for the entire bioassay test conducted, with physico-chemical properties shown in Appendix I. Before the commencement of bioassay, preliminary range finding tests were carried to determine the concentrations of toxicant used in acute toxicity tests. Sediments were first spread to form a uniform layer in the bioassay container. Thereafter, pre-determined concentrations of drill cuttings were added followed by the addition of 1 liter of water less the volume of the drill cuttings. The contents of the container were then gently mixed to achieve even distribution of cuttings on the sediment before the introduction of test species. This procedure was adopted in all cases of bioassay.

3.3.4 Bioassay method

Static bioassay was adopted for acute toxicity tests while static-renewal procedure was used during the sublethal tests.

3.3.4.1 Acute Toxicity Tests

Selection of Periwinkles for Bioassay

Periwinkles of unknown age but of approximately the same size (*Tympanotonus fuscatus* (length 32-35 mm , diameter of aperture 0.7 – 9.0 mm) and *Pachymelania aurita* (length 35 – 40 mm, diameter of aperture 0.8 – 1.0 mm)) were handpicked from the acclimatized batch and then introduced into the test media.

Assessment of Quantal Response (Mortality) of S. huzardi

This animal was considered dead when no movement of movable parts was observed and if its cheliped did not respond to stimulus when probed several times with a glass rod. Mortality was monitored and recorded every 24 hours for 4 days.

3.3.4.2 Sublethal Toxicity Tests

New specimens of test species were collected from field at the end of the acute toxicity study for use in sublethal studies. Static-renewal bioassay method was adopted. In this case, test solution and sediment was replaced on day 4, 8, 16 and 32. The bioassay lasted for 32 days. Periwinkles *T. fuscatus* and *P. aurita* were picked with forceps and randomly assigned to bioassay container containing treated or untreated test media prepared as described above and containing standardised substrate. For the series of bioassays, one hundred and twenty (120) specimens of each test species were exposed per treatment including untreated control in two replicates (40 animals per replicate). Sixty (60) specimen of the mangrove crabs, i.e. 20 crabs per replicate were used per treatment including control (untreated). The organisms were sexed and only sexually immature adults were used.

The concentrations of drill cuttings used were deduced based on the LC_{50} value as determined from the acute toxicity studies, i.e. 10 % and 1 % of the LC_{50} values and are shown below. At pre-determined intervals of day 0, 4, 8, 18 and 32, specimens of the periwinkles and crabs were randomly selected both from drill cuttings exposed groups and control groups for biomarker analysis.

Drill cuttings was tested against *T. fuscatus* as follows:

- 10.24ml (i.e. 0.01 of the 96hLC$_{50}$) plus 989.76 ml lagoon water,
- 1.024ml (i.e. 0.1 of the 96hLC$_{50}$) plus 998.976 ml lagoon water, and
- 1000 ml lagoon water for the control.

The test concentrations for *P. aurita* were as follows:

- 12.2ml/l (i.e. 0.01 of the 96hLC$_{50}$) plus 987.78 ml lagoon water,
- 1.22 ml/l (i.e. 0.1 of the 96hLC$_{50}$) plus 998.78 ml lagoon water, and
- 1000 ml lagoon water for the control.

S. huzardi were exposed to the following concentrations of drill cuttings:

- 81.50 ml/l (i.e. 0.01 of the 96hLC$_{50}$) plus 918.50 ml lagoon water,
- 8.15 ml/l (i.e. 0.1 of the 96hLC$_{50}$) plus 991.50 ml lagoon water, and
- 1000 ml lagoon water for the control.

3.4 SUBLETHAL STUDIES

The Micro MT Spec and Lyso-Tox test kits used for this study were purchased from Ikzus Environment, Alessandria Italy. Chemicals required (section 3.4.3.1; Appendix II) but not provided in the kit were obtained from the Biochemistry Department of University of Lagos, Nigeria. Chemicals (see section 3.4.4.4 - 3.4.4.7) for determination of antioxidants were also obtained from Biochemistry Department, University of Lagos, while chemicals (see section 3.4.2.2) for heavy metal determination were obtained from the Department of Chemistry, University of Lagos, and were of analytical grade.

3.4.1 Equipment and glass wares

3.4.1.1 Major equipment used:

The major equipment used during the experiment include:

- Atomic Absorption Spectrophotometer (AAS) (A-Analyst 200, Perkin Elmer).
- pH Meter (Mettler, Switzerland)
- Spectrophotometer (Thermospectronic Genesys 4001/1, USA; Spectonic Genesys TMS, USA)
- Ultra Cenrifuge (Superspeed RC-B, Sorvall Inc., Newton Connecticut, USA)
- Vortex (Fisons scientific equipment)
- Weighing Balance (Mettler Toledo Ab204, Switzerland)
- Centrifuge (Uniscope Sm112, Springfriend Medicals, England)
- Microscopes (Light microscope, Carl Zeiss, Germany; Swift Instruments International, South Africa)

3.4.1.2 Glass ware and other materials used:

Other glass wares and materials used during the experiment include: Erlenmeyer flasks, Separating funnel, Bunsen burners, Glass rods, Test tubes, Racks, Beakers, Pipette, Conical flasks, membrane filter, Eppendorf tubes and tips, Retort stand, Measuring cylinders, Pipettes, Microscopic slides, Cover slips, Calibrated Meter rule, Aluminum foils, Whatman No.1 filter paper, Cotton wool, Hand gloves and Face masks.

3.4.2 Tissue preparation

For lysosomal membrane stability tests, the haemolymph was used for determination. while the digestive gland was used for metallothionein content, lysosomal membranes stability determination, enzymes studied, lipid peroxidation activity, total hydrocarbon content and heavy metals determinations in all the species studied. For each replicate, 10 specimens of periwinkles and 5 crabs were collected from bioassay chambers on day 4, 8 16 and 32 for analysis. Specimens were also analysed on day 0 (before the start of experiment) to determine the basal level of stress indices chosen for study.

3.4.2.1 Periwinkle

Haemolymph was collected from the foot of the specimens using micro surgical syringes The collected haemolymph were pooled to minimise individual variability. Samples were immediately frozen and analysis carried out within one month. In removing the digestive gland, the posterior shell was gently broken and the digestive gland removed. The collected samples were also pooled and immediately frozen. They were analysed within one month of sampling.

3.4.2.2 Mangrove crabs

Haemolymph samples were collected from the visceral cavity using micro surgical syringe. Digestive glands were removed after careful removal of the ventral carapace. Samples were also pooled to minimise individual variability and thereafter frozen. Analyses were carried within one month of collection of samples.

3.4.3 Body burden indices

3.4.3.1 Analysis of Heavy Metals in Digestive Gland of Test Species and in Drill Cuttings Sample

Heavy metal analysis of all samples was also determined in the Chemistry Department, University of Lagos. Metal concentrations were determined using Atomic Absorption method after digestion of samples in acid solution. The concentration of heavy metals in the digestive gland of treatment groups and control groups were monitored over a period of 16 days. Samples were analysed before exposure to determine the basal level, and also on day 4, 8 and 16.

In each case where heavy metal analysis was carried out, one gramme (1 g) of the sample was weighed into a porcelain crucible and heated with 1:1 mixture of nitric acid and perchloric acid (each addition was 25 ml). The resulting residue was dissolved in dilute hydrochloric acid, filtered into a 50 ml volumetric flask and the volume made up to the mark. The resulting digested solution was aspirated into the flame of the Atomic Absorption Spectrophotometer (AAS) (A-Analyst 200, Perkin Elmer) using air-acetylene or nitrous oxide-acetylene gas mixture for the metal analysis against standard metal solution.

3.4.3.2 Analysis of Total Hydrocarbon Content (THC) in Digestive Gland of Test Species and in Drill Cuttings Sample

THC was analysed in the Chemistry Department of University of Lagos. Total hydrocarbons content in the digestive gland of treatment groups and control groups were

monitored over a period of 16 days. Samples were analysed before exposure to determine the basal level, and also on day 4, 8 and 16.

Three gramme (3 g) of sample was put into a round bottom flask. Then 3 g of KOH dissolved in 100 ml redistilled methanol was added into the flask with two seeds of anti bumping. The whole content was thereafter refluxed for 2½ hours. The cooled methanolic fraction was extracted with 25 ml n-hexane twice, pooled and dried with 10 g of anhydrous Na_2SO_4. The hexane extract was first evaporated to about 1 ml, and drying was completed in a dessicator. The resulting residue was then weighed using a chemical balance to obtain the concentration of total hydrocarbon in the digestive gland of the sample.

3.4.4 Stress indices (Biomarker responses)

Biomarker response of test animals were analysed in the Department of Biochemistry, University of Lagos. For lysosomal membrane stability, the haemolymph was used while the digestive gland was used for metallothionein content, enzyme activities and lipid peroxidation activity determinations in all the species studied. Digestive gland cell disruption took place in cold buffer A (tissue extraction buffer provided in the kit from Ikzus Environment) homogenisation. Cells debris was removed by centrifugation (10,000× g, 20 min, 4°C) and the supernatant removed by decanting. The supernatant was used as the crude enzyme extract.

3.4.4.1 Measurement of Metallothionein Concentration

Metallothionein levels were assessed on the digestive gland homogenates content of the samples by evaluating the sulphyhydryl (-SH) residue (Ellman, 1959; Viarengo *et al.*, 1997). Due to the high cysteine content of MT when compared to other proteins eventually present in ethanolic extract, MT determination based on detection of sulphyhydryl allows a more selective evaluation of the metalloproteins (Domouhtsidou, *et al.*, 2004). The amount of MT was defined assuming a cysteine content of 23. The MT concentrations of 3 replicates for each measurement were calculated using reduced glutathione (GSH) as a standard and expressed as (n mol MT).g^{-1} (Appendix II). The concentration of metallothionein may be expressed as a fraction of the total protein present in the cytosol or as a fraction of the weight of 0.1 g of tissue.

Reagent and working solutions preparation:

Sulfhydryl reference (reduced glutathione) standard was prepared by adding 0.725 Sol C_2 (resuspension buffer Component 2) to a microtube. The reaction buffer (sol D) was obtained by mixing 200ml stabilised Ellman's buffer and 3.4ml concentrated Ellman's reagent and equilibrated at 23°C. Absolute ethanol was also equilibrated at –20°c. The homogenating buffer was obtained by adding 1 part of sol B (protease inhibitor) to 99 parts of sol A extraction buffer. The resuspension buffer was prepared by mixing equal amounts of sol C_1 (resuspension buffer, Component 1) and sol C_2 (resuspension buffer, Component 2). The blank solution was obtained by mixing 50µl Sol C (resuspension buffer, Component 1) and 1.950 µl Sol D (reaction buffer).

Procedure for the extraction and evaluation of metallothionein content

0.5 g of tissue was homogenised at 0-4°C using the already prepared homogenating buffer and centrifuged at 30,000g at 4°C for 20min. The protein content was then determined from the supernatant by means of Bradford assay. The supernatant was collected in a 2 ml tube, then 1.5 ml of cold absolute ethanol was added to the tube and incubated at 20°c for 30-60min. The mixture was then centrifuged at 12-16,000g at 4°c for 5 minutes and 1.95 ml of the previously prepared reaction buffer, which was equilibrated at room temperature (RT) was then added. The resulting mixture was mixed, incubated for 2 minutes at RT and then centrifuged at 12-16,000g. Absorbance was thereafter read at 412nm against the blank solution, and a standard curve was obtained by plotting absorbance against the concentration. Absorbance was also read for samples at 412. To calculate metallothionein concentration, the ABS^{MT}_{412} value of sample was interpolated over the standard curve to obtain the concentration (nmol) of sulfhydryl groups, i.e. cysteine residues, due to metallothionein present in the sample: (nmol Cys^{MT}) (Appendix II).

3.4.4.2 Lysosomal Membrane Stability Determination:

This was evaluated using the neutral red retention assay (Lowe *et al.*, 1992). The assay was performed on the haemolymph of the test species. The working solution, Sol D Hank's balanced salt solution (HBSS) was prepared by diluting 10g of vial D (HBSS powder), in 990 ml distilled and adjusting the pH to between pH 7.1- 7.3. The neutral red solution was prepared by adding 5 µl neutral red concentrate to 995 µl sol D. Samples were prepared by adding 40 µl of Sol C (0.5 M di-sodium EDTA) to a 1.5 ml

(siliconised) microcentrifuge tube. A hypodermic syringe was then filled with 0.5 ml of salt adjust Sol D. 0.5 ml of haemolymph then collected and the whole syringe content was thereafter transferred to the normalised microtube.

Thereafter 40 µl prepared cells were placed on poly-lysinated slide and incubated for 30 minutes at 16-20°C in a humidity chamber to allow cell attachment. Excess solution was gently tapped off by turning the slide on a side. To this was added 40 µl of 1X Neutral Red solution and the slide was then incubated in a humidity chamber for 15 minutes at 16-20°C. The slide was transferred to a humidity chamber after 3 minutes to avoid excessive evaporation. The samples were thereafter examined under the microscopic intervals for the first 15 minutes and every other minute thereafter for another 90 minutes (Appendix III).

3.4.4.3 Determination of Antioxidant Activity

The absorbance values for each enzyme activity including lipid peroxidation were read spectrophotometrically, and the specific enzyme activity was obtained using the formula below.

$$\text{Specific activity of enzyme} = \frac{OD/min}{\Sigma} \times \frac{v}{V}$$

Where OD = absorbance value

v = volume of crude enzyme extract sample used

V = total volume of reagents used

Σ = extinction coefficient; for catalase = $40 M^{-1}cm^{-1}$

for SOD = $4020 M^{-1}cm^{-1}$

for GST = $9.6 M^{-1}cm^{-1}$

for LPO = $1.5 \times 10^{5} M^{-1}cm^{-1}$

3.4.4.4 Catalase Enzyme Activity Assay

The activity of the enzyme catalase was analyzed by measuring the initial rate of H_2O_2 (50 mM) decomposition at 240 nm (Beutler, 1975; Cohen *et al.*, 1990). The results were expressed in CAT units/mg protein, where one unit is the amount of enzyme that hydrolyzes 1 µmol of H_2O_2 per minute per milligram of protein at 30 °C and pH 8.0.

To 0.3ml of extract sample, 1.8ml of 30mM H_2O_2 was added. Thereafter, phosphate buffer was used as the blank and absorbance read at 240nm, at 60s intervals for 5 minutes.

3.4.4.5 Superoxide Dismutase (SOD) Enzyme Activity

SOD enzyme activity was determined according to the method of Sun and Zigman. (1978). Here, the SOD enzyme assay determined the difference between superoxide anion decomposition and production i.e. its ability to inhibit the auto -oxidation of epinephrine.

The assay was performed in 3.0ml of 50mM Na_2CO_3 buffer (in 2 different test tubes) to which 0.02ml of extract was added. Then 0.03ml of the epinephrine stock solution was added to the above before taking absorbance at 480nm for 3-5 minutes. A blank devoid of the sample (but having all the reagents) was used for background correction. Enzyme activity was expressed as SOD units, where one unit is defined as the amount of enzyme needed to inhibit 50 % epinephrine reduction per minute and per milligram of protein at 25 °C and pH 7.8.

3.4.4.6 *Determination of Glutathione-S-Transferase (GST) Activity*

The activity of GST was determined according to the method of Habig and Jakoby, (1981) and Habig *et al.* (1974). GST activity was determined by monitoring at 340 nm the formation of a conjugate between 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB). The results were expressed in GST units/mg protein, where one unit is defined as the amount of enzyme that conjugates 1 µmol of CDNB per minute and per milligram of protein at 25°C and pH 7.4.

During the process, 0.1ml of CDNB solution was pipetted into a conical flask before adding 1ml of phosphate buffer and 1.7ml of distilled water. The mixture was then incubated at 37°C for 5mins. After incubation, 0.1ml of the sample and also 0.1ml of GSH solution were added. A blank devoid of the crude enzyme extract was prepared for correction of reagent blank. Absorbance readings at 340nm were taken for 5 minutes at 60 seconds interval using a UV-VIS spectrophotometer.

3.4.4.7 *Lipid Peroxidation Analysis*

Lipid peroxidation analysis was determined using thiobarbituric acid (TBARS) assay as described by Ohkawa (1979). This assayed for total free radical damage in the digestive gland of the test organisms. Malondialdehyde, the end product of lipid peroxidation served as a convenient index, and was measured spectrophotometrically at 535nm to assay for the extent of lipid peroxidation in the samples.

To 2ml of trichloroacetic acid- thiobarbituric acid- hydrochloric acid (TCA-TBA-HCl) reagent in a test tube, 1ml of sample was added and mixed thoroughly. Thereafter, the mixture was heated for 15mins in a boiling water bath, and then cooled. After cooling, the flocculent precipitate was removed by centrifugation at 1000 xg for 10mins. The absorbance of the sample was then determined at 535nm against a blank that contains all the reagents minus the sample.

3.5 STATISTICAL ANALYSIS

Dose Response Data Analysis

Toxicity dose response data were analyzed by probit analysis as adopted by Don-Pedro (1989) to estimate the LC_{50} concentration of the drill cuttings. The indices of toxicity measurement derived from this analysis were:

LC_{50} = Median lethal concentration that causes 50 % mortality of exposed organisms.

LC_{95} = Median Lethal concentration that causes 95 % mortality of exposed organisms.

LC_5 = Median lethal concentration that causes 5 % mortality of exposed organisms.

TF = Toxicity factor of relative potency measurement e.g. 24-h LC_{50} of another compound tested against same species.

$$TF = \frac{\text{LC50 of test compound at 24 hours}}{\text{LC50 of test compound at other hours (48,72,96 hours)}}$$

SF = Susceptibility/Sensitivity Factor

$$= \frac{\text{96h LC50 of other test animal}}{\text{96h LC50 of most sensitive test animal}}$$

Regression analysis and Pearson correlation were used to test for linear relationship between time of exposure and results obtained, and between the results obtained for

control and that of the drill cuttings exposed groups respectively. Descriptive statistics gave the degree of dispersion around the mean. One-way analysis of variance (ANOVA) was used to compare the means of results obtained, and where a significant difference ($P < 0.05$) was obtained, Duncan test was used to detect the source of difference. Unpaired Sample t-test was also used, where applicable to test for significant difference between means of biomarker results obtained. All statistics were first determined at $P < 0.05$ significant level, and where high significant difference was observed, a significant level of $P < 0.01$ or $P < 0.001$ is applied. All statistics were calculated using SPSS 15.0 software except for Unpaired t-test which was calculated using Microsoft Excel 2007.

4.0 RESULTS

4.1 TOTAL HYDROCARBON AND HEAVY METALS CONTENT OF THE DRILL CUTTINGS

The result of the laboratory analysis of the THC and heavy metals of the drill cuttings used in this study is presented in **Table 1**. The values of heavy metals ranged from non detectable µg/g to 2.581µg/g, while the concentration of total hydrocarbon was 25.14µg/g.

Table 1: Chemical Properties of Drill Cuttings (n = 3)

Chemical Parameter	Level Detected (mean ± standard deviation)
Ba (µg/g)	0.013 ± 0.06
Na (µg/g)	0.182 ± 0.06
K (µg/g)	0.142 ± 0.25
Fe (µg/g)	2.581 ± 0.10
Cu (µg/g)	0.344 ± 0.00
Zn (µg/g)	1.851 ± 0.06
Cd (µg/g)	0.002 ± 0.25
Ag (µg/g)	ND
Pb (µg/g)	0.015 ± 0.15
V (µg/g)	ND
Mn (µg/g)	0.056 ± 0.25
THC (µg/g)	25.14 ± 0.12
Salinity (‰)	1.22 ± 0.10
pH	9.28 ± 0.06

4.2 ACUTE TOXICITY OF DRILL CUTTINGS

Toxicity was based on lack of mobility response of the test species on being prodded with a forcep. An animal is taken for dead if it failed to respond to gentle prodding with a glass rod. Mortality was monitored and recorded every 24 hours for 96 hours.

4.2.1 Toxicity of Drill Cuttings against Test Species

The results of toxicity based on mortality response of test organisms on exposure to drill cuttings calculated using probit method at 24hrs, 48hrs, 72hrs and 96hrs of exposure are shown in **Table 2**. The results indicate that concentrations that will cause 50% mortality in the organisms after 96 hour exposure ($96hLC_{50}$) were 102.43 ml/l, 122.15 ml/l and 814.72 ml/l respectively for *T. fuscatus*, *P. aurita* and *S. huzardi*. There was also increase in toxicity as time of exposure increased as indicated by the values of toxicity factor (TF) which increases with exposure time **(Table 2, Figure 1)**.

Table 2: Acute toxicity of drill cuttings against *T. fuscatus*, *P. aurita* and *S. huzardi* at 24, 48, 72 and 96 hours of exposure

Tympanotonus fuscatus							
Time (hr)	LC_{50} (95% CL)	LC_{95} (95% CL)	LC_5 (95% CL)	Slope ± S.D.	DF	Probit Equation	TF
24	3808.8	414203	35.02	0.81 ± 0.49	4	y=2.11 + 0.81x	1
48	660.89 (298.65 - 124.93)	62239.02 (3456.77-1.037)	7.02 (1.181475E-15 - 32.86)	0.83 ± 0.39	4	y=2.70 + 0.83x	5.7
72	302.28 (187.890 - 3323.54)	15843.6 (2062.91 -76214787029)	5.77 (0.0001 - 25.1182	0.96 ± 0.38	4	y=2.63 + 0.96x	12.6
96	102.43 (65.80 - 134.06)	1010.42 (531.71 - 5107.57)	10.38 (1.19 - 24.18)	1.65 ± 0.38	4	y=2.67 + 1.65x	37.2
Pachymelania aurita							
Time (hr)	LC_{50} (95% CL)	LC_{95} (95% CL)	LC_5 (95% CL)	Slope ± S.D.	DF	Probit Equation	TF
24	2057.93	57400.94	73.78	1.14 ± 0.69	4	Y = 1.23 + 1.14X	1
48	384.11 (293.19947 – 1021.58)	2970.33 (1093.51 – 150892.72)	52.29 (5.84056 – 92.67)	1.88 ± 0.55	4	y = 0.13 ± 1.88x	5.2
72	321.85 (248.76910 – 646.26)	2623.14 (1012.43 – 105169.47)	39.49 (3.17058 - 76.560)	1.81 ± 0.55	4	y = 0.47 + 1.81x	6.4
96	122.15	1149.34	12.98	1.68 ± 0.53	4	y = 1.47 + 1.69	16.8
Sesarma huzardi							
Time (hr)	LC_{50} (95% CL)	LC_{95} (95% CL)	LC_5 (95% CL)	Slope ± S.D.	DF	Probit Equation	TF
24	-	-	-	-	-	-	-
48	871.42 (857.88 – 891.64)	984.99 (940.99 – 1119.68)	770.95 (693.23 – 801.08)	30.92 ± 7.64	4	Y = -85.90 + 30.92X	1
72	827.5 (759.47 – 847.26)	985.69 (928.99 – 1336.80)	694.7 (439.89 – 757.94)	21.65 ± 7.56	4	Y = -58.17 + 21.65X	1.05
96	814.72 (778.67 – 830.25)	903.23 (882.57 – 958.82)	734.88 (639.79 – 771.96)	36.72 ± 9.06	4	Y = -101.90 + 36.72X	1.07

CL – Confidence Limit SD – Standard Deviation DF – Degree of Freedom

$$\text{TF – Toxicity Factor} = \frac{\text{Toxicity at 24h } LC_{95}}{\text{Toxicity at 48,72 96 h } LC_{50}}$$

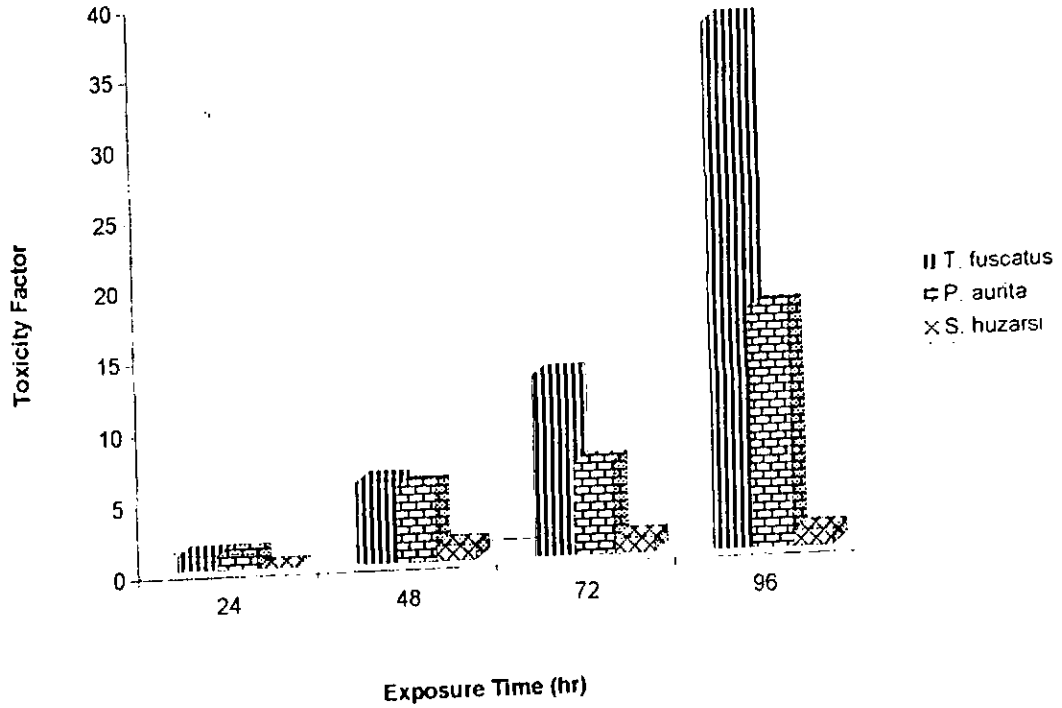

Figure 1: Variation of Toxicity Factor with time among the test organisms

4.2.2 Relative Sensitivity of Test Species to Drill Cuttings Exposure

Sensitivity of test species to drill cuttings was calculated on the basis of 96hLC$_{50}$ concentration of the different organisms. The result shows that *T. fuscatus* was the most susceptible species of the three benthic invertebrates, while *S. huzardi* was the most resistant to the lethal effects of drill cuttings among the test species.

Table 3: Susceptibility of test estuarine animals against drill cuttings based on 96 h LC_{50} data

Test Organism	LC_{50} (95% CL)	Slope ± S.D.	DF	Probit Line Equation	SF
T. fuscatus	102.43 (65.80 - 134.06)	1.65 ± 0.38	4	y=2.67 + 1.65x	1
P. aurita	122.15(88.50-142.13)	1.68 ± 0.53	4	y = 1.47 + 1.69	1.19
S. huzardi	814.72 (778.67–830.25)	36.72±9.06	4	Y = -101.90+36.72X	7.95

CL - Confident Limit SD – Standard Deviation DF – Degree of Freedom

SF – Susceptibility/Sensitivity Factor = $\frac{\text{e.g. } LC_{50} \text{ value of other test animal}}{\text{e.g. } LC_{50} \text{ of the most sensitive test animal}}$

4.3 ACCUMULATION OF HYDROCARBONS IN THE DIGESTIVE GLAND OF THE TEST ORGANISMS ON EXPOSURE TO SUBLETHAL CONCENTRATIONS OF DRILL CUTTINGS

4.3.1 Accumulation of Hydrocarbons in the Digestive gland of *Tympanotonus fuscatus* exposed to Sublethal Concentrations of Drill Cuttings

The basal level of measured hydrocarbon in the digestive gland of *T. fuscatus* was 0.003µg/g THC. In all groups including control, this value was found to have reduced by the end of 16 day exposure. In control group the basal level was maintained on day 4 of exposure, but was found to have reduced to 0.001 µg/g THC on day 8, which later increased to 0.002µg/g THC on day 16. In the group exposed to 1.02 ml/l, a reduction to 0.002µg/g THC on day 4 was observed, and no further decrease or increase was found till the end of the exposure period. Organisms exposed to 10.24 ml/l first showed a decrease in the amount of total hydrocarbon present in the digestive gland from 0.003 µg/g THC to

0.002µg/g THC on day 4. A further decrease to 0.001 µg/g THC was also observed at the end of the exposure period **(Table 4)**.

Descriptive statistics revealed low dispersion around the mean value in the total hydrocarbon concentrations both for control and each of the drill cuttings exposed group (SD = ± 0.0010 for control, ± 0.0005 for *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration and ± 0.0008 for 0.1 96hLC$_{50}$ drill cuttings concentration exposed *T. fuscatus*. With a Pearson correlation (r) of 0.522 (p-value of 0.478), the correlation between control and *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration was not statistically significant ($P > 0.05$). Pearson correlation between control and 0.1 96hLC$_{50}$ *T. fuscatus* was 0.426 (p-value of 0.574) and was also not significant. Correlation ($r = 0.816$, p-value = 0.184) in total hydrocarbon concentrations between the two drill cuttings exposed groups was also not statistically different. Analysis of variance (ANOVA) was used to compare the means of control and the drill cuttings exposed groups, and revealed no significant ($P < 0.05$) difference among the control and the two treatments exposed to drill cuttings.

Table 4: Concentrations of Total Hydrocarbon Content (THC) (µg/g) in *T. fuscatus* exposed to Sublethal Drill Cuttings Concentrations

Treatments	Total Hydrocarbon (THC) (µg/g)				
	Day 0	Day 4	Day 8	Day 16	overall net gain
Control	0.003	0.003	0.001	0.002	-0.001
1.024 ml/l	0.003	0.002	0.002	0.002	-0.001
10.24 ml/l	0.003	0.002	0.002	0.001	-0.002

4.3.2 Accumulation of Hydrocarbon in the Digestive gland of *P aurita* exposed to Sublethal Concentrations of Drill Cuttings

In all groups including control, no reduction in the amount of total hydrocarbons was observed on any sampling day in the digestive gland of *P. aurita* (**Table 5**). In control and the group exposed to 12.22 ml/l, the basal level of 0.002 µg/g THC initially rose to 0.003 µg/g THC, then fell to the basal level on day 8, a concentration that was maintained also on day 16 of experimental period. On the other hand the basal level of total hydrocarbon in the group exposed to 1.22 ml/l was also observed on day 4, before an increase to 0.004 µg/g THC was found on day 8. This value later fell to 0.003µg/g THC on day 16.

Descriptive analysis revealed that dispersion around the mean value was higher in *P. aurita* exposed to 0.01 96h LC_{50} drill cuttings concentration compared to control *P. aurita* and *P. aurita* exposed to 0.1 96h LC_{50} drill cuttings concentration groups (SD = ± 0.0005 for control, ± 1.9988 for *P. aurita* exposed to 0.01 96hLC_{50} drill cuttings concentration and ± 0.0006 for 0.1 96hLC_{50} drill cuttings concentration exposed *P. aurita*. There was no significant correlation in total hydrocarbon between the control and

each of the drill cuttings exposed group concentration (r = -0.333, p-value = 0.667 for control and *P. aurita* exposed to 0.01 96h LC_{50} drill cuttings concentration; (r = 0.577, p-value = 0.423 between control and control and 0.01 96h LC_{50} drill cuttings concentration *P. aurita*). Correlation was also not significant between the two drill cuttings exposed groups (r = -0.577, p-value = 0.423) ($P > 0.05$). There was no significant difference in the accumulation of hydrocarbon in the digestive gland of *P. aurita* among the control and the drill cuttings exposed groups (ANOVA, $P > 0.05$).

Table 5: Concentrations of Total Hydrocarbon Content (THC) (µg/g) in *P. aurita* exposed to Sublethal Drill Cuttings Concentrations

Treatments	Total Hydrocarbon (THC) (µg/kg)				
	Day 0	Day 4	Day 8	Day 16	overall net gain
Control	0.002	0.003	0.002	0.002	0
1.22 ml/l	0.002	0.002	0.004	0.003	0.001
12.22 ml/l	0.002	0.003	0.002	0.003	0.001

4.3.3 Accumulation of Hydrocarbon in the Digestive gland of *Sesarma huzardi* exposed to Sublethal Concentrations of Drill Cuttings

Samples were analysed on day 0, 4, 8 and 16 during the study. In control, the basal level of 0.001 µg/g THC was maintained throughout the sampling day in both male and female crabs (**Table 6**). In the male group exposed to 8.15 ml/l the basal level first rose to 0.003 µg/g THC and fell to 0.002µg/g THC on day 8, a value that was also observed on day 16 of exposure. In female on the other hand, THC concentration rose to 0.002µg/g with no further change to the 16 day exposure. Both male and female crabs exposed to 81.5 ml/l exhibited similar trend in THC levels during the study. Relative high concentration of

THC was observed in the digestive gland on day 4, which later fell to the background value of 0.001 µg/g on day 8 and rising again on day16 (**Table 6**).

There was low dispersion around the mean value in the concentration of total hydrocarbon content in the digestive gland of both male and female crab in each treatment group including as indicated by the descriptive statistics. (SD = ± 0.0000 for male control, ± 0.0005 for male *S. huzardi* exposed to 0.01 96hLC$_{50}$ drill cuttings concentrations and ± 0.0008 for 0.1 96hLC$_{50}$ drill cuttings concentration exposed male *S. huzardi*. SD = ± 0.0000 for female control, ± 0.0005 for female *S. huzardi* exposed to 0.01 96hLC$_{50}$ drill cuttings concentrations and ± 0.0014 for 0.1 96hLC$_{50}$ drill cuttings concentration exposed for female *S. huzardi*).

There was no significant difference among the control and the drill cuttings exposed groups both for male and female crabs (ANOVA, $P > 0.05$). Total hydrocarbon content concentration in the digestive gland was not affected by sex of the crab as comparison in total hydrocarbon content concentration of any male group with the corresponding female treatment group revealed no significant difference (Unpaired t-test, $P > 0.05$).

Table 6: Concentrations of Total Hydrocarbon (THC) (µg/g) in *S. huzardi* exposed to drill cuttings

Treatments	Mean Concentration of THC (µg/g) in Male *S. huzardi*					Mean Concentration of THC (µg/g) in Female *S. huzardi*				
	Day 0	Day 4	Day 8	Day 16	Overall net gain	Day 0	Day 4	Day 8	Day 16	Overall net gain
Control	0.001	0.001	0.001	0.001	0	0.001	0.001	0.001	0.001	0
8.15 ml/l	0.001	0.003	0.002	0.002	0.001	0.001	0.002	0.002	0.002	0.001
81.5 ml/l	0.001	0.003	0.001	0.002	0.001	0.001	0.004	0.001	0.002	0.001

4.4 ACCUMULATION OF HEAVY METALS (Fe, Cu, Zn, Cd, Ag) BY TEST ANIMAL SPECIES EXPOSED TO SUBLETHAL CONCENTRATIONS OF DRILL CUTTINGS

The concentration of heavy metals in the digestive gland of the test species, namely *Tympanotonus fuscatus*, *Pachymelania aurita* and *Sesarma huzardi* were monitored over an exposure period of 16 days. Sample analyses were carried out before exposure of the organisms to drill cuttings and also on day 4, 8 and 16. The variations in concentrations of heavy metals in the digestive gland of the test animals are shown in Tables 7, 9 and 11. In all cases, Cadmium and Silver were not detectable in the gland studied.

4.4.1 Accumulation of Heavy Metals in the Digestive gland of *T. fuscatus* exposed to Sublethal Concentrations of Drill Cuttings

The post treatment analyses of digestive gland of *T. fuscatus* revealed that heavy metal concentrations fluctuated in all treatment samples including controls (**Table 7**). In some cases also, heavy metal concentrations were less than the background value.

4.4.1.1 Accumulation of Iron in the Digestive gland of T. fuscatus exposed to Sublethal Concentrations of Drill Cuttings

Accumulation of Iron in the digestive gland of *T. fuscatus* from drill cuttings was neither time nor concentration dependent (b = -0.618 for control, -0.693 for organisms exposed to both 0.01 96hLC_{50} and -0.670 for organisms exposed to 0.1 96hLC_{50} drill cuttings concentration). The concentration of Iron steadily decreased in all sampling day in both

control and treatment groups such that by the end of the 16 day exposure, there was a negative concentration of -0.11 µg/g compared to the basal level in the two treated groups. The reduction in the amount of Iron found in the digestive gland was also observed in the control which decreased from 0.16 µg/g Fe to 0.06 µg/g Fe, 0.10 µg/g Fe and 0.07 µg/g Fe respectively on day 4, 8 and 16 in the studied tissue (**Table 7**). BAF values were very low and similar for all treatment groups including control (**Table 8**).

The spread around the mean value in Iron concentration was low as indicated by descriptive statistics (SD = ± 0.045 for control, ± 0.052 for *T. fuscatus* exposed to 0.01 $96hLC_{50}$ drill cuttings concentration and ± 0.052 for 0.1 $96hLC_{50}$ drill cuttings concentration exposed *T. fuscatus*. Pearson correlation in Iron accumulation between control and each drill cuttings exposed group was positive (r = 0.474 between control *T. fuscatus* and *T. fuscatus* exposed to 0.01 $96hLC_{50}$ drill cuttings concentration, r = 0.977 between control *T. fuscatus* and 0.01 $96hLC_{50}$ drill cuttings concentration exposed *T. fuscatus*).

There was no significant difference in Iron accumulation in the digestive gland of *T. fuscatus* among the control and the drill cuttings exposed groups at all exposure concentration (ANOVA, Duncan, $P > 0.05$).

Table 7: Concentrations of Heavy Metals (µg/g) in *T. fuscatus* exposed to Sublethal Concentrations of drill cuttings

Treatments	Mean Concentration of Heavy Metals in *Tympanotonus fuscatus* (µg/g)				
	Day 0	Day 4	Day 8	Day 16	Overall net gain
Iron (Fe) (µg/g)					
Control	0.16	0.06	0.1	0.07	-0.09
1.024 ml/l	0.16	0.05	0.06	0.05	
	0	-0.01	-0.04	-0.02	-0.11
Control	0.16	0.06	0.1	0.07	
10.24 ml/l	0.16	0.05	0.07	0.05	
	0	-0.01	-0.03	-0.02	-0.11
Copper (Cu) (µg/g)					
Control	0.014	0.018	0.026	0.007	-0.007
1.024 ml/l	0.014	0.024	0.018	0.021	
	0	0.006		0.014	0.007
Control	0.014	0.018	0.026	0.007	
10.24 ml/l	0.014	0.006	0.020	0.012	
	0	-0.012	-0.006	0.005	-0.002
Zinc (Zn) (µg/g)					
Control	0.21	0.54	0.24	0.18	
1.024 ml/l	0.21	0.08	0.15	0.21	
	0	-0.46	0.09	0.03	0
Control	0.21	0.54	0.24	0.18	
10.24 ml/l	0.21	0.26	0.23	0.14	
	0.196	0.242	0.01	0.133	-0.07
Cadmium (Cd) (µg/g)					
Control	ND	ND	ND	ND	
1.024 ml/l	ND	ND	ND	ND	
10.24 ml/l	ND	ND	ND	ND	
Silver (Ag) (µg/g)					
Control	ND	ND	ND	ND	
1.024 ml/l	ND	ND	ND	ND	
10.24 ml/l	ND	ND	ND	ND	

Table 8: Bioaccumulation factor of heavy metals for *T. fuscatus*

Treatment	Mean Metal Concentration in Sediment			Mean Metal Concentration in Dig Gland			Bioaccumulation Factor		
	Fe	Cu	Zn	Fe	Cu	Zn	Fe	Cu	Zn
Control	1.273	0.060	1.733	0.098	0.016	0.293	0.077	0.271	0.169
0.01 $96hLC_{50}$ drill cuttings concentration	0.898	0.103	3.970	0.080	0.019	0.163	0.089	0.188	0.041
0.1 $96hLC_{50}$ drill cuttings concentration	1.000	0.043	3.340	0.083	0.013	0.210	0.083	0.306	0.063

4.4.1.2 Accumulation of Copper in the Digestive gland of T. fuscatus Exposed to Sublethal Concentrations of Drill Cuttings

Also a transition metal, its accumulation followed neither a dose nor a time dependent manner. The concentration of Copper in control organisms reduced by -0.007 µg/g Cu and by -0.002µg/g Cu in the 10.24ml/l organisms but increased by 0.007µg/g Cu in the 1.024 ml/l exposed organisms at the end of the experimental period (**Table 7**). Prior to the loss in Copper concentration, there was elevated concentration of Copper on day 4 in both control organisms and the group exposed to 1.024 ml/l, but a reduction in Copper concentration occurred in the 10.24 m/l drill cuttings exposed group during the same period. A subsequent increase to 0.012 µg/g observed on the 16th day in organisms exposed to 10.24 ml/l was still lower than the basal 0.014 µg/g Cu level. There was little correlation between exposure period and the amount of Copper accumulated in the digestive gland (b = -0.388 for control organisms, 0.423 for *T. fuscatus* exposed to 0.01 $96hLC_{50}$ drill cuttings concentration and 0.101 for 0.1 $96hLC_{50}$ drill cuttings concentration exposed *T. fuscatus*). The order for BAF was as follows: *T. fuscatus*

exposed to 0.1 96hLC$_{50}$ drill cuttings concentration > control *T. fuscatus* > *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration. The low BAF for *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration compared to other exposure concentration was a reflection of the relative higher concentration of Copper in the medium compared to the media of other exposure concentration **(Table 8)**.

There was low spread around the mean value in Copper accumulation in the digestive gland of *T. fuscatus* as indicated by descriptive statistics (SD = ± 0.010 for control and ± 0.005 for both *T. fuscatus* groups exposed to drill cuttings). Pearson correlation also revealed non-significant (P > 0.05) and positive correlation in Copper accumulation between control and each of the drill cuttings exposed groups as well as between the two exposed groups (r = 0.522, p-value = 0.367 between control and *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and r = 0.870, p-value = 0.055 between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed *T. fuscatus*). Exposure to drill cuttings did not have a significant effect on the accumulation of Copper in the digestive gland of the organism as shown by ANOVA and Duncan test (P > 0.05) among the control and drill cuttings exposed groups.

4.4.1.3 Accumulation of Zinc in the Digestive gland of T. fuscatus Exposed to Sublethal Concentrations of Drill Cuttings

The trend in Zinc concentrations was the same for both control and 10.24 ml/l exposed organisms. The amount of Zinc in control organisms reduced by -0.03µg/g Zn and by -0.07µg/g Zn in the 10.24ml/l exposed organisms by the end of the 16th exposure period. On the other hand, the concentration of Zinc at the end of exposure period in the 1.024

ml/l exposed organisms was the same as was recorded at the start of the experiment **(Table 7)**. Bioaccumulation factor was low **(Table 8)** and is reflected in the regression analysis between exposure period and the amount of Zinc accumulated in the digestive gland during the exposure period (b = -0.360 for control organisms, 0.260 for *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration and -0.727 for 0.1 96hLC$_{50}$ drill cuttings concentration exposed *T. fuscatus*. Zinc level increased above the basal level on day 4 in control and 10.24 ml/l exposed organisms, but a reduction in Zinc value was observed in 1.024 ml/l exposed organisms during the same period. While a fall in Zinc value was found on day 16 in control and 10.24 ml/l exposed organisms, the concentration of Zinc increased in the 1.024 ml/l exposed organisms during the same period.

There was low spread around the mean value in the concentrations of Zinc in the digestive gland of *T. fuscatus* as indicated by the descriptive statistics (SD = 0.062 ± for control, ± 0.051 for *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration and 0.10 for *T. fuscatus* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration). Pearson correlation revealed negative and non-significant (P > 0.05) correlation of Zinc levels in the digestive gland of control and each of the drill cuttings exposed groups (r = -0.814, p-value = 0.186 between control and *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and r = -0.239, p-value = 0.761 between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed *T. fuscatus*). Positive and non-significant (P > 0.05) difference in correlation was also observed between the two drill cuttings exposed groups (r = 0.194, p-value = 806). *T. fuscatus* showed no significant difference in Zinc accumulation in the digestive gland on exposure to drill cuttings among the control and

the drill cuttings exposed group at all exposure concentration (ANOVA, Duncan. P > 0.05).

4.4.2 Accumulation of Heavy Metals in the Digestive gland of *P. aurita* exposed to Sublethal Concentrations of Drill Cuttings

The post treatment analyses of heavy metals level in the digestive gland of *P. aurita* subjected to sublethal concentrations of drill cuttings also revealed fluctuations in the concentration of heavy metals in the digestive gland of this species. In some instances, higher concentrations of heavy metals control samples than in organisms exposed to drill cuttings, and accumulation was not dose-dependent or time dependent **(Table 9)**

4.4.2.1 Accumulation of Iron in the Digestive gland of P. aurita exposed to Sublethal Concentrations of Drill Cuttings

In contrast to the decrease in concentration of Iron in the digestive gland of *T. fuscatus* over the exposure period, there was an increase in the level of Iron in the digestive gland of *P. aurita* above the background value in all groups on all sampling days. Both control and 12.22 ml/l exposed organisms showed an increase of 0.03 µg/g Fe above the basal value, while 1.22 ml/l exposed organisms accumulated 0.01 µg/g Fe above the background concentration of 0.003 µg/g Fe at the end of the exposure period **(Table 9)**. This is reflected in positive correlation observed between exposure period and concentration of Iron in the digestive gland of *P. aurita* (b = 0.507 for control group, 0.683 for *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, 0.969 for 0.1 96hLC$_{50}$ drill cuttings concentration). BAF for Iron was also relatively higher in *P. aurita* compared to *T. fuscatus* (Table 10). In control the initial increase on day 4 was followed

by a reduction on day 8 and the subsequent value was observed at the end of the experiment. In 1.22 ml/l exposed organisms, no further change was observed after the initially increase on day 4, while in the 12.22 ml/l exposed organisms no change in Iron level was observed on day 8 after the increase on day 4, but a further rise in Iron concentration was observed on day 16.

The spread around the mean value of Iron level in the digestive gland was high in control group compared to drill cuttings exposed group as shown by descriptive statistics (SD = ± 6.831 for control, ± 0.017 for *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentrations and 0.005 for 0.1 96hLC$_{50}$ drill cuttings concentration exposed *P. aurita*). Pearson correlation of Iron concentration between control and *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration was positive and significant ($P < 0.05$) ($r = 0.963$, p-value = 0.038), while Pearson correlation was positive and not significant between control and *P. aurita* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration ($r = 0.535$, p-value = 0.465). Pearson correlation was also positive and not significant ($P > 0.05$) between the two drill cuttings exposed groups ($r = 0.662$, p-value = 0.338). Accumulation of Iron from drill cuttings was not significant ($P > 0.05$) compared to control as indicated by the result of ANOVA.

4.4.2.2 Accumulation of Copper in the Digestive gland of P. aurita exposed to Sublethal Concentrations of Drill Cuttings

P. aurita accumulated in its digestive gland 0.003µg/g Cu in control, 0.013µg/g Cu in 1.22ml/l exposed organisms and 0.010µg/g Cu in 12.22ml/l exposed organisms above the background level of 0.005µg/g Cu **(Table 9)**. All treatment groups including control

showed elevated Copper concentration on day 4, with the highest Copper level being observed in control organisms on the same day. Reduction in Copper concentration was observed on day 8 in both control and the group exposed to 1.22 ml/l, but while there was no further change in Copper value in control organisms, a subsequent increase in Copper value was observed on day 16 in 12.22 ml/l exposed organisms.

Organisms exposed to 1.22 ml/l on the other hand showed a consistent increase in Copper concentration throughout the exposure period. This trend is reflected in the linear regression analysis (b = -0.153 for control animals, 0.989 for *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and 0.308 for 0.1 96hLC$_{50}$ drill cuttings concentration exposed organisms). Bioaccumulation factor was low with higher values being recorded for organism exposed to 0.1 96hLC$_{50}$ drill cuttings concentration (**Table 10**).

Descriptive statistics showed low dispersion around the mean value of Copper levels in the digestive gland of *P. aurita* (SD = ± 0.009 for control, ± 0.005 for *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentrations and ± 0.007 for 0.1 96hLC$_{50}$ drill cuttings concentration exposed *P. aurita*). Pearson correlation was not significant ($P > 0.05$) between the control and each drill cuttings exposed group (r = -0.009, p-value = 0.991 between control *P. aurita* and *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and r = -0.893, p-value = 0.107 between control *P. aurita* and 0.1 96hLC$_{50}$ drill cuttings concentration exposed *P. aurita*) as well as between the two drill cuttings exposed groups (r = 449, p-value = 551). ANOVA and post hoc Duncan test showed no

statistical difference ($P > 0.05$) in accumulation of Copper in the digestive gland of *P. aurita* among the control and drill cuttings exposed groups at all exposure concentrations.

Table 9: Concentrations of Heavy Metals (µg/g) in *P. aurita* exposed to sublethal concentrations of drill cuttings

Treatments	Mean Concentration of Heavy Metals in *Tympanotonus fuscatus* (µg/g)				
	Day 0	Day 4	Day 8	Day 16	Overall net gain
Iron (Fe) (µg/g)					
Control	0.03	0.07	0.06	0.06	0.03
1.22ml/l	0.03	0.04	0.04	0.04	
net gain	0	-0.03	-0.02	-0.02	0.01
Control	0.03	0.07	0.06	0.06	
12.22 ml/l	0.03	0.04	0.04	0.06	
net gain		-0.03	-0.02	0	0.03
Copper (Cu) (µg/g)					
Control	0.005	0.024	0.008	0.008	0.003
1.22 ml/l	0.005	0.01	0.012	0.018	
net gain		-0.014	0.004	0.01	0.013
Control	0.005	0.024	0.008	0.008	
12.22 ml/l	0.005	0.022	0.011	0.015	
net gain		-0.002	0.003	0.007	0.01
Zinc (Zn) (µg/g)					
Control	0.13	0.22	0.18	0.24	0.11
1.22 ml/l	0.13	0.27	0.22	0.1	
net gain		0.05	0.04	-0.14	-0.03
Control	0.13	0.22	0.18	0.24	
12.22 ml/l	0.13	0.22	0.26	0.16	
net gain		0	0.08	-0.08	0.03
Cadmium (Cd) (µg/g)					
Control	ND	ND	ND	ND	
1.22 ml/l	ND	ND	ND	ND	
12.22 ml/l	ND	ND	ND	ND	
Silver (Ag) (µg/g)					
Control	ND	ND	ND	ND	
1.22 ml/l	ND	ND	ND	ND	
12.22 ml/l	ND	ND	ND	ND	

Table 10: Bioaccumulation factor of heavy metals for *P. aurita*

Treatment	Mean Metal Concentration in Sediment			Mean Metal Concentration in Dig Gland			Bioaccumulation Factor		
	Fe	Cu	Zn	Fe	Cu	Zn	Fe	Cu	Zn
Control	0.080	0.060	2.765	0.055	0.011	0.193	0.688	0.188	0.070
0.01 96hLC$_{50}$ drill cuttings concentration	0.180	0.103	3.920	0.038	0.011	0.180	0.208	0.110	0.046
0.1 96hLC$_{50}$ drill cuttings concentration	0.230	0.043	2.645	0.043	0.013	0.193	0.185	0.312	0.073

4.4.2.3 Accumulation of Zinc in the Digestive gland of P. aurita exposed to Sublethal Concentrations of Drill Cuttings

Zinc level increased by 0.11µg/g Zn in control and by 0.003µg/g Zn in organisms exposed to 12.22 ml/l, and reduced by -0.003 µg/g Zn in organisms exposed to 1.22 ml/l at the end of the 16 days experimental period **(Table 9)**. There was however elevated Zinc concentration above the background value of 0.13 µg/g Zn in all groups on day 4. The highest 0.27µg/g Zn and lowest 0.10 µg/g Zn Zinc values were observed in the 1.22 ml/l exposed group on day 4 and 16 respectively. While Zinc values fell on day 8 in both control and 1.22ml/l exposed organisms, there was an increase in Zinc concentration in 12.22ml/l exposed group during the same period, which was followed a reduction in Zinc values on day 16. Control organisms on the other hand showed elevated Zinc value on day 16 of the experimental period. There was a strong positive correlation between exposure period and Zinc concentration in control animal (b = 0.774), while the correlation between exposure time and concentration in animals was low in animals

exposed to drill cuttings (b = -0.372 *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and 0.108 for 0.1 96hLC$_{50}$ drill cuttings concentration). Bioaccumulation factor was very low for all treatment groups including control **(Table 10)**.

As was observed for Copper, dispersion around the mean value in Zinc concentration in the digestive gland of *P.* aurita was low among all the treatment and the control groups as indicated by the descriptive statistics (SD = ± 0.049 for control, ± 0.079 for *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration and 0.059 for 0.1 96hLC$_{50}$ drill cuttings concentration exposed *P. aurita*). Pearson correlation was not significant (P > 0.05) between the control and each drill cuttings exposed group (r = 0.113, p-value = 0.887 between control and *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and r = 0.267, p-value = 0.733 between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed *P. aurita*) as well as between the two drill cuttings exposed groups (r = 788, p-value = 212). Analysis of variance showed that there was no significant difference (P > 0.05) in accumulation of Zinc in the digestive gland when control group is compared together with the drill cuttings exposed groups at all exposure concentrations.

4.4.3 Accumulation of Heavy Metals in the Digestive gland of *S. huzardi* exposed to Sublethal Concentrations of Drill Cuttings

The post treatment analysis of heavy metals in *S. huzardi* also revealed fluctuations in the concentrations of heavy metals in the digestive gland of both treatment and control groups **(Table 11)**.

4.4.3.1 *Accumulation of Iron in the Digestive gland of S. huzardi exposed to Sublethal Concentrations of Drill Cuttings*

The background level of Iron was found to be lower in female than in the male of *S. huzardi*. In the controls, Iron level was also always lower in the female compared to the male except on day 8 when the value was the same for both male and female. The trends in the accumulation pattern of male and female were the same for any exposure group.

In male all exposure groups followed similar pattern of Iron concentration. There was an initial increase above the 0.07 µg/g Fe background value on day 4 in all treatment groups including control, which was followed by a reduction in Iron values on day 8 before a final increase on day 16 of the experiment (b = 0.255 for control, 0.418 for male *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentration, and 0.153 for 0.1 96hLC_{50} drill cuttings concentration exposed male crabs) showing little correlation between exposure time and concentration of Iron in the digestive gland of male *S. huzardi*. This is reflected also in the very low BAF recorded for Iron during the experiment (Table 12). At the end of experiment, both control and 81.5 ml/l exposed organisms accumulated 0.03µg/g Fe, while 8.15 ml/l exposed organisms accumulated 0.01 µg/g Fe in the digestive gland.

Female crabs accumulated 0.03 µg/g Fe in the control, 0.05 µg/g Fe in organisms exposed to 8.15 ml/l and 0.01 µg/g Fe in the 81.5 ml/l exposed organisms at the end of the exposure period above the basal 0.04 µg/g Fe. Trend in Iron concentration was similar to that of the male, and correlation between exposure time and concentration in

the digestive gland of female *S. huzardi* was stronger in control and 8.15 ml/l exposed group compared to the similar treatment group in male, and almost nonexistent in 81.5 ml/l exposed group (b = 0.690 in control, 0.697 in female *S. huzardi* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and 0.090 for 0.1 96hLC$_{50}$ drill cuttings concentration exposed female crabs.

There was low dispersion around the mean value in Iron concentration in the digestive gland of both male and female *S. huzardi* as indicated by descriptive statistics (SD = ± 0.010 for male control, ± 0.022 for male *S. huzardi* exposed to 0.01 96hLC$_{50}$ drill cuttings concentrations and ± 0.019 for 0.1 96hLC$_{50}$ drill cuttings concentration exposed male *S. huzardi*. For female crab, SD = ± 0.014 for control, ± 0.024 for female *S. huzardi* exposed to 0.01 96hLC$_{50}$ drill cuttings concentrations and ± 0.022 for 0.1 96hLC$_{50}$ drill cuttings concentration exposed female *S. huzardi*).

A comparison of Iron accumulation in digestive gland revealed a non significant difference in Iron accumulation in the digestive gland among control and the drill cuttings exposed groups (ANOVA, $P < 0.05$). Sex related difference in accumulation of Iron was investigated using Unpaired t-test and revealed no significant difference ($P > 0.05$) in Iron accumulation between the male and female crab.

Table 11: Concentrations of Heavy Metals (µg/g) in *S. huzardi* exposed to sublethal concentrations of drill cuttings over 16 days

	Mean Concentration of Heavy Metals (µg/g) in Male *S. huzardi*					Mean Concentration of Heavy Metals (µg/g) in Female *S. huzardi*				
	Day 0	Day 4	Day 8	Day 16	Overall net gain	Day 0	Day 4	Day 8	Day 16	Overall net gain
Iron (Fe) (µg/g)										
Control	0.07	0.08	0.06	0.08	0.01	0.04	0.07	0.06	0.07	0.03
8.15 ml/l	0.07	0.09	0.05	0.1		0.04	0.08	0.05	0.09	
Net gain		0.01	-0.01	0.02	0.03	0	0.01	-0.01	0.02	0.05
Control	0.07	0.08	0.06	0.08		0.04	0.07	0.06	0.07	
81.5 ml/l	0.07	0.11	0.07	0.09		0.04	0.08	0.03	0.05	
Net gain		0.03	0.01	0.01	0.02		0.01	-0.03	-0.02	0.01
Copper (Cu) (µg/g)										
Control	0.021	0.019	0.022	0.023	0.02	0.017	0.02	0.02	0.02	0.003
8.15 ml/l	0.021	0.019	0.008	0.014		0.017	0.017	0.017	0.018	
Net gain		0	-0.014	-0.009	-0.007		-0.003	-0.003	-0.002	0.001
Control	0.021	0.019	0.022	0.023		0.017	0.02	0.02	0.02	
81.5 ml/l	0.021	0.028	0.019	0.022		0.017	0.02	0.028	0.023	
Net gain		0.009	-0.003	-0.001	0.001		0	0.008	0.003	Net gain
Zinc (Zn) (µg/g)										
Control	0.19	0.22	0.18	0.24	0.05	0.23	0.19	0.17	0.21	-0.02
8.15 ml/l	0.19	0.22	0.26	0.27		0.23	0.27	0.2	0.23	
Net gain		0	0.08	0.03	0.08		0.08	0.03	0.02	0
Control	0.19	0.22	0.18	0.24		0.23	0.19	0.17	0.21	
81.5 ml/l	0.19	0.24	0.22	0.27		0.23	0.22	0.24	0.27	
Net gain		0.02	0.04	0.03	0.08		0.03	0.07	0.06	0.04
Cadmium (Cd) (µg/g) and Silver (Ag) (µg/g)										
Control	ND	ND	ND	ND		ND	ND	ND	ND	
8.15 ml/l	ND	ND	ND	ND		ND	ND	ND	ND	
81.5 ml/l	ND	ND	ND	ND		ND	ND	ND	ND	

Table 12: Bioaccumulation factor of heavy metals for *S. huzardi*

Male *S. huzardi*									
Treatment	**Mean Metal Concentration in Sediment**			**Mean Metal Concentration in Dig Gland**			**BAF**		
	Fe	**Cu**	**Zn**	**Fe**	**Cu**	**Zn**	**Fe**	**Cu**	**Zn**
Control	10.03	1.62	18.91	0.07	0.02	0.21	0.0070	0.0123	0.0111
1/100 96hLC$_{50}$	9.02	1.45	15.01	0.08	0.02	0.23	0.0089	0.0138	0.0153
1/10 96hLC$_{50}$	10.78	1.69	17.89	0.09	0.02	0.23	0.0083	0.0118	0.0129
Female *S. huzardi*									
Treatment	**Mean Metal Concentration in Sediment**			**Mean Metal Concentration in Dig Gland**			**BAF**		
	Fe	**Cu**	**Zn**	**Fe**	**Cu**	**Zn**	**Fe**	**Cu**	**Zn**
Control	7.24	1.23	16.00	0.06	0.02	0.20	0.0083	0.0163	0.0125
1/100 96hLC$_{50}$	9.14	1.26	15.43	0.07	0.02	0.23	0.0077	0.0159	0.0149
1/10 96hLC$_{50}$	10.08	1.27	15.83	0.05	0.02	0.24	0.0050	0.0157	0.0152

4.4.3.2 Accumulation of Copper in the Digestive gland of S. huzardi exposed to Sublethal Concentrations of Drill Cuttings

The background level of Copper in the digestive gland of *S. huzardi* was also lower in the female than in the male, and a comparison of the control groups also revealed that Copper levels were higher in male than in the female at the end of the exposure, but while the male control accumulated 0.02µg/g Cu the female accumulated 0.03µg/g Cu in the digestive gland. In the control group, Copper level was initially depressed on day 4 before rising on day 8 and 16. Correlation between exposure time and Copper concentration in the digestive gland was positive for control (b = 0.714 for male and 0.690 for female). Depression of Copper level was also observed in the 8.15 ml/l exposed organisms on day 4 which continued to day 8, before an increase on day16, though the level of Copper was still lower than the background level. Organisms exposed to the 81.5 ml/l showed elevated Copper levels on day 4 which fell in value on day 8 and

subsequently rose on day 16. At the end of 16 day exposure period, the male group exposed to 81.5 ml/l accumulated 0.01 µg/g Cu (b = -0.176), while in the 8.15 ml/l exposed organisms Copper concentration was -0.07 µg/g Cu (b = -0.589) less than background level of 0.021 µg/g Cu (**Table 12**).

In female *S. huzardi* there was an accumulation of 0.03 µg/g Cu in the control, 0.010 µg/g Cu in organisms exposed to 8.15 ml/l , and 0.060 µg/g Cu in the 81.5 ml/l exposed organism at the end of the 16 day exposure period. Copper concentrations in the digestive gland of control group remained unchanged after the initial rise on day 4 to the end of the experiment (b = 0.683), while in the 8.15 ml/l exposed group, the background 0.017 µg/g Cu was unchanged but for the marginal increase on day 16 (b = 0.878). *S. huzardi* exposed to the 81.5 ml/l showed elevated Copper levels on day 4 and 8, which decreased on day 16 (b = 0.583). Highest Copper concentration in the female was observed on day 8 in the 81.5 ml/l exposed group, unlike in the male, where a sharp fall was observed during the same period.

There was low dispersion around the mean value in the concentration of Copper in the digestive gland of the test species as indicated by descriptive statistics (SD = ± 0.002 for male control, ± 0.006 for male *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentrations and 0.004 for 0.1 96hLC_{50} drill cuttings concentration exposed male *S. huzardi*. For female, SD = ± 0.002 for control, and ± 0.005 for both female *S. huzardi* exposed to drill cuttings).

Bioaccumulation of Copper in the digestive gland on exposure to drill cuttings was not significantly different (ANOVA, $P > 0.05$) from control, each for both male and female *S. huzardi*. Accumulation was also not significant (Unpaired, $P > 0.05$) between male and female crabs for each treatment.

4.4.3.3 Accumulation of Zinc in the Digestive gland of S. huzardi exposed to Sublethal Concentrations of Drill Cuttings

The background level of this transition metal was higher in the female than in the male, however by the end of the experiment, the concentration of Zinc in the male control rose above that of the female control group.

In male, there was an accumulation of 0.05µg/g Zn in control organisms while each of the treatment group accumulated 0.08µg/g Zn in the digestive gland at the end of exposure period **(Table 12)**. There was a strong positive correlation between exposure period and Zinc concentration in drill cuttings exposed male crabs despite the fall in Zinc concentration observed in the 81.5 ml/l exposed organisms showed a fall in Zinc concentration on day 8 ($b = 0.620$ for control, 0.923 for male crabs exposed to 8.15 ml/l and 0.870 for male crabs exposed to 81.5 ml/l).

In female Zinc concentration decreased by 0.02 µg/g Zn in control group, was unchanged in the 8.15 ml/l exposed organism, while increasing by 0.04 µg/g Zn in the group exposed to 81.5 ml/l at the end of the exposure period. Regression analysis revealed a strong negative correlation between exposure time and concentration of Zinc in the digestive gland of control and 8.15 ml/l exposed female crabs, while there was a strong

positive correlation for 81.5 ml/l exposed female crabs (b = -0.223 for control, -0.255 for 8.15 ml/l exposed female crabs and 0.904 for 81.5 ml/l exposed female crabs. On each sampling day in the control group, Zinc concentration was always less than the 0.23 µg/g Zn background value. The values of control group were also always lower than those observed in treatment groups on each sampling day.

Using descriptive statistics, it was possible to show that dispersion around the mean value in Zinc accumulation in the digestive gland of both male and female *S. huzardi* was low (SD = ± 0.028 for male control, ± 0.037 for male *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentrations and ± 0.034 for 0.1 96hLC_{50} drill cuttings concentration exposed male *S. huzardi*. For female, SD = ± 0.026 for control, ± 0.029 for female *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentrations and ± 0.022 for 0.1 96hLC_{50} drill cuttings concentration exposed female *S. huzardi*.). Exposure to drill cuttings did not have any significant effect on the accumulation of Zinc in the digestive gland of the crabs compared to control (ANOVA, $P > 0.05$) for both male and female crabs. There was also no statistical difference in Zinc accumulation between male and female crabs for any exposure groups when tested with Unpaired t-test at 0.05 significant level.

4.5 METALLOTHIONEIN INDUCTION IN THE DIGESTIVE GLANDS OF TEST SPECIES ON EXPOSURE TO SUBLETHAL CONCENTRATIONS OF DRILL CUTTINGS

The induction of this protein was monitored in all treatment groups including control over an exposure period of 32 days for the periwinkles and 16 days for the crab.

4.5.1 Metallothionein induction in the Digestive Gland of *T. fuscatus* on exposure to Sublethal Concentrations of Drill Cuttings

Cysteine levels present due to the presence of metallothionein increased in all treatment groups by the end of the experiment. The average background cysteine value observed in *T. fuscatus* was 1.7657 $nmol^{MT}$ and rose to an average 2.2408 $nmol^{MT}$ in control, 5.3731 $nmol^{MT}$ in 0.01 96hLC_{50} exposed group and 3.0859 $nmol^{MT}$ in 0.1 96hLC_{50} drill cuttings exposed group **(Table 13)**. Pearson correlation revealed strong positive and significant correlation ($P < 0.01$) in cysteine concentrations between control and each drill cuttings exposed groups ($r = 0.905$, p-value = 0.000 between control and *T. fuscatus* exposed to 0.01 96hLC_{50} drill cuttings concentration, and $r = 0.902$, p-value = 0.000 between control and 0.1 96hLC_{50} drill cuttings concentration exposed *T. fuscatus*), as well as between the two drill cuttings exposed groups ($r = 0.0981$, p-value = 0.000). Analysis of variance (ANOVA) showed significant difference ($P < 0.001$) in each treatment group, as well as among control the drill cuttings exposed groups. Post hoc analysis using Duncan test also showed that there was statistical difference on all sampling days for control *T. fuscatus* and *T. fuscatus* exposed to 0.01 96hLC_{50} drill cuttings concentration. The values observed on day 0 and day 4 in 0.1 96h LC_{50} exposed *T. fuscatus* were not significantly different ($P > 0.05$) from each other but were significantly different ($P < 0.05$) from the values observed on subsequent sampling days.

Expressed per gram of tissue, the average background value recorded for metallothionein (MT) was 0.7677 nmol g^{-1}. At the end of 32 day exposure period the values of MT ranged from 0.7568 nmol g^{-1} to 1.5510 nmol g^{-1} (mean = 0.9742 nmol g^{-1}) in control organisms, 0.7677 nmol g^{-1} to 4.3571 nmol g^{-1} (mean = 2.3361 nmol g^{-1}) in organisms exposed to

1.024 ml/l, and 0.7677 nmol g^{-1} to 2.0959 nmol g^{-1} (mean = 1.3417 nmol g^{-1}) in 10.24 ml/l exposed organisms **(Table 13, Figure 2)**. The control group always has lower values compared to drill cuttings exposed groups on any sampling day **(Table 13)**.

Regression analysis showed a strong positive correlation between exposure time and concentration of metallothionein in the digestive gland of *T. fuscatus*. MT levels fell marginally on day 4 and on day 8 before rising above the background level on day 16 and 32 (b = 0.951). In the 1.024 ml/l exposed organisms, there was a consistent rise in MT levels but for the dip on day 8 though MT value was above the basal level, which was followed by a sharp increase in MT values on day 16 with further increase on day 32 (b = 0.887). In organisms exposed to 10.24 ml/l, MT was positively correlated with time (b b = 0.926), though the concentrations were lower than those found in organisms exposed to 1.024 ml/l, but greater than those found in control organism. Pearson correlation showed strong positive and significant correlation ($P < 0.01$) between control and each drill cuttings exposed group ($r = 0.9058$, p-value = 0.000 between control and *T. fuscatus* exposed to 0.01 96hLC_{50} drill cuttings concentration, and $r = 0.902$, p-value = 0.000 between control and 0.1 96hLC_{50} drill cuttings concentration exposed *T. fuscatus*), as well as between the two drill cuttings exposed groups ($r = 0.981$, p- value = 0.000).

MT concentrations were significant for all treatment groups (ANOVA, $P < 0.001$) between control and the drill cuttings exposed groups, as well as between the two drill cuttings exposed groups (ANOVA, $P < 0.001$). Post hoc treatment using Duncan test indicated that for all treatment groups there was significant difference ($P < 0.05$) in MT concentration on all sampling days.

Table 13: Protein and metallothionein concentrations in *T. fuscatus* on sublethal exposure to drill cuttings (mean ± SD)

Control *T. fuscatus*						
Incubation Time (Day)	**n° Cys**	**Wt of tissue (g)**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmol MT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1	5.483 ± 0.014^{d}	1.766 ± 0.00^{d}	0.7677 ± 0.00^{e}	0.014 ± 0.00^{b}
4	23	0.1	4.958 ± 0.014^{e}	1.741 ± 0.00^{d}	0.7568 ± 0.00^{f}	0.0153 ± 0.00^{a}
8	23	0.1	9.299 ± 0.000^{c}	1.567 ± 0.00^{e}	0.6812 ± 0.00^{g}	0.0073 ± 0.00^{c}
16	23	0.1	21.790 ± 0.014^{b}	2.564 ± 0.00^{b}	1.1146 ± 0.00^{b}	0.0051 ± 0.00^{e}
32	23	0.1	22.574 ± 0.108^{a}	3.567 ± 0.01^{a}	1.551 ± 0.00^{a}	0.0069 ± 0.00^{d}
***T. fuscatus* subjected to 0.01 96H LC_{50} drill cuttings concentration**						
Incubation Time (Day)	**n° Cys**	**Wt of tissue (g)**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmolMT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1	5.483 ± 0.014^{e}	1.766 ± 0.00^{e}	0.7677 ± 0.00^{e}	0.014 ± 0.00^{a}
4	23	0.1	4.237 ± 0.020^{e}	2.894 ± 0.00^{c}	1.2581 ± 0.00^{c}	0.0297 ± 0.00^{a}
8	23	0.1	4.508 ± 0.014^{d}	2.471 ± 0.00^{d}	1.0745 ± 0.00^{d}	0.0238 ± 0.00^{b}
16	23	0.1	20.761 ± 0.012^{b}	9.713 ± 0.00^{b}	4.2232 ± 0.00^{b}	0.0203 ± 0.00^{c}
32	23	0.1	22.269 ± 0.026^{a}	10.021 ± 0.00^{a}	4.3571 ± 0.00^{a}	0.0196 ± 0.00^{d}
***T. fuscatus* subjected to 0.196H LC_{50} drill cuttings concentration**						
Incubation Time (Day)	**n° Cys**	**Wt of tissue (g)**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmolMT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1 ± 0.014	5.483^{e}	1.766 ± 0.00^{e}	0.7677 ± 0.00^{e}	0.014 ± 0.00^{a}
4	23	0.1 ± 0.013	7.112^{d}	1.898 ± 0.00^{d}	0.8254 ± 0.00^{d}	0.0116 ± 0.00^{d}
8	23	0.1 ± 009	9.23^{c}	2.486 ± 0.00^{c}	1.0809 ± 0.00^{c}	0.0117 ± 0.00^{c}
16	23	0.1 ± 0.014	16.516^{b}	4.459 ± 0.00^{b}	1.9387 ± 0.00^{b}	0.0117 ± 0.00^{c}
32	23	0.1 ± 0.063	16.738^{a}	4.821 ± 0.00^{a}	2.0959 ± 0.00^{a}	0.0125 ± 0.00^{b}

Mean ± SD; n =3 (mean and SD are calculated from three measurements, considering three pooled samples at each concentration. Means not sharing the same superscript (a, b, c, d or e) in each column are significantly different ($P < 0.001$, Duncan).

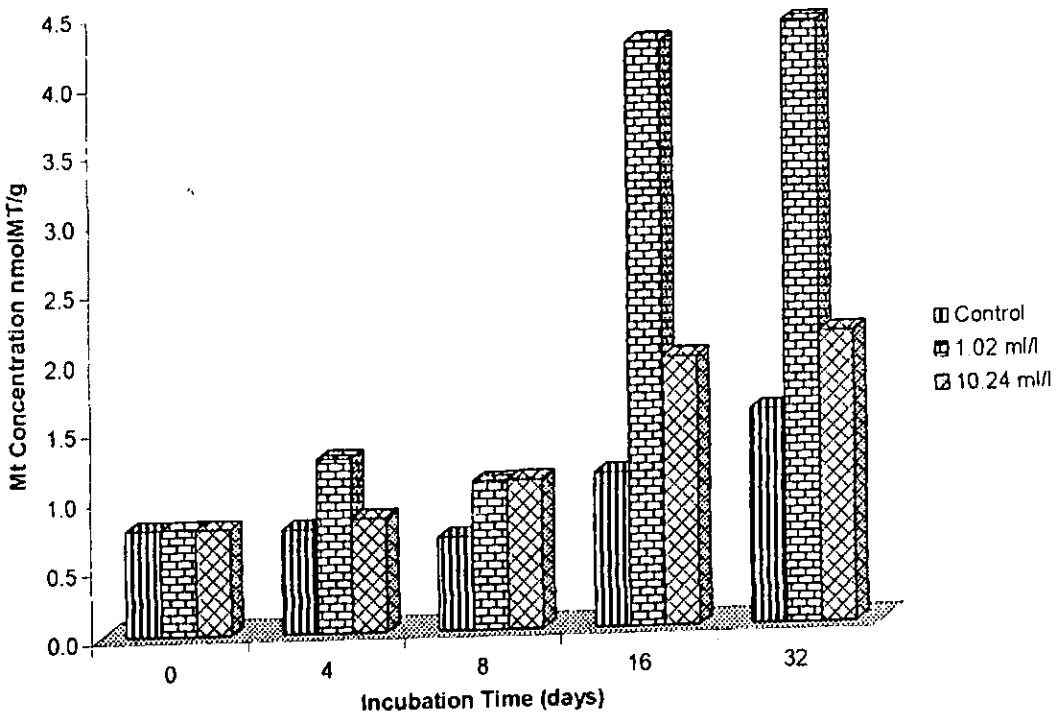

Figure 2: Metallothionein induction in *T. fuscatus*

There was increase in protein levels in all treatment groups by the end of the exposure period. The average protein value observed after 32 days exposure was 12.8210 mg/l from an average background level of 5.4829 mg/l in control, 11.4515 mg/l in *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration and 11.0157 in 0.1 96hLC$_{50}$ drill cuttings concentration. Pearson correlation showed that the correlation of protein in the digestive gland was positively significant ($P < 0.01$) between control and each of the drill cuttings exposed group ($r = 0.978$, p-value = 0.000 between control and *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and $r = 0.991$, p-value = 0.000 between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed *T. fuscatus*), as well as between the two drill cuttings exposed groups ($r = 0.957$, p- value = 0.000).

Test of ANOVA showed that protein levels were significant ($P < 0.001$) for each treatment group, and the control was also significantly different from the drill cuttings exposed groups. Post hoc test using the Duncan test showed that there was significant difference ($P < 0.001$) in protein concentration on all sampling day in all treatment groups.

The average background MT concentration relative to total protein in the supernatant fraction obtained from the digestive gland of *T. fuscatus* was 0.0140nmol^{MT}/mg. During the experiment the value of MT relative to total protein in control group ranged from 0.0051 nmolMT/mg protein to 0.0153 nmol^{MT}/mg protein (mean = 0.0097 nmol^{MT}/mg protein). In 1.02 ml/l exposed organisms, MT values ranged from 0.0140 nmol^{MT}/mg protein to 0.0297 nmolMT/mg protein (mean = 0215 nmol^{MT}/mg protein), and from 0.0116 nmolMT/mg protein to 0.0140 nmolMT/mg protein (mean = 0. 0123 nmol^{MT}/mg protein) in organisms exposed to 10.04 ml/l **(Table 13, Figure 3)**.

In control organisms, MT synthesis in relation to other proteins was initially elevated above the background value on day 4, but thereafter, fell below the basal level on the remaining sampling days, with the lowest value of MT occurring in this group on day 16. Reduction during these periods reflected the increased production of other proteins relative to MT, and is shown in the negative correlation between MT concentration and exposure time during the study (b = -0.696). Ratio of MT level to other proteins was sharply elevated on day 4 in organisms exposed to 1.024 ml/l, while there was a reduction in the total concentration of proteins during the same period. Thereafter relative

concentrations of MT to total proteins started to dip till the end of the exposure period with total protein concentration rising simultaneously. There was virtually no correlation between concentration of MT per total protein and exposure time in this group (b= 0.096). Depression in MT concentration relative to total proteins on day 4 was observed only in organisms exposed to 10.24 ml/l as the concentration of other proteins rose over that of MT. MT values thereafter remained more or less unchanged but for the marginal rise on day 32 as rate of change of all proteins including MT became equivalent. Correlation between ratio of MT to other proteins and time of exposure was weakly negative (b = -0.202). Generally, MT levels per total protein were always highest in the group exposed to 1.02 ml/l, and generally lowest in the control organisms.

Pearson correlation showed positive and non-significant correlation in MT concentration relative to other protein between control and drill cuttings exposed groups (r = 0.174, p-value = 0.534 between control and *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and r = 0.371, p-value = 0.173 between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed *T. fuscatus*). However correlation was strongly significant ($P < 0.01$) between the two drill cuttings exposed groups (r = -832, p-value = 0.000).

Analysis of variance revealed that for each treatment, MT per total proteins was significantly different ($P < 0.001$) by the end of the 32 day exposure. Post hoc test with the Duncan showed that the difference was significant ($P < 0.05$) on all sampling days in control and in *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration. However, in 0.1 96hLC$_{50}$ drill cuttings concentration exposed *T. fuscatus*, Duncan test revealed that

the value of MT relative to total proteins observed on day 8 and day 16 were not significantly different ($P > 0.01$) between each other, but was significantly different from the values observed on other sampling days.

ANOVA and Duncan test also revealed that there was a high significant difference ($P < 0.001$) among control and the drill cuttings exposed groups at all concentrations

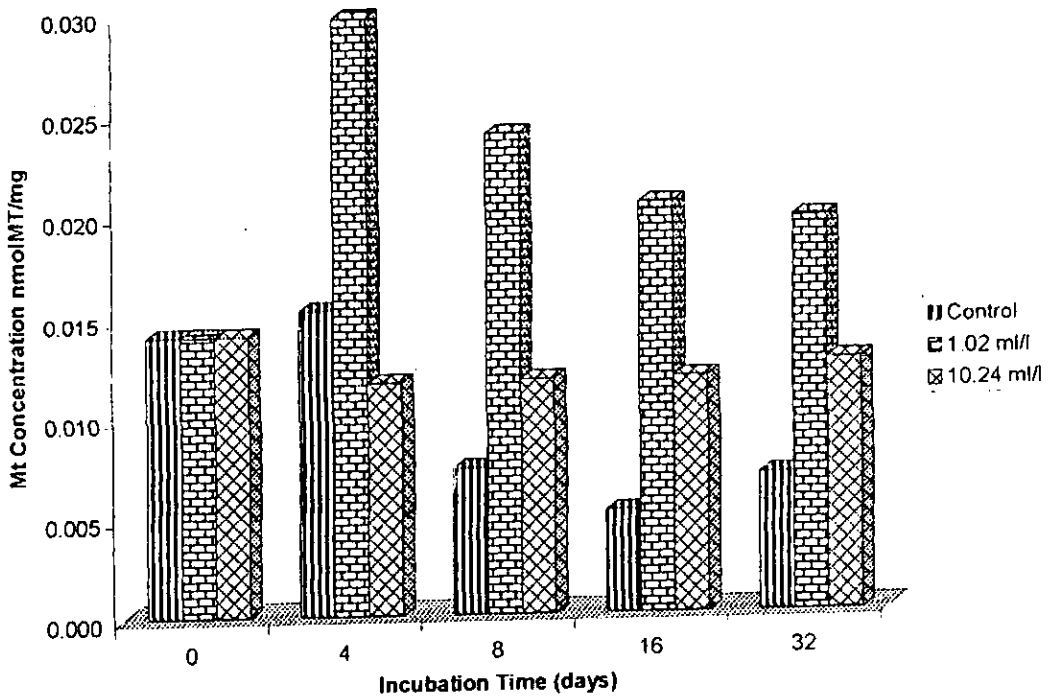

Figure 3: Metallothionein per total protein levels in the digestive gland of *T. fuscatus*

4.5.2 Metallothionein induction in the Digestive Gland of *P. aurita* on exposure to Sublethal Concentrations of Drill Cuttings

The average background cysteine value of 7.789 nmolCys^{MT} recorded in the digestive gland of *P. aurita* during the study was higher than that found in *T. fuscatus*. During the study, the mean value recorded was 8.0495 nmolCys^{MT} in control group, 5.1849

nmolCys^{MT} in *P. aurita* exposed to 0.01 96hLC$_{50}$ concentration and 3.3473 nmolCys^{MT} **(Table 14)**. Pearson correlation showed that correlation in cysteine levels in the digestive gland of *P. aurita* was positively significant ($P < 0.05$) between control and *P. aurita* exposed to 0.01 96LC$_{50}$ drill cuttings concentration ($r = 0.558$, p-value = 0.031). However, there was a poor and non significant correlation ($P > 0.05$) between control and 0.1 96hLC$_{50}$ drill cuttings exposed *P. aurita* ($r = 0.038$, p-value = 0.892). Correlation between the two exposed groups was positive (r 0.752, p-value = 0.001) and significant ($P < 0.01$). There was significant difference in the cysteine concentration of each treatment group during the study and on all the sampling days (ANOVA, Duncan, $P < 0.001$). There was also significant difference in cysteine concentration in the digestive gland among *P. aurita* control group and the drill cuttings exposed *P. aurita* (ANOVA, $P < 0.001$).

The Background MT level in *P. aurita* was higher than that of *T. fuscatus*. The values of MT recorded during the experiment ranged from 3.4430 nmol^{MT} g^{-1} to 3.8865 nmol^{MT} g^{-1} in control organisms (mean = 3.4998 nmol^{MT}g^{-1}), 0.1902 nmol^{MT}g^{-1} to 3.8807^{MT} nmol g^{-1} (mean = 2.2543 nmol^{MT}g^{-1}) in organisms exposed to 1.22 ml/l, and 0.3301 nmol^{MT}g^{-1} (mean = 1.4554 nmol^{MT}g^{-1}) to 3.3865^{MT} nmol g^{-1} in 12.22 ml/l exposed organisms **(Table 14)**.

In the control, the concentration of MT was comparatively unchanged with time (correlation coefficient, 0.965), and the values were always higher than those of exposed groups on all sampling days but for day 32 when the level of the group exposed 1.22 ml/l was essentially equal to the control value. Both exposed groups exhibited similar trend

of MT fluctuation. In both groups, MT was severely depressed on day 4. and thereafter started to rise. Induction of MT was higher in organisms exposed to 1.22 ml/l compared to organisms exposed to 12.22 ml/l, such that by the end to the end of the exposure period, MT concentration in organisms exposed to 1.22 ml/l was higher than the background value. Induction of MT in the 12.22 ml/l exposed organisms continued to be depressed as the concentration remained lower than the background value **(Table 14, Figure 4)**. Correlation between MT concentration in the digestive gland of *P. aurita* and exposure time was positive for *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings (b = 0.523) and weakly negative for *P. aurita* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration (b = -0.096).

Pearson correlation revealed that correlation in MT concentration was positive (r = 0.558, p-value = 0.031) and significant ($P < 0.05$) between control *P. aurita* and the group exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, while correlation was not significant ($P > 0.05$) between control and *P. aurita* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration (r = 0.038, p-value = 0.859). Strong significant correlation ($P < 0.01$) in MT concentrations was also observed between the two drill cuttings exposed groups (r = 0.752, p-value = 0.001).

ANOVA revealed that there was statistical difference ($P < 0.001$) in MT concentration for each treatment in the digestive gland of *P. aurita* during the study. Post hoc analysis with Duncan test showed that MT concentration was significantly different ($P < 0.001$) on each sampling day for all treatment. Comparison of MT levels in control *P. aurita*

with that of the drill cuttings exposed groups showed that there was significant difference (ANOVA, $P < 0.001$) among them.

Comparing MT induction in *P. aurita* with that of *T. fuscatus* shows the difference in the induction pattern of these two gastropod species. In the control group of *P. aurita*, induction of MT was very gradually increasing with time, while in the control group of *T. fuscatus*, there was initial depression of MT on day 4 and day 8, which was followed by elevated concentration of MT on day 16 and day 32. By the end of the 32 day experimental period, MT induction in the control organisms of *T. fuscatus* was double the background value. However, the absolute value of MT on any sampling day was higher in *P. aurita* than in *T. fuscatus*. High significant difference ($P < 0.001$) in MT concentration in the digestive gland was observed between the control group of *T. fuscatus* and that of *P. aurita*.

MT concentration pattern was similar in drill cuttings exposed groups of *P. aurita*, where initial depression of MT on day 4 was followed by increase in concentration of MT on subsequent sampling days. On the other hand, there was elevation of MT on day 4 in *T. fuscatus* exposed organisms, followed by a reduction in MT concentration on day 8 in the group exposed to 1.024 ml/l, which was followed by increases on subsequent sampling days. The similarity observed in induction of MT between *P. aurita* and *T. fuscatus* relates to the higher concentration of MT in the organisms exposed to 0.01 $96hLC_{50}$ concentration of drill cuttings compared to organisms exposed to 0.1 $96hLC_{50}$ drill cuttings concentration. Test of comparison of means in the drill cuttings exposed groups revealed no significant difference (Unpaired t-test, $P < 0.05$) *T. fuscatus* and *P. aurita*

exposed to 0.01 96hLC$_{50}$ drill cuttings exposed groups as well as in the 0.1 96hLC$_{50}$ drill cuttings exposed groups .

Table 14: Induction of metallothionein in *P. aurita* on sublethal exposure to drill cuttings (mean ± SD)

Control P. aurita						
Incubation Time (Day)	**n° Cys**	**Wt of tissue (g)**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmol MT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1	11.147 ± 0.025^{d}	7.789 ± 0.00^{e}	3.3865 ± 0.00^{a}	0.0304 ± 0.00^{a}
4	23	0.1	10.915 ± 0.013^{e}	7.809 ± 0.00^{d}	3.3952 ± 0.00^{b}	0.0311 ± 0.00^{c}
8	23	0.1	20.906 ± 0.012^{c}	7.919 ± 0.00^{c}	3.443 ± 0.00^{c}	0.0165 ± 0.00^{f}
16	23	0.1	38.688 ± 0.028^{b}	8.01 ± 0.00^{b}	3.4828 ± 0.00^{d}	0.009 ± 0.00^{g}
32	23	0.1	42.666 ± 0.145^{a}	8.72 ± 0.00^{a}	3.7914 ± 0.00^{e}	0.0089 ± 0.00^{h}
***P. aurita* subjected to 1.22 ml/l**						
Incubation Time (Day)	**n° Cys**	**Wt of tissue (g)**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmol MT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1	11.147 ± 0.025^{e}	7.789 ± 0.00^{b}	3.3865 ± 0.00^{d}	0.0304 ± 0.00^{a}
4	23	0.1	13.246 ± 0.025^{d}	0.438 ± 0.00^{e}	0.1902 ± 0.00^{a}	0.0014 ± 0.00^{e}
8	23	0.1	21.931 ± 0.054^{c}	1.478 ± 0.00^{d}	0.6426 ± 0.00^{b}	0.0029 ± 0.00^{d}
16	23	0.1	23.266 ± 0.029^{b}	7.294 ± 0.00^{c}	3.1714 ± 0.00^{c}	0.0136 ± 0.00^{c}
32	23	0.1	27.940 ± 0.014^{a}	8.926 ± 0.00^{a}	3.8807 ± 0.00^{e}	0.0139 ± 0.00^{b}
***P. aurita* subjected to 12.22 ml/l**						
Incubation Time (Day)	**n° Cys**	**Wt of tissue (g)**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmol MT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1	11.147 ± 0.025^{e}	7.789 ± 0.00^{a}	3.3865 ± 0.00^{e}	0.0304 ± 0.00^{a}
4	23	0.1	24.431 ± 0.0356^{a}	0.759 ± 0.00^{e}	0.3301 ± 0.00^{d}	0.0014 ± 0.00^{e}
8	23	0.1	21.323 ± 0.064^{b}	1.284 ± 0.00^{d}	0.5583 ± 0.00^{b}	0.0026 ± 0.00^{d}
16	23	0.1	16.106 ± 0.052^{d}	2.939 ± 0.00^{c}	1.2778 ± 0.00^{c}	0.0079 ± 0.00^{c}
32	23	0.1	16.766 ± 0.029^{c}	3.965 ± 0.02^{b}	1.724 ± 0.00^{d}	0.0103 ± 0.00^{b}

Mean ± SD: n =3 (mean and SD are calculated from three measurements, considering three pooled samples at each concentration. Means not sharing the same superscript (a, b, c, d or e) in each column are significantly different (Duncan, P < 0.001).

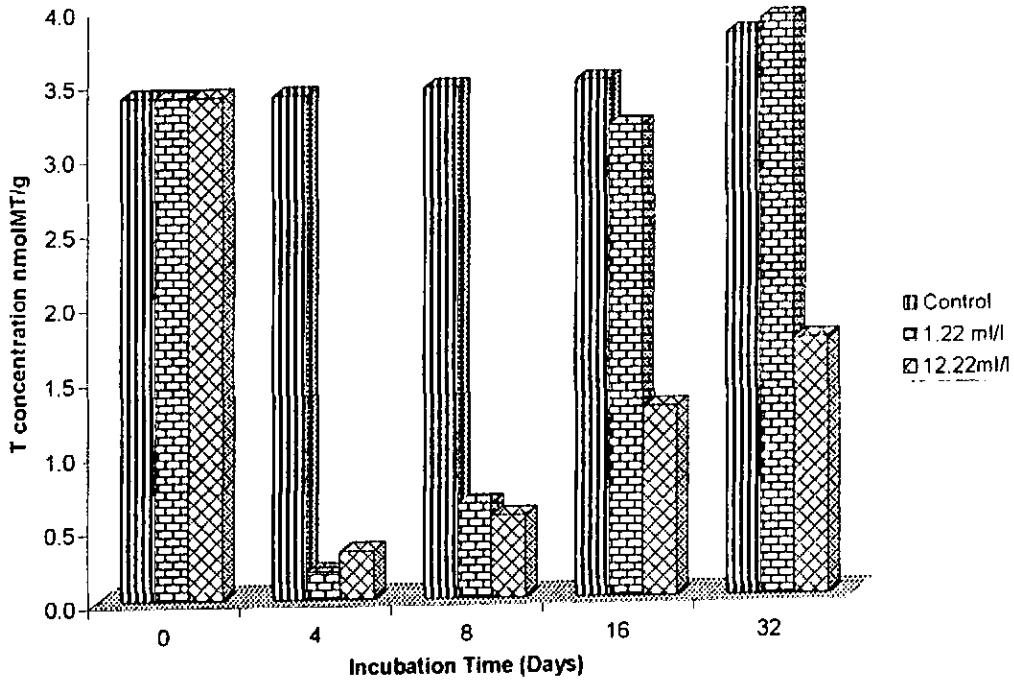

Figure 4: Metallothionein levels in the digestive gland of *P. aurita*

Protein levels increased from the average background level of 11.1467 mg/l protein to 24.8643 mg/l protein in control *P. aurita*, 19.5062 in *P. aurita* exposed to 0.01 $96hLC_{50}$ drill cuttings concentration and 17.9547 in *P. aurita* exposed to 0.1 $96hLC5_0$ concentration. Pearson correlation showed that there was a significant positive correlation ($P < 0.05$) between control and 0. 01 $96hLC_{50}$ drill cuttings concentration exposed *P. aurita* ($r = 0.926$, p-value = 0.000) but not between control and *P. aurita* exposed to 0.1 $96hLC_{50}$ drill cuttings concentration. ($r = -0.185$, p-value = 0.509) and between the two drill cuttings exposed groups ($r = 0.052$, p-value = 0.853).

Analysis of variance showed that protein concentration was significantly different ($P <$ 0.001) in all treatment groups, while post hoc test using Duncan showed that the

difference was significant ($P < 0.001$) on all the sampling days for each treatment. The protein levels in the digestive gland of *P. aurita* among the control and the drill cuttings exposed groups was statistically different (ANOVA, $P < 0.001$).

When expressed as a fraction of total protein of the supernatant, the background MT concentration per total protein in the digestive gland of *P. aurita* was 0.0304 $nmol^{MT}$/mg protein. During the study, MT levels ranged in the digestive gland of *P. aurita* from 0.0090 $nmol^{MT}$/mg protein to 0.0304 $nmol^{MT}$/mg protein (mean = 0. 0192 $nmol^{MT}$/mg protein) in control organisms, and 0.0014 $nmol^{MT}$/mg protein to 0.0304 $nmol^{MT}$/mg protein in both 1.22 ml (mean = 0.0125 $nmol^{MT}$/mg protein) and 12.22 ml/l (mean = 0.0105 $nmol^{MT}$/mg protein) exposed organisms **(Table 14, Figure 5)**.

MT values relative to other proteins were significantly (ANOVA, $P < 0.001$) reduced by the end of the experimental period in all the groups. In the control, the ratio of MT in relation to other proteins was marginally elevated in control group on day 4, followed by a fall below the background level on day 8 and 16 and then remained essentially unchanged on day 32. Regression analysis revealed a strong positive correlation between total protein concentration and metallothionein concentration in control *P. aurita* ($b = 0.812$).

Drill cuttings exposed organisms exhibited similar trend in relative importance of MT concentration to other proteins. In both cases, MT ratio to other proteins fell by the same factor on day 4, and thereafter the ratio started to increase to the end of the exposure

period, but the background level was never achieved during the experiment (**Table 15**) Regression analysis showed a negative correlation between total protein concentration and metallothionein concentration (b = -0.346 for *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings and b = -0.944 for *P. aurita* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration). The result of correlation analysis between exposure time and MT relative to other protein was strongly negative for control organisms (b = -0.836), and weakly negative for exposed organisms (b = -0.095 for *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings, and -0.251 for *P. aurita* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration).

Pearson correlation showed that correlation of MT relative to total proteins in the digestive gland of *P. aurita* was low and not significant ($P > 0.05$) between control and each of the drill cuttings exposed groups, while a significant correlation ($P > 0.05$) was observed between the two drill cuttings exposed groups.

Pearson correlation revealed very strong and high significant correlation ($P < 0.005$) in MT relative to total proteins between control and each of the drill cuttings exposed groups (r = 0.975, p-value = 0.000 between control and *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and r = 1.000, p-value = 0.000 between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed *P. aurita*), as well as between the two treatments exposed to drill cuttings (= 0.975, p-value = 0.000).

There was significant difference in MT concentration relative to other proteins in all treatment groups during the experiment as shown using ANOVA ($P < 0.001$), while post

hoc treatment using Duncan test showed that the difference was significant ($P < 0.001$) on all the sampling day in all the treatment groups. ANOVA was also used to establish significant difference in MT concentration relative to other proteins among control *P. aurita* and drill cuttings exposed *P. aurita* ($P < 0.001$).

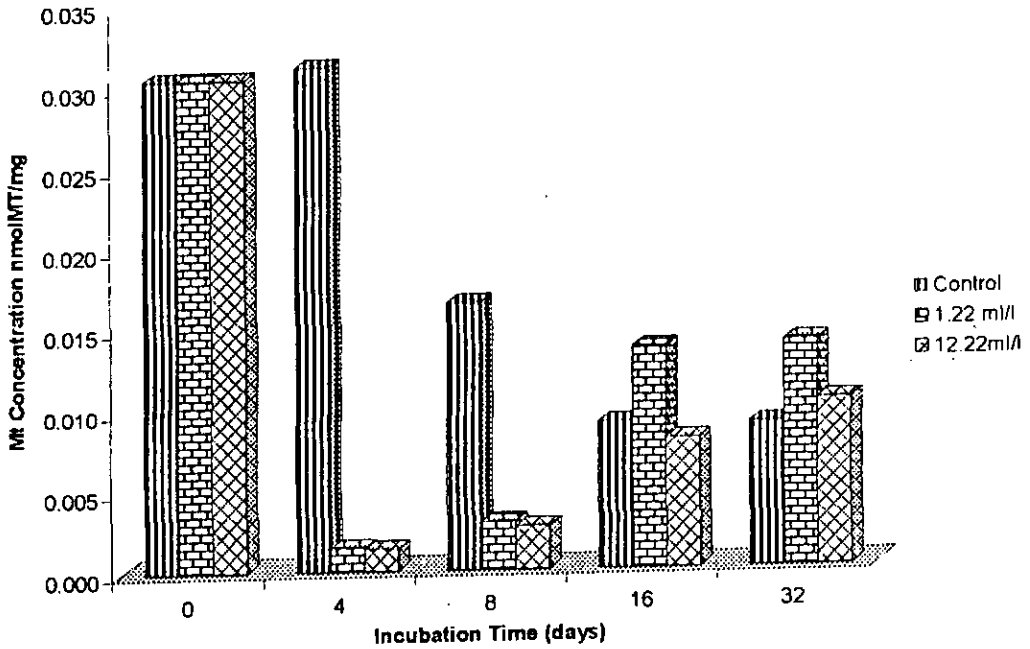

Figure 5: Varying levels of Metallothionein as fraction of total protein in the digestive gland of *P. aurita*

Comparing MT values as fractions of the total protein present between of *P. aurita* and *T. fuscatus* showed differences and similarities in MT fluctuations in the two periwinkles. As a fraction of the total protein, the background value was higher in the control *P. aurita* than in the control of *T. fuscatus*. MT values was initially elevated on day 4 followed by a fall on day 8 in both organisms. While the fall continued to the end of the experiment in *P. aurita*, some increases were observed in *T. fuscatus* on day 32. There was no significant difference ($P > 0.05$) in MT concentration per total protein between the control groups of the two periwinkle species as shown using Unpaired t-test.

Organisms exposed to 0.01 96hLC$_{50}$ drill cuttings concentration exhibited different responses in the two periwinkles. MT fraction in relation to other proteins was significantly reduced below the background values on day 4, thereafter the ratio increased but the background value was never attained in *P. aurita*. In *T. fuscatus* on the other hand, the ratio was more than doubled on day 4, and subsequently gradually reduced during the exposure period. However, the values were also higher than the background value on any sampling of the days. Unpaired t-test showed the response pattern to be significantly different ($P < 0.05$) between *T. fuscatus* and *P. aurita* on exposure to 0.01 96hLC$_{50}$ drill cuttings concentration.

P. aurita exposed to 0.1 96hLC$_{50}$ drill cuttings concentration showed a similar response to *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration. The ratio of MT in relation to other proteins in *T. fuscatus* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration initially fell below the background value on day 4, and thereafter remained more or less unchanged till day 32 when some marginal increase was observed. There was no significant difference ($P > 0.05$) in MT concentration per total protein between *T. fuscatus* exposed to 0.1 96hLC$_{50}$ drill cuttings exposed group and the similarly exposed *P. aurita* using Unpaired t-test.

4.5.3 Metallothionein induction in the Digestive Gland of *S. huzardi* on exposure to Sublethal Concentrations of Drill Cuttings

The average cysteine residue value observed in male *S. huzardi* at the beginning of the experiment was 0.44 nmolCyt^{MT} **(Table 15)**. At the end of the experiment, the mean cysteine residue values due to metallothionein detected in the digestive gland were

0.4525 nmolCyt^{MT} in the control, 5.7983 nmolCyt^{MT} in male crab exposed to 0.01 96hLC$_{50}$ and 6.0850 nmolCys^{MT} in 0.1 96hLC$_{50}$ drill cuttings exposed crabs. Pearson correlation showed that there was non-significant correlation ($P > 0.05$) between male control crabs and each of the drill cuttings exposed groups, ($r = 0.287$, p-value = 0.365 between control and male *S. huzardi* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and $r = 0.372$, p-value = 0.233 between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed male *S. huzardi*) but significant correlation ($P < 0.01$) was observed between male crabs exposed to 0.01 96hLC$_{50}$ drill cuttings concentration and 0.1 96hLC$_{50}$ drill cuttings exposed crabs ($r = 0.951$, p-value = 0.000). ANOVA showed that there was statistical difference ($P < 0.001$) in all treatment during the experiment, while post hoc test showed that in male control crab the value observed on day 0, day 8 and day 16 were not significantly different (Duncan, $P > 0.01$) from each other, but was significantly different from the mean value observed on day 4 (Duncan, $P < 0.01$). In male crabs exposed to drill cuttings, the difference was significant ($P < 0.001$) on all the sampling days. ANOVA was used to confirm significant difference ($P < 0.05$) in cysteine residue concentration among the control and the drill cuttings exposed group. The difference in cysteine concentration between the two drill cuttings exposed male crabs was also not significant (ANOVA, $P > 0.05$).

The average background concentration of cysteine in the digestive gland of female crab was 0.41 nmolCyt^{MT}. The mean cysteine value observed during the experiment was 0.4054 nmolCyt^{MT} in control, 5.4525 nmolCyt^{MT} in female crabs exposed to 0.01 96hLC$_{50}$ drill cuttings concentration and 4.9875 nmolCyt^{MT} in 0.1 96hLC$_{50}$ drill cuttings exposed female crabs (Table 15). Pearson correlation showed that correlation in cysteine

concentration was not significant ($P > 0.05$) between the control female crabs and each of the female group exposed to drill cuttings, ($r = -0.504$, p-value = 0.095 between control and female *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentration, and $r = -0.509$, p-value = 0.091 between control and 0.1 96hLC_{50} drill cuttings concentration exposed female *S. huzardi*). However, correlation was significant ($P < 0.01$) in cysteine concentration between the two drill cuttings exposed groups ($r = 0.971$, p-value = 0.000.

Analysis of variance revealed that there was no significant difference ($P > 0.05$) in cysteine levels in the digestive gland of control female crab, while high significant difference ($P < 0.001$) existed in all the drill cuttings exposed groups during the experiment. Post hoc test using Duncan showed that the difference was highly significant ($P < 0.001$) on each sampling day in the drill cuttings exposed groups. Differences in induction of cysteine in the digestive gland of female crabs were also highly significant (Duncan, $P < 0.001$) among control and the drill cuttings exposed groups. Sex related difference in cysteine production was observed only between control male and female control crab (Unpaired t-test, $P < 0.001$), while influence of sex was absent in the drill cuttings exposed groups (Unpaired t-test, $P > 0.05$).

At the start of the experiment, the mean background 0.1913 $nmol^{MT}g^{-1}$. MT level of male was higher than the 0.1783 $nmol^{MT}g^{-1}$ observed in female *S. huzardi*. The concentration of MT in male *S. huzardi* ranged from 0.1913 $nmol^{MT}g^{-1}$ to 0.2042$nmol^{MT}g^{-1}$ (mean = 0.1967 $nmol^{MT}g^{-1}$) in control organisms, 0.1913$nmol^{MT}g^{-1}$ to 4.30$nmol^{MT}g^{-1}$ (mean = 2.5206$nmol^{MT}g^{-1}$) in organisms exposed to 8.15ml/l, and 0.1913 $nmol^{MT}g^{-1}$ to 3.8696

$nmol^{MT}g^{-1}$ (mean = 2.6457$nmol^{MT}g^{-1}$) in 81.5 ml/l exposed organisms (**Table 15, Figure 6**).

Correlation between exposure time and metallothionein concentration in the digestive gland of male *S. huzardi* was practically absent for control *S. huzardi* (b = 0.045), and strongly positive for drill cuttings exposed male *S. huzardi* (b =0.682 for male *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentration and 0.826 for male *S. huzardi* exposed to 0.1 96hLC_{50} drill cuttings concentration).

Pearson correlation was not significant between control and each of the treatment exposed to drill cuttings both for male and female crabs (male r = 0.290, p-value = 0.361; female r = -0.502, p-value = 0.096, between control and *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentration; and male r = 0.375, p-value = 0.229, female r = 0.509, p-value = 0.091, between control and 0.1 96hLC_{50} drill cuttings concentration exposed *S. huzardi*). However, Pearson correlation was positive (r = 0.951 for male, r = 0.974 for female) and strongly significant ($P < 0.01$) between the two drill cuttings exposed groups both for male and female *S. huzardi*.

ANOVA revealed that there was a significant difference ($P < 0.05$) in MT concentration in control male *S. huzardi*, post hoc using Duncan test showed the source of variation to have occurred on day 4, which was significantly different from the values observed on day 0, day 8 and day 16 while there was no statistical difference ($P > 0.05$) in male control crab on day 0, 8 and 16. Statistical differences ($P < 0.001$) were also observed in the two treatments exposed to drill cuttings using ANOVA. Post hoc test with Duncan

revealed that the differences observed in the drill cuttings exposed groups were significant on all sampling days ($P < 0.001$). Exposure to drill cuttings has an effect on MT induction in male crab as indicated by the statistical difference among control crabs and crabs exposed to drill cuttings (ANOVA, $P < 0.001$).

In female *S. huzardi*, MT concentrations ranged from 0.1739 $nmol^{MT}g^{-1}$ to 0.1783 $nmol^{MT}g^{-1}$ (mean = 0.1761 $nmol^{MT}g^{-1}$) in control organisms, 0.1783 $nmol^{MT}g^{-1}$ to 3.6522 $nmol^{MT}g^{-1}$ (mean = 2.3707 $nmol^{MT}g^{-1}$) in organisms exposed to 8.15 ml/l, and 0.1783 $nmol^{MT}g^{-1}$ to 3.2609 $nmol^{MT}g^{-1}$ (mean = 2.1685 $nmol^{MT}g^{-1}$) in 81.5 ml/l exposed organisms **(Table 15, Figure 7)**. Correlation between exposure time and metallothionein concentration in the digestive gland of female *S. huzardi* was strongly negative for control *S. huzardi* (b =-0.845), and strongly positive for drill cuttings exposed female *S. huzardi* (b = 0.733 for female *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentration and 0.851 for female *S. huzardi* exposed to 0.1 96hLC_{50} drill cuttings concentration).

ANOVA showed that there was no significant difference ($P > 0.05$) in the control female crabs during the study, while significance differences ($P < 0.001$) were observed in the two treatments exposed to drill cuttings. Post hoc treatment with Duncan showed that the difference observed in the drill cuttings exposed group was significant ($P < 0.001$) on all the sampling days. Test using ANOVA revealed that MT induction in the digestive gland of female *S. huzardi* was affected by exposure to drill cuttings as indicated by the statistical difference ($P < 0.001$) observed among the control group and the drill cuttings exposed groups.

Table 15: Induction of metallothionein in *S. huzardi* on sublethal exposure to drill cuttings (mean ± SD)

Male *Sesarma huzardi* Control						
Incubation Time (day)	**n° Cys**	**wt of tissue (g)**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmol MT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1	8.2 ± 0.10^{d}	0.44 ± 0.10^{b}	0.1913 ± 0.00^{b}	0.0023 ± 0.00^{a}
4	23	0.1	12.6 ± 0.10^{c}	0.47 ± 0.10^{a}	0.2043 ± 0.00^{a}	0.0016 ± 0.00^{b}
8	23	0.1	17.5 ± 0.06^{b}	0.45 ± 0.06^{b}	0.1957 ± 0.00^{b}	0.0011 ± 0.00^{c}
16	23	0.1	20.8 ± 0.21^{a}	0.45 ± 0.21^{b}	0.1957 ± 0.00^{b}	0.0009 ± 0.00^{d}
Male *Sesarma huzardi* subjected to 8.15 ml/l						
Incubation Time (day)	**n° Cys**	**wt of tissue**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmol MT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1	8.2 ± 0.10^{d}	0.44 ± 0.10^{d}	0.1913 ± 0.00^{d}	0.0023 ± 0.00^{d}
4	23	0.1	10.5 ± 0.0^{c}	5.57 ± 0.00^{c}	2.4217 ± 1.26^{c}	0.0231 ± 0.01^{b}
8	23	0.1	15.7 ± 0.10^{b}	9.89 ± 0.10^{a}	4.3000 ± 0.00^{a}	0.0274 ± 0.00^{a}
16	23	0.1	25.4 ± 0.26^{a}	7.29 ± 0.26^{b}	3.1710 ± 0.00^{b}	0.0125 ± 0.00^{c}
Male *Sesarma huzardi* subjected to 81.5 ml/l						
Incubation Time (day)	**n° Cys**	**wt of tissue**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmol MT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1	8.2 ± 0.10^{d}	0.44 ± 0.10^{d}	0.1913 ± 0.00^{d}	0.0023 ± 0.00^{d}
4	23	0.1	11.3 ± 0.10^{c}	6.4 ± 0.10^{c}	2.7826 ± 0.01^{c}	0.0246 ± 0.00^{a}
8	23	0.1	16.2 ± 0.20^{b}	8.6 ± 0.20^{b}	3.7391 ± 0.02^{b}	0.0231 ± 0.00^{b}
16	23	0.1	25.5 ± 0.00^{a}	8.9 ± 0.00^{a}	3.8696 ± 0.01^{a}	0.0152 ± 0.00^{c}
Female *Sesarma huzardi* Control						
Protein (mg/l)	**n° Cys**	**wt of tissue**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmol MT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1	7.3 ± 0.00^{d}	0.41 ± 0.00^{a}	0.1783 ± 0.00^{a}	0.0024 ± 0.00^{a}
4	23	0.1	8.4 ± 0.20^{c}	0.41 ± 0.20^{a}	0.1783 ± 0.00^{a}	0.0021 ± 0.00^{b}
8	23	0.1	10.2 ± 0.10^{b}	0.4 ± 0.10^{a}	0.1739 ± 0.00^{a}	0.0017 ± 0.00^{c}
16	23	0.1	18.9 ± 0.26^{a}	0.4 ± 0.26^{a}	0.1739 ± 0.00^{a}	0.0009 ± 0.00^{d}
Female *Sesarma huzardi* subjected to 8.15 ml/l						
Incubation Time (day)	**n° Cys**	**wt of tissue**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmol MT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1	7.3 ± 0.00^{d}	0.41 ± 0.00^{d}	0.1783 ± 0.00^{d}	0.0024 ± 0.00^{d}
4	23	0.1	9.5 ± 0.00^{c}	5.8 ± 0.00^{c}	2.5217 ± 0.00^{c}	0.0265 ± 0.00^{b}
8	23	0.1	12.4 ± 0.17^{b}	8.4 ± 0.17^{a}	3.6522 ± 0.00^{a}	0.0295 ± 0.00^{a}
16	23	0.1	20.6 ± 0.26^{a}	7.2 ± 0.26^{b}	3.1304 ± 0.01^{b}	0.0152 ± 0.00^{c}
Female *Sesarma huzardi* subjected to 81.5 ml/l						
Incubation Time (day)	**n° Cys**	**wt of tissue**	**Protein (mg/l)**	**nmol Cys^{MT}**	**nmol MT g^{-1}**	**nmol MT mg^{-1} protein**
0	23	0.1	7.3 ± 0.00^{d}	0.41 ± 0.00^{d}	0.1783 ± 0.00^{d}	0.0024 ± 0.00^{d}
4	23	0.1	9.3 ± 0.10^{c}	5.24 ± 0.10^{c}	2.2783 ± 0.02^{c}	0.0245 ± 0.00^{b}
8	23	0.1	10.4 ± 0.10^{b}	6.8 ± 0.10^{b}	2.9565 ± 0.01^{b}	0.0284 ± 0.00^{a}
16	23	0.1	14.6 ± 0.0^{a}	7.5 ± 0.00^{a}	3.2609 ± 0.01^{a}	0.0223 ± 0.00^{c}

Mean ± SD; n =3 (mean and SD are calculated from three measurements, considering three pooled samples at each concentration. Means not sharing the same superscript (a, b, c or d) in each column are significantly different (Duncan, $P < 0.001$).

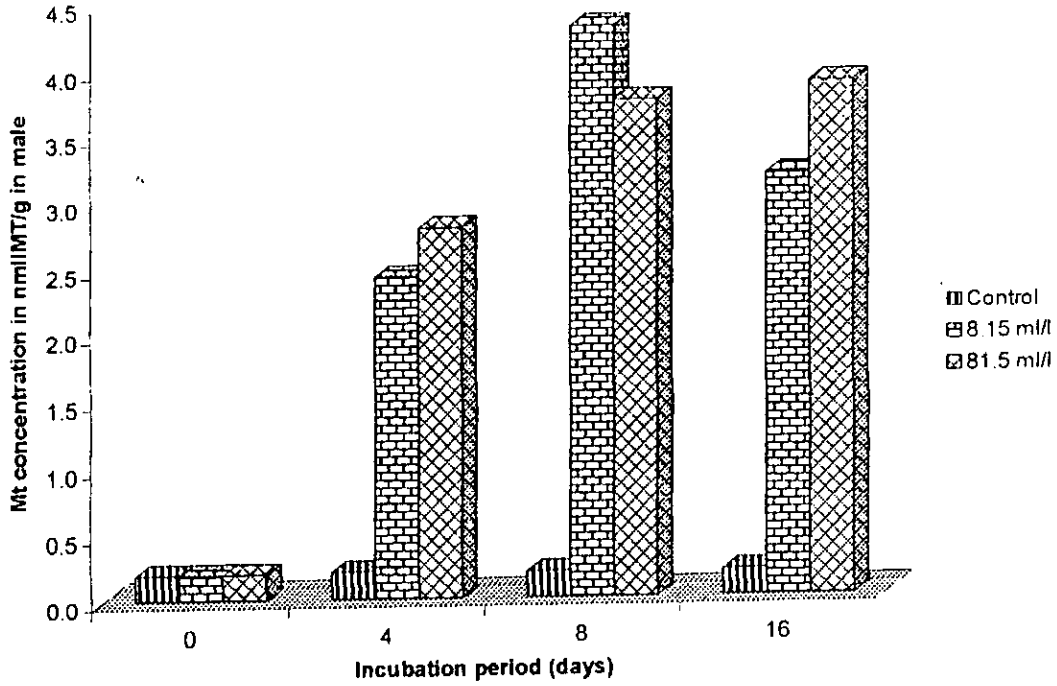

Figure 6: Metallothionein levels in the digestive gland of male *S. huzardi*

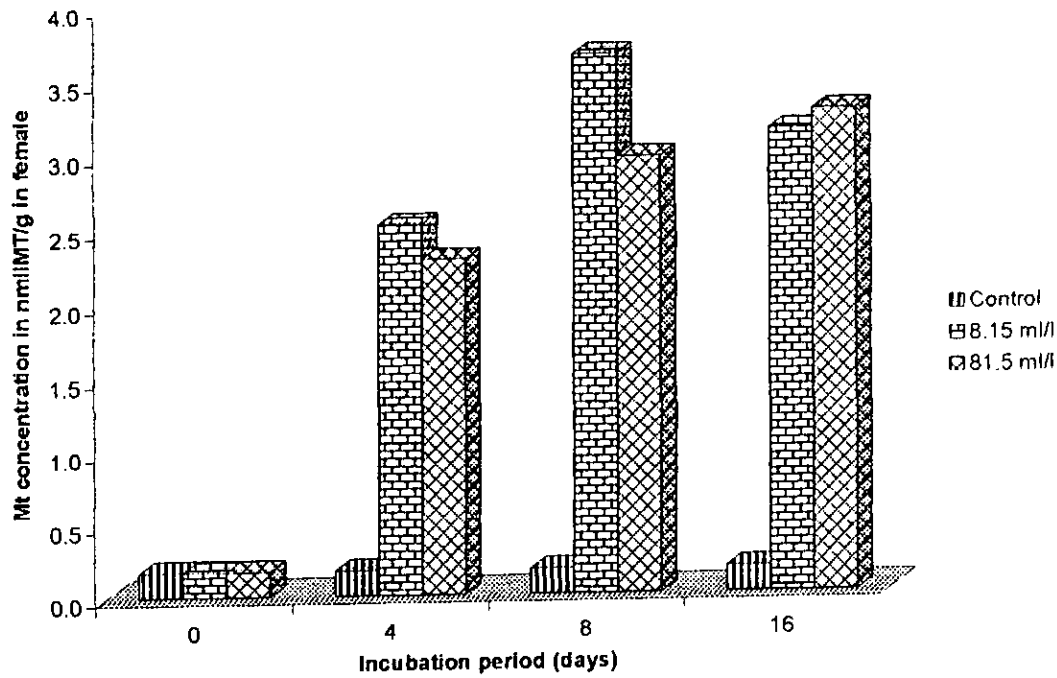

Figure 7: Metallothionein levels in the digestive gland of female *S. huzardi*

Comparing the concentration of MT in control male and female *S. huzardi* showed differences and similarities in their response. MT levels remained very low in both groups, but in male, some increase was observed on day 4, and thereafter the value fell on day 8 to a value that was still above the background level. In female on the other hand, the background value was unchanged on day 4, and only fell marginally on day 8 (Table 16). Sexually related differences in MT concentration between control male and female crabs were also established using Unpaired t-test ($P < 0.05$).

Induction pattern of MT was similar in both male and female exposed to drill cuttings. In the organisms exposed to 8.15 ml/l, exposure to drill cuttings resulted in significant increase in concentrations of MT, with peak values occurring on day 8, which thereafter dipped on day 16. On the other hand, there was consistent increase in MT concentration in 81.5 ml/l exposed organisms ($b = 0.83$ for male, $b = 0.85$ for female), and on day 16, MT concentration was higher in 81.5 ml/l exposed crabs than was observed in the 8.15 ml/l exposed organisms. Unpaired t-test ($P > 0.05$) showed that there were no sexually related differences in MT induction in both groups exposed drill cuttings.

The average background protein level observed at the beginning of the study in male *S. huzardi* was 8.2 mg/l protein. The mean values observed during the study in male crab were 14.80 mg/l protein in control, 14.95 mg/l protein in 0.01 $96hLC_{50}$ exposed organisms and 15.30 mg/l protein in crabs exposed to 0.1 $96hLC_{50}$. In female crabs, the average background value observed was 7.3 mg/l protein, while the mean protein observed during the study were11.20 mg/l protein in female control, 12.45 mg/l protein in 0.01 $96hLC_{50}$ drill cuttings exposed female crabs and 10.40 mg/protein in female crabs

exposed to 0.1 96hLC$_{50}$ drill cuttings concentration. Pearson correlation revealed that correlation of protein in the digestive gland was positive and significant ($P < 0.01$) between control and each of the drill cuttings exposed crabs both for male and female crabs (male r = 0.939, p-value = 0.000; female r = 0.989, p-value = 0.000, between control and *S. huzardi* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration; and male r = 0.975, p-value = 0.000, female r = 0.975, between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed *S. huzardi*). Pearson correlation was also positive (r = 0.999 for male, r = 0.993 for female) and highly significant ($P < 0.01$) between the two drill cuttings exposed groups both for male and female *S. huzardi*.

ANOVA revealed significant difference in protein levels in all treatment groups for both male and female crabs ($P < 0.001$), while post hoc test using Duncan revealed that the difference was significant ($P < 0.001$) on all the sampling days in both male and female crabs. Comparing the protein concentrations among all treatments also revealed significant difference among (ANOVA, $P < 0.001$). Test using of significance with Unpaired t-test revealed no sexually related difference between male and female crabs at all treatment ($P > 0.5$).

As a fraction of the total protein present, the mean value observed in male crab was 0.0023 nmol^{MT}mg-^{1}, and during the study in male *S. huzardi* ranged from 0.0009 nmol^{MT}mg-^{1} to 0.0023 nmol^{MT}mg-^{1} (mean = 0.0015 nmol^{MT}mg-1) in control organisms, 0.0023 nmol^{MT}mg-^{1} to 0.0274 nmol^{MT}mg-^{1} (mean = 0.0163 nmol^{MT}mg-^{1}) in organisms exposed 8.15 ml/l, and 0.0023 nmol^{MT}mg-^{1} to 0.0246 nmol^{MT}mg-^{1} (mean = 0.0163 nmol^{MT}mg-^{1}) in 81.5 ml/l exposed organisms **(Table 15, Figure 8)**.

With a value of 0.0024nmol^{MT}mg-^{1} MT value relative to total proteins in female was marginally higher than that of the male. During the study the value ranged from 0.0009 nmol^{MT}mg-^{1} to 0.0024 (mean = 0.0018 nmol^{MT}mg-1) in control organisms, 0.0024 nmol^{MT}mg-^{1} to 0.0295 nmol^{MT}mg-^{1} (mean = 0.0184 nmol^{MT}mg-1) in organisms exposed 8.15 ml/l, and 0.0024 nmol^{MT}mg-^{1} to 0.0284 nmol^{MT}mg-^{1} (mean = 0.0194 nmol^{MT}mg-1) in 81.5 ml/l exposed organisms **(Table 15, Figure 9)**.

The ratio of MT to total protein was strongly negatively correlated with exposure time in the control organisms of both groups (correlation coefficient, male = -0.908, female = -0.999). Regression analysis between total protein and metallothionein concentration in control crabs was also weakly positively correlated for male crabs (b = 0.107) and strongly negatively correlated for female crabs (b = -0.734).

In drill cuttings exposed groups, MT ratio to other proteins increased above the background value on all the sampling days. In organisms exposed to 8.15 ml/l, MT ratio trend was similar for both male and female groups. In this group, the ratio increased significantly above the background value on day 4 and 8, and sharply dropped on day 16. Correlation between total protein and metallothionein concentrations was positively correlated (b = 0.611 for males exposed to 0.01 96hLC_{50} drill cuttings concentration, and 0.634 for females exposed to 0.01 96hLC_{50} drill cuttings concentration. Also in this treatment group, regression analysis between exposure time and MT concentration relative to other protein was weakly positively correlated (b = 0.235 for male crabs and 0.276 for female crabs).

In crabs exposed 81.5 ml/l, MT concentration relative to other proteins increased on day 4 in male crabs, and thereafter fell on subsequent sampling days. In female on the other hand, the trend was similar to that of the female exposed to 0.01 96hLC$_{50}$ drill cuttings concentration. Here the concentration of MT relative to other proteins steadily increased to day 8 before a decrease was observed on day 16. Correlation between total protein and MT concentrations was strongly positive (b= 0.785 for male *S. huzardi* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration and 0.829 for female *S. huzardi* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration). Positive correlation was also observed between exposure time and MT concentration relative to other protein for crabs exposed to 0.1 96hLC$_{50}$ drill cuttings concentration (b = 0.332 for male and 0.584 for male). In general, MT concentration relative to the total protein present was higher in female than in male *S. huzardi* exposed to 81.5 ml/l concentration as shown by the results of their regressive analysis.

Pearson correlation revealed significant correlation ($P < 0.05$) for MT relative to other proteins between control male crabs and male crabs exposed to drill cuttings (r = -0.608, p-value = 0.036 between control and male *S. huzardi* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and r = -0.652, p-value = 0.022 between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed male *S. huzardi*. Pearson correlation was also significant ($P < 0.01$) between the two drill cuttings exposed male crabs (r = 0.963, p-value = 0.000). In female crabs on the other hand, Pearson correlation showed that there was no significant correlation ($P > 0.05$) in MT concentration relative to other proteins between control and female crabs exposed to 0.01 96hLC$_{50}$ drill cuttings concentrations (r

= -0.213, p-value = 0.506), as well as between control and crabs exposed to 0.1 96hLC$_{50}$ drill cuttings concentration (r = -0.529, p-value = 0.077), while significant correlation ($P < 0.01$) existed between the two male groups exposed to drill cuttings (r = 0.941, p-value = 0.000)

There was high significant difference (ANOVA, $P < 0.001$) in MT per total protein concentration for each treatment both for male and female crabs. Post hoc test with Duncan showed that the differences were highly significant ($P < 0.001$) on all the sampling days for all treatments including control both for male and female crabs.

ANOVA was used to investigate the difference in MT per total protein concentration between control and drill cuttings exposed groups. The difference was found to be significant both in male and female crabs. However, in male crabs, the control was significantly different (Duncan, $P < 0.001$) from the drill cuttings exposed groups, while the two drill cuttings treatments were not significantly different ($P > 0.05$) from each other. In female crabs on the other hand, both control and each of the drill cuttings exposed group are significantly different from one another (ANOVA, Duncan, $P < 0.001$).

Influence of sex on the concentration of MT relative to total protein present was investigated using Unpaired t-test, and was found to be absent at all treatment concentration during the study ($P > 0.05$).

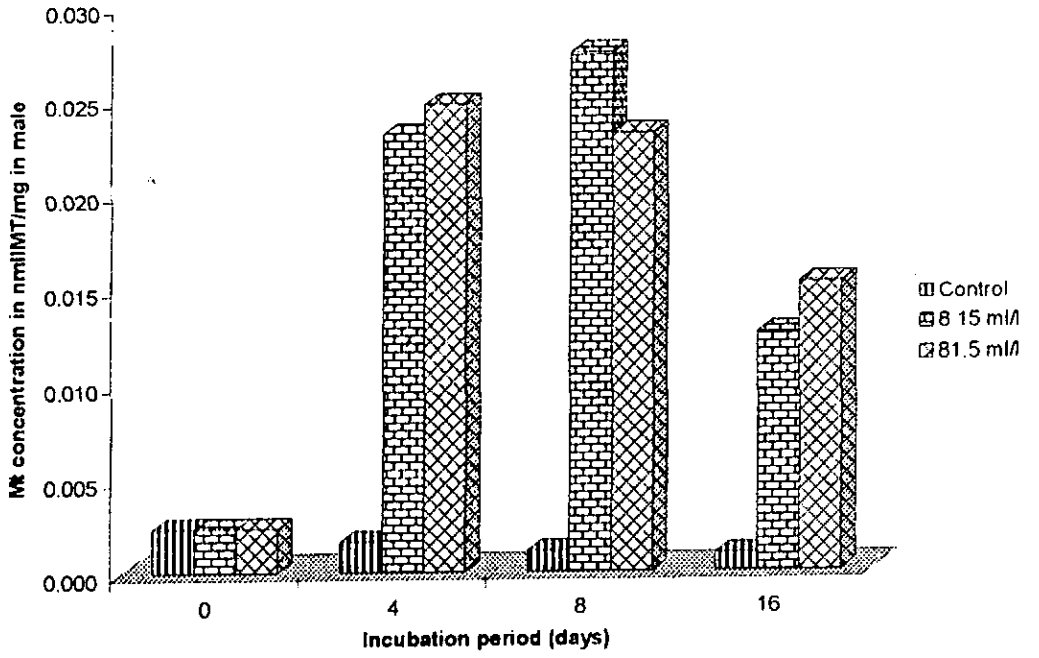

Figure 8: Varying levels of Metallothionein as fraction of total protein in male *S. luzardi*

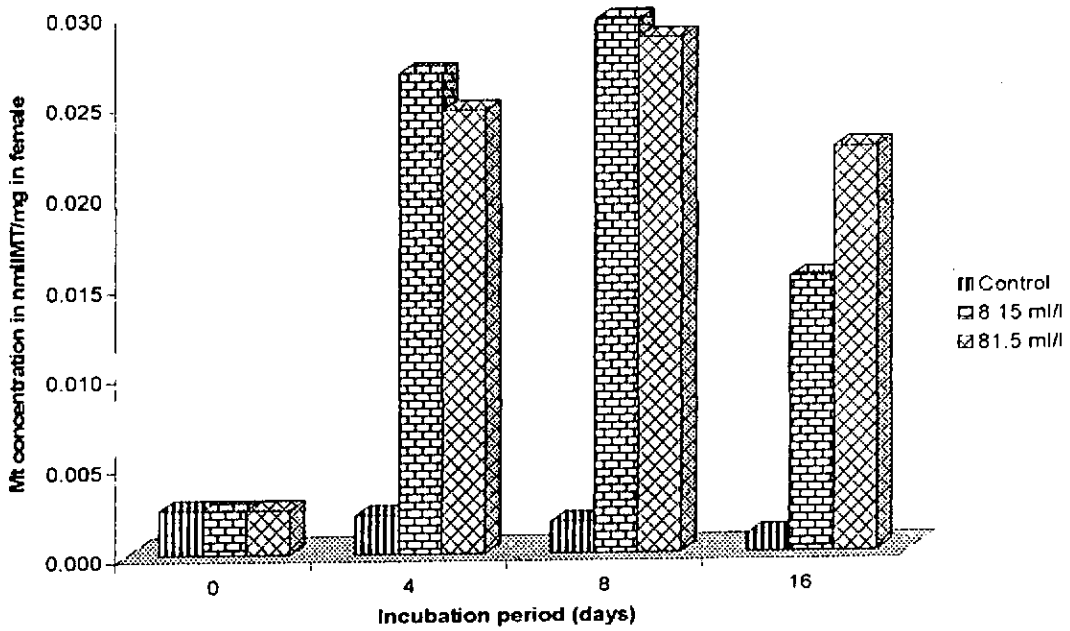

Figure 9: Varying levels of Metallothionein as fraction of total protein in female *S. luzardi*

4.6 LYSOSOMAL MEMBRANE STABILITY IN TEST SPECIES EXPOSED TO SUBLETHAL CONCENTRATIONS OF DRILL CUTTINGS

The ability to retain neutral red (NR) dye is dependent on cell viability. Stressed membranes are more likely to release the contents of the lysosome including the neutral red dye, into the cytosol. The findings here show that control cells were able to retain the NR dye the longest throughout the experimental period in both gastropod and crab species studied.

In *T. fuscatus*, the average observed background neutral red retention (NRR) time was 17.0 minutes. The mean NRR time observed during the experiment was 16.97 minutes in control, 12.15 minutes in 0.01 96hLC$_{50}$ exposed drill cuttings *T. fuscatus* and 11.62 minutes in 0.1 96hLC$_{50}$ drill cuttings exposed *T. fuscatus*. Pearson correlation revealed that linear relationship for NRR time between control and each of the drill cuttings exposed treatment was not significant ($P > 0.05$) in *T. fuscatus* ($r = 0.138$, p-value = 0.623 between control and *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and $r = 0.175$, p-value = 0.534 between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed *T. fuscatus*), while the relationship was significant between the two drill cuttings exposed groups ($r = 0.995$, p-value = 0.000).

Test using ANOVA revealed significant differences ($P < 0.05$) in all treatment for *T. fuscatus*, while post hoc analysis with Duncan showed that the source of variation in control group was on day 8 which was statistically difference ($P < 0.05$) from background value and values observed on all other sampling days, while the values obtained on day 0,

day 4, day 16 and day 32 were not significantly different from each other ($P > 0.05$). Duncan test also revealed that NRR time was significantly different ($P < 0.001$) on all sampling days in drill cuttings exposed groups. ANOVA confirmed that exposure to drill cuttings affected NRR time in *T. fuscatus* since significant difference ($P < 0.001$) was observed among control the drill cuttings exposed groups.

In *P. aurita*, the observed background NRR time was 16.3 minutes. The mean values recorded during the study period was 16.08 minutes in control, 10.86 minutes in *P. aurita* exposed to 0.01 $96hLC_{50}$ exposed drill and 10.41 minutes in *P. aurita* exposed to 0.1 $96hLC_{50}$ drill cuttings concentration. Pearson correlation revealed a non-significant ($P > 0.05$) linear relationship between control *P. aurita* and *P. aurita* exposed to 0.01 $96hLC_{50}$ drill cuttings concentration ($r = 0.500$, p-value = 0.058). However Pearson correlation was significant ($P < 0.05$) between control *P. aurita* and *P. aurita* group exposed to 0.1 $96hLC_{50}$ drill cuttings concentration ($r = 0.518$, p-value = 0.048). A positive ($r = 0.998$, p-value = 0.000) and highly significant relationship ($P < 0.01$) in NRR time was observed between the two treatments exposed to drill cuttings in *P. aurita*.

Test of significance using ANOVA showed that there were significant differences ($P < 0.001$) in all the treatments during the study. Post hoc test using Duncan showed that in control *P. aurita* the values obtained on day 0, day 4 and day 16 were not significantly different ($P > 0.05$) from one another. Day 4 was also not significantly different from day 8, while day 32was significantly different ($P < 0.001$) from the values obtained on other sampling days. In *P. aurita* exposed to 0.01 $96hLC_{50}$ drill cuttings concentration, post

hoc test with Duncan revealed that the background value was significantly different ($P < 0.001$) from the values obtained on other sampling days during the study. The test also revealed that day 8 and day 16 were not significantly different ($P > 0.05$) from each other but are significantly different ($P < 0.001$) from the values obtained on other sampling days. The values obtained on day 4 and day 32 were also each significantly different from the values observed on other sampling days. In 0.1 96hLC_{50} drill cuttings concentration exposed *P. aurita*, Duncan test revealed that the values obtained are significantly different ($P < 0.001$) on all sampling days.

Difference in NRR time attributable to exposure to drill cuttings was confirmed by ANOVA, which revealed that significant difference in among control and the drill cuttings exposed groups ($P < 0.001$).

A comparison of NRR time in *T. fuscatus* and *P. aurita* revealed that though the background NRR time was higher in *T. fuscatus*, by the end of the 32 day exposure period, NRR time was found to be lower in *T. fuscatus* than in P. *aurita* in drill cuttings exposed organisms. In control organisms on the other hand, NRR time remained higher in *T. fuscatus* compared to *P. aurita*. Unpaired t-test revealed that the difference in NRR time between control *T. fuscatus* and control *P. aurita* was significant ($P < 0.001$) during the study, however, by the end of the study the difference was not significant ($P > 0.05$) between *T. fuscatus* and *P. aurita* exposed to 0.01 96hLC_{50} drill cuttings concentration as well as between 0.1 96hLC_{50} drill cuttings concentration exposed *T. fuscatus* and *P. aurita*.

NRR time in *S. huzardi* also showed both time and concentration dependent trend when monitored over a study period of 16 days. The average background NRR time observed in male crab was 20.83 minutes against 20.42 minutes recorded for female crabs. The average NRR time observed during the study was 20.31 minutes for male control, 17.65 minutes for male crab exposed to 0.01 96hLC_{50} drill cuttings concentration and 14.61 for male crabs exposed to 0.1 96hLC_{50} drill cuttings concentration. In female crab on the other hand, the average NRR time observed during the study was 20.26 minutes for female control, 17.14 minutes female crab exposed to 0.01 96hLC_{50} drill cuttings concentration and 14.84 for female crabs exposed to 0.1 96hLC_{50} drill cuttings concentration.

In male crab, Pearson correlation revealed positive and significant correlation ($P < 0.05$) in NRR time between control male *S. huzardi* and male *S. huzardi* exposed to 0.01 96hLC50 drill cuttings concentration ($r = 0.645$, p-value = 0.023). High significant correlation ($P < 0.01$) also existed between male control *S. huzardi* and 0.1 96hLC50 drill cuttings exposed male *S. huzardi* ($r = 0.714$, p-value = 0.009) as well between the two male groups exposed to drill cuttings ($r = 0.945$, p-value = 0.000). In female crab, correlation in NRR time between control and each of the drill cuttings treated groups was not significant ($P > 0.05$) ($r = 0.521$, p-value = 0.082, for female control *S. huzardi* and *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentration; $r = 0.561$, p-value = 0.058. for female control *S. huzardi* and female *S. huzardi* exposed), while high significant correlation ($P < 0.01$) was observed between the two female groups exposed to drill cuttings ($r = 0.954$, p-value = 0.000).

ANOVA was used to test for difference in NRR time in all treatment for the crab during the study and revealed that there was no statistical difference ($P > 0.05$) in control of both control male and control female *S. huzardi*. However significant difference was observed in each of the drill cuttings exposed groups of both male and female *S. huzardi*. Post hoc test with Duncan showed that in male control crab, day 4, day 8 and day 16 were not significantly different ($P > 0.05$) from one another, while day 0, day 4 and day 16 were also not significantly different ($P > 0.05$) from one another.

In male crabs exposed to drill cuttings, post hoc test with Duncan showed that there was significant difference ($P < 0.001$) on each sampling day for the group exposed to 0.01 $96hLC_{50}$ drill cuttings concentration. In 0.1 $96hLC_{50}$ drill cuttings concentration exposed male crabs on the other hand, while there were significant differences ($P < 0.001$) in NRR time on all other sampling days relative to day 0, NRR time on day 8 and 16 were not statistically different ($P > 0.05$) from each other, but were significantly different ($P < 0.001$) from day 0 and day 4.

In female crabs exposed to drill cuttings, post hoc tests with Duncan confirmed significant difference ($P < 0.001$) in NRR time on all sampling days.

Differences in NRR time attributable to exposure to drill cuttings were confirmed using ANOVA which revealed significant difference ($P < 0.001$) in NRR time among the control and the drill cuttings exposed groups both for male and female crabs.

Though the background NRR time was marginally higher in male compared to female in control crabs by the end of experiment, both male and female control crabs had the same NRR time (**Table 16**). There was no sexually related difference in the observed NRR time between male and female crabs at all exposure concentrations (Unpaired t-test, $P > 0.05$) (**Table 16**).

Table 16: Summary of Neutral Red Retention Time in Test Species on Exposure to Sublethal Drill Cuttings Concentrations Expressed in minutes (mean ± SD)

Tympanotonus fuscatus			
Incubation Time (Day)	**Control**	**1.02 ml/l**	**10.24 ml/l**
0	17.0 ± 0.06^{a}	17.0 ± 0.06^{a}	17.0 ± 0.06^{a}
4	17.0 ± 0.00^{a}	16.0 ± 0.01^{b}	15.1 ± 0.06^{b}
8	16.7 ± 0.20^{b}	10.3 ± 06^{c}	9.5 ± 0.06^{c}
16	17.1 ± 0.06^{a}	10.1 ± 0.10^{d}	9.4 ± 0.10^{d}
32	17.0 ± 0.06^{a}	7.2 ± 0.06^{e}	7.1 ± 0.06^{e}
Pachymelania aurita			
Incubation Time (Day)	**Control**	**1.22 ml/l**	**12.22 ml/l**
0	16.3 ± 0.00^{a}	16.3 ± 0.00^{a}	16.3 ± 0.00^{a}
4	16.2 ± 0.02^{ab}	12.6 ± 0.32^{b}	12.2 ± 0.06^{b}
8	16.0 ± 06^{b}	8.6 ± 0.10^{c}	8.2 ± 0.06^{c}
16	16.3 ± 0.06^{a}	8.6 ± 0.05^{c}	8.1 ± 0.06^{d}
32	15.6 ± 0.32^{c}	8.2 ± 0.10^{d}	7.4 ± 0.06^{e}
Male *Sesarma huzardi*			
Incubation Time (day)	**Control**	**8.15 ml/l**	**81.5 ml/l**
0	20.87 ± 1.08^{a}	20.87 ± 1.08^{a}	20.87 ± 1.08^{a}
4	20.17 ± 0.06^{ab}	18.6 ± 0.10^{b}	14.97 ± 0.12^{b}
8	20.1 ± 0.10^{b}	16.63 ± 0.21^{c}	11.33 ± 1.10^{c}
16	20.17 ± 0.06^{ab}	14.53 ± 0.35^{d}	10.93 ± 0.67^{c}
Female *Sesarma huzardi*			
Incubation Time (day)	**Control**	**8.15 ml/l**	**81.5 ml/l**
0	20.43 ± 25^{a}	20.43 ± 25^{a}	20.43 ± 25^{a}
4	20.23 ± 0.06^{a}	18.23 ± 0.06^{b}	16.63 ± 0.15^{b}
8	20.17 ± 0.06^{a}	16.27 ± 0.15^{c}	11.6 ± 0.27^{c}
16	20.2 ± 0.06^{a}	13.63 ± 0.15^{d}	10.7 ± 0.20^{d}

Mean ± SD; n =3 (mean and SD are calculated from three measurements, considering three pooled samples at each concentration. Means not sharing the same superscript (a, b, c, d or e) in each column are significantly different ($P < 0.001$, Duncan).

4.7 ANTIOXIDANT BIOMARKERS IN TEST SPECIES EXPOSED TO SUBLETHAL CONCENTRATIONS OF DRILL CUTTINGS

Indicators of oxidative damage studied included catalase activity, superoxide, glutathione *S*-transferase and lipid peroxidation. Activities were monitored over 16 day exposure period.

4.7.1 Catalase Activity in Test Species Exposed to Sublethal Concentrations of Drill Cuttings

The background catalase (CAT) activity observed in *T. fuscatus* and *P. aurita* was 6.70 CAT μmol/min/mg protein, which in the two periwinkle species increased in all treatment groups including control by the 4th day, with drill cuttings exposed organisms having higher activity than the control groups during this period. In the control organisms no further change in catalase activity was observed to the end of the experimental period. This response held true for both *P. aurita* and *T. fuscatus*. Regression analysis between catalase concentration and exposure period was positive (b = 0.712 for control *T. fuscatus*, b = 0.679 for control *P. aurita*).

On exposure to drill cuttings, both *T. fuscatus* and *P. aurita* responded in similar manners. Peak catalase activity was observed on day 8, which on day 16 fell to comparable control values in *T. fuscatus*. Regression analysis between catalase concentration and exposure period for *T. fuscatus* exposed to sublethal drill cuttings concentration was weakly positively correlated (b = 0.109 for *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and 0.124 for *T. fuscatus* exposed to 0.1 96hLC$_{50}$

drill cuttings concentration). In *P. aurita* on the other hand, catalase activity returned to background level on day 16 in *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, while catalase activity in 0.1 96hLC$_{50}$ drill cuttings concentration group was lower than the control value during the same period. Regression analysis between catalase activity and exposure period for *P. aurita* exposed to sublethal drill cuttings concentration showed virtually no correlation (b = -0.032 for *P.* aurita exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and -0.089 for *P. aurita* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration).

The mean CAT activity detected during the study was 9.28 CAT µmol/min/mg protein in control *T. fuscatus*, 17.26 CAT µmol/min/mg protein in *T. fuscatus* treated to 0.01 96hLC$_{50}$ drill cuttings concentration and 13.62 CAT µmol/min/mg protein in *T. fuscatus* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration. Pearson correlation showed that correlation of CAT activity between the control group and each of the drill cuttings treated groups was significant ($P < 0.05$) ($r = 0.597$, p-value = 0.040 between control and *T. fuscatus* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and $r = 0.662$, p-value = 0.019 between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed *T. fuscatus*). High significant correlation ($p < 0.01$) was also observed between the two drill cuttings exposed group ($r = 0.992$, p-value = 0.000).

Analysis of variance revealed that there was significant ($P < 0.05$) difference in CAT activity in all treatment groups during the study. Post hoc test with Duncan revealed that the differences were significant ($P < 0.001$) on all the sampling days in all the groups

except for day 8 and day 16 when the observed difference was not significant ($P > 0.05$) between each other in the control *T. fuscatus*. Differences in CAT activity among control and the drill cuttings exposed groups was also indicated using ANOVA ($P < 0.001$).

The mean CAT level observed in *P. aurita* during the experiment was 9.25 CAT µmol/min/mg protein in control, 14.57 CAT µmol/min/mg protein *P. aurita* exposed to 0.01 96hLC_{50} drill cuttings concentration and 13.65 CAT µmol/min/mg protein 0.1 96hLC_{50} drill cuttings concentration. Pearson correlation revealed that linear relation for CAT was not significant ($P > 0.05$) between the control *P. aurita* and *P. aurita* exposed to 0.01 96hLC_{50} drill cuttings concentration ($r = 0.518$, p-value = 0.087) while significant correlation was observed between control *P. aurita* and 0.1 96hLC_{50} drill cuttings concentration exposed *P. aurita* ($r = 0.655$, p-value = 0.021). The correlation ($r = 0.983$, p-value = 0.000) observed between the two groups exposed to drill cuttings was also highly significant ($P < 0.01$).

ANOVA showed that there was significant difference ($P < 0.05$) in all the treatment groups in *P. aurita*. While CAT activity tended to stabilise with time in control *P. aurita*, its activities fluctuated on all sampling days in drill cuttings exposed organisms (**Table 17**). This was verified using post Duncan test which revealed that on day 4, 8 and 16 CAT activity was not statistically different ($P > 0.05$) among one another, but activity on each of these days was significantly different ($P < 0.001$) from background value in control *P. aurita*. In drill cuttings exposed *P. aurita* on the other hand post hoc test with Duncan showed that there was significant difference ($P < 0.0501$) in CAT activity on each sampling day except between day 0 and day 16 in *P. aurita* exposed to 0.01

$96hLC_{50}$ drill cuttings concentration since CAT activity on day 16 in this group was the same as the observed background value in this treatment group.

There was also statistical difference in CAT activity attributable to drill cuttings exposure as revealed by the significant difference ($P < 0.001$) observed among control *P. aurita* and *P. aurita* groups exposed to drill cuttings.

Comparing CAT activity in the two periwinkle species using Unpaired t-test confirmed that CAT activity was similar between both controls groups as well as between the groups exposed to drill cuttings concentration of the two periwinkle species ($P > 0.05$).

Table 17: Catalase Activity in the Test Species on Exposure to Sublethal Drill Cuttings Concentration Expressed as µmol/min/mg protein (Mean ± SD)

T. fuscatus Activity expressed as CAT µmol/min/mg protein			
Incubation Time (day)	**Control**	**1.02 ml/l**	**10.24 ml/l**
0	6.7 ± 0.02^{c}	6.7 ± 0.20^{d}	6.7 ± 3.27^{d}
4	10 ± 0.06^{b}	18.2 ± 0.06^{b}	16.5 ± 0.03^{b}
8	10.2 ± 0.02^{a}	33.7 ± 0.10^{a}	24.8 ± 0.02^{a}
16	10.2 ± 0.06^{a}	10.5 ± 0.10^{c}	10.24 ± 0.02^{c}
P. aurita Activity expressed as CAT µmol/min/mg protein			
Incubation Time (day)	**Control**	**1.22 ml/l**	**12.22 ml/l**
0	6.7 ± 0.05^{b}	6.7 ± 0.05^{c}	6.7 ± 0.05^{d}
4	10 ± 0.30^{a}	16.3 ± 0.15^{b}	15.8 ± 0.25^{b}
8	10.1 ± 0.35^{a}	28.7 ± 0.25^{a}	22.7 ± 0.29^{a}
16	10 ± 0.23^{a}	6.7 ± 0.10^{c}	9.4 ± 0.06^{c}
Male *Sesarma huzardi* Activity expressed as CAT µmol/min/mg protein			
Incubation Time (day)	**Control**	**8.15 ml/l**	**81.5 ml/l**
0	1.0 ± 0.04	1.0 ± 0.04^{b}	1.0 ± 0.04^{c}
4	0.5 ± 0.01^{c}	0.72 ± 0.06^{c}	3.71 ± 0.01^{a}
8	2.78 ± 0.03^{a}	0.72 ± 0.02^{c}	3.7 ± 0.04^{a}
16	2.78 ± 0.03^{a}	3.0 ± 0.17^{a}	1.2 ± 0.02^{b}
Female *Sesarma huzardi* Activity expressed as CAT µmol/min/mg protein			
Incubation Time (day)	**Control**	**8.15 ml/l**	**81.5 ml/l**
0	1.0 ± 0.06^{c}	1.0 ± 0.06^{b}	1.0 ± 0.06^{c}
4	0.5 ± 0.01^{c}	0.72 ± 0.02^{c}	3.7 ± 0.01^{a}
8	2.78 ± 0.03^{a}	0.69 ± 0.02^{c}	3.68 ± 0.03^{a}
16	2.78 ± 0.03^{a}	3 ± 0.06^{a}	1.19 ± 0.06^{b}

Mean ± SD; n =3 (mean and SD are calculated from three measurements, considering three pooled samples at each concentration. Means not sharing the same superscript (a, b, c or d) in each column are significantly different ($P < 0.05$, Duncan).

For crabs, the mean background value of 1.0 CAT µmol/min/mg protein activity level was same in both male and female *S. huzardi*. Also, catalase activities in the female *S. huzardi* followed a similar trend to the one observed in the male, with almost the same values as shown in **Table 17**. However, responses differed among the treatment groups.

In control crabs, CAT activity was initially depressed on day 4. Depression in CAT activity level on day 4 was also observed in crabs exposed to 0.01 96hLC_{50} drill cuttings concentration, which continued to day 8 in this exposure group. This response was contrary to the increase in CAT activity levels observed in similar treatments for periwinkles on day 4. Increase in CAT activity level was observed on day 8 in control *S. huzardi* and on day 16 in *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentration. Regression analysis between catalase activity level and exposure period for *S. huzardi* was positively correlated (b = 0.792 for both control male and female *S. huzardi*, b = 0.822 for male crabs exposed to 0.01 96hLC_{50} drill cuttings concentration, b = 0.817 for female crabs exposed to 0.01 96hLC_{50} drill cuttings concentration).

In 0.1 96hLC_{50} drill cuttings concentration exposed *S. huzardi* on the other hand, CAT activity level was significantly elevated on day 4 and 8. However, on day 16 CAT activity level decreased to values very close to background values, while the values were still relatively higher in control and 0.01 96hLC_{50} drill cuttings concentration exposed crabs during the same period. Regression analysis showed that correlation between CAT activity level and exposure period was weakly negatively correlated (b = -0.118 for male crabs and 0.-121 for female crabs exposed to 0.1 96hLC_{50} drill cuttings concentration).

The mean CAT levels observed for male was 1.76 CAT µmol/min/mg protein in control, 1.35 CAT µmol/min/mg protein in male crab exposed to 0.01 96hLC$_{50}$ drill cuttings concentration and 2.39 CAT µmol/min/mg protein in 0.1 96hLC$_{50}$ drill cuttings concentration exposed male crabs. Pearson correlation revealed no significant ($P > 0.05$) correlation between male control crabs and male crabs exposed to 0.01 96hLC$_{50}$ drill cuttings concentration ($r = 0.537$, p-value = 0.072) as well as between control and male crabs exposed to 0.1 96hLC$_{50}$ drill cuttings concentration ($r = -0.081$, p-value = 0.803. However, there was significant correlation ($P < 0.05$) between the two male crab groups exposed to drill cuttings ($r = -0.609$, p-value = 0.0036).

In female crabs, the mean CAT activity observed during the study was 1.76 CAT µmol/min/mg protein in female control, 1.34 CAT µmol/min/mg protein in female crab exposed to 0.01 96hLC$_{50}$ drill cuttings concentration and 2.40 CAT µmol/min/mg protein in female crab exposed to 0.1 96hLC$_{50}$ drill cuttings concentration. As was observed in male crab, Pearson correlation revealed no significant correlation ($P > 0.05$) in CAT activity between control group and each female group exposed drill cuttings ($r = 0.522$, p-value = 0.082 for female control *S. huzardi* and female *S. huzardi* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, and $r = -0.088$, p-value = 0.786 for female control crab and 0.1 96hLC$_{50}$ drill cuttings concentration exposed female *S. huzardi*). Significant correlation ($P < 0.05$), however, existed between the two drill cuttings exposed groups ($r = -0.638$, p-value = 0.026).

Analysis of variance revealed significant difference ($P < 0.05$) in all treatment groups Post hoc test using Duncan showed that in both control male and female *S. huzardi* that there was significant difference ($P < 0.05$) on all the sampling days compared to background value, however no statistical difference ($P > 0.05$) was observed in CAT activity between day 8 and 16 for male and female crabs. In drill cuttings exposed crabs, post hoc test Duncan indicated that the background values was significantly difference ($P < 0.001$) from the values obtained on other sampling days, however there was no significant difference ($P > 0.05$) in CAT activity between day 4 and day 8.

Difference relating to drill cuttings exposure was also observed as indicated by the result of ANOVA which showed significant difference ($P < 0.001$) among control and the drill cuttings exposed groups. T-test was used to investigate influence of sex on CAT activity and revealed that CAT was not significantly different ($P > 0.05$) between male and female crabs at all exposure concentrations.

4.7.2 Superoxide dismutase in male and female *S. huzardi* exposed to sub-lethal concentrations of drill cuttings

The result of superoxide dismutase (SOD) activity in the crabs measured over the experimental period of 16 days is presented in **Table 18**. The background level of the enzyme as well as the pattern of fluctuation of the enzyme was similar in both male and female crabs. For any treatment however, male crabs have slightly higher values than female on all the sampling days.

The average background SOD activity of 5.26 SOD µmol/min/mg protein fell significantly in all groups on day 4 and on subsequent days, and the fall was negatively correlated with time. In control, the correlation coefficient was -0.795, in organisms exposed to 8.15 ml/l correlation coefficient was -0.842 for male and -0.837 for female, and in 81.5 ml/l exposed organisms correlation coefficient was -0.916 for male and -0.907 for female. Pearson correlation revealed that correlation (r = 0. 988, p-value = 0.000 both for male and female control *S. huzardi* and *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentration, and male r = -0.972, p-value = 0.000, female r = 0.977, p-value = 0.000 between control crab and 0.1 96hLC_{50} drill cuttings concentration exposed *S. huzardi*) was positive and highly significant ($P < 0.01$) for both male and female crabs. Correlation was also highly significant ($P < 0.01$) between the two groups exposed to drill cuttings for both male and female *S. huzardi* (r = 0.984, p-value = 0.000 for male, and r = 0.987, p-value = 0.000 for female).

The mean value observed during the study was 3.01 SOD µmol/min/mg protein in male control, 2.72 SOD µmol/min/mg protein in male crabs exposed to 0.01 96hLC_{50} drill cuttings concentration and 2.78 SOD µmol/min/mg protein in male crabs exposed to 0.1 96hLC_{50} drill cuttings concentration. In female crabs the mean SOD activity was 2.99 SOD µmol/min/mg protein in female control crabs, 2.71 SOD µmol/min/mg protein in male crabs exposed to 0.01 96hLC_{50} drill cuttings concentration and 2.75 SOD µmol/min/mg protein in male crabs exposed to 0.1 96hLC_{50} drill cuttings concentration.

Analysis of variance revealed statistical difference in SOD activity for all treatment while post hoc tests with Duncan showed that the difference was significant on all sampling day

for all treatment. The findings hold true for both male and female crabs. ANOVA also confirmed significant difference ($P < 0.001$) among control crab and crabs exposed to drill cuttings. Unpaired Sample t-test also indicated that sexually related difference in SOD induction was also absent at all exposure concentrations ($P > 0.05$).

Table 18: Superoxide (SOD) activity of *S. huzardi* on Exposure to Sublethal Drill Cuttings Concentration Expressed as USOD/mg protein (mean ± SD)

Incubation time (day	Control	8.15 ml/l	81.5 ml/l
Male *Sesarma huzardi*			
0	5.26 ± 0.06^{a}	5.26 ± 0.06^{a}	5.26 ± 0.06^{a}
4	2.73 ± 0.03^{b}	2.31 ± 0.01^{b}	2.81 ± 0.03^{b}
8	2.08 ± 0.03^{c}	2.04 ± 0.03^{c}	2.0 ± 0.06^{c}
16	2.0 ± 0.03^{d}	1.29 ± 0.02^{d}	1.04 ± 0.03^{d}
Female Sesarma huzardi			
0	5.26 ± 0.04^{a}	5.26 ± 0.04^{a}	5.26 ± 0.04^{a}
4	2.73 ± 0.03^{b}	2.28 ± 0.03^{b}	2.72 ± 0.00^{b}
8	2.08 ± 0.03^{c}	2.01 ± 0.03^{c}	1.97 ± 0.03^{c}
16	2.0 ± 0.03^{d}	1.29 ± 0.02^{d}	1.04 ± 0.04^{d}

Mean ± SD; n =3 (mean and SD are calculated from three measurements, considering three pooled samples at each concentration. Means not sharing the same superscript (a, b, c or d) in each column are significantly different ($P < 0.05$, Duncan).

4.7.3 Glutathione S-Transferase Activity in Test Species exposed to Sublethal Concentrations of Drill Cuttings

The background level of GST activity was 5.7 GST nmol/min/mg protein (**Table 19**). The mean GST activity detected during the study were 5.70 GST nmol/min/mg protein for control *T. fuscatus*, 5.40 GST nmol/min/mg protein for *T. fuscatus* exposed to 1.024 ml/l and 5.86 in *T. fuscatus* exposed to 10.24 ml/l. GST activity in *T. fuscatus* was

comparatively constant in the control throughout the experimental period. Appreciable increase was observed on day 4 only in the group exposed to 10.24 ml/l, but this was followed by a fall in GST values on subsequent sampling days **(Table 19)**.

Regression analysis between GST activity level and exposure time showed a positive correlation for control *T. fuscatus* (b = 0.722 for control *T. fuscatus)* and weak correlation for drill cuttings exposed *T. fuscatus* (b = 0.015 for *T. fuscatus* exposed to 0.01 96hLC_{50} drill cuttings concentration and b = -0.474 for 0.1 96hLC_{50} drill cuttings concentration exposed *T. fuscatus*). Pearson correlation revealed that the linear relationship (r = -0.160, p-value = 0.570) between control and *T. fuscatus* exposed to 0.01 96hLC_{50} drill cuttings concentration was not significant ($P > 0.05$), while the linear relationship (r = -0.527, p-value = 0.44) between control and 0.1 96hLC_{50} drill cuttings concentration exposed *T. fuscatus* was significant ($P < 0.05$). Correlation (r = -0.233, p-value = 0.425) was also not significant ($P > 0.05$) between two drill cuttings exposed groups.

Analysis of variance showed that GST activity was not significant ($P > 0.05$) in the control *T. fuscatus*, while significant difference ($P < 0.05$) was observed in both drill cuttings exposed groups. Post hoc test with Duncan revealed that in *T. fuscatus* exposed to 0.01 96hLC_{50} drill cuttings concentration the background value (day 0) was significantly different ($P < 0.01$) from the values observed on other sampling days, while day 4 and day 8 were not significantly different ($P > 0.05$) from each but significantly different from the values observed on other sampling days. The observed values on day 16 and day 32 were also not significantly different ($P > 0.05$) from each other but were significantly different ($P < 0.01$) from the values obtained on other sampling days. In *T.*

fuscatus exposed to 0.1 96hLC$_{50}$ drill cuttings concentration, the background GST value as well as the value obtained on day 4, each was significantly different ($P < 0.001$) from the values obtained on other sampling days. GST activity values obtained on day 8, 16 and day 32 were not significantly different ($P > 0.05$) from each other.

The effect of exposure to drill cuttings on GST activity was tested using ANOVA and revealed that GST activity was significant ($P < 0.001$) among control and the drill cuttings exposed *T. fuscatus*.

The background GST activity in *P. aurita* was similar to that of *T. fuscatus* (Table 19). Mean GST activity detected during the study was 5.71 GST nmol/min/mg protein in control *P. aurita*, 5.40 GST nmol/min/mg protein in *P. aurita* exposed to 1.22 ml/l drill cuttings concentration and 6.20 GST nmol/min/mg protein in *P. aurita* exposed to 12.22 ml/l drill cuttings concentration. The response of the organisms for each exposure concentration was also generally identical to that observed in *T. fuscatus* over the experimental time. The only difference in response observed in the two periwinkle species was the second rise above background value observed in 0.1 96hLC$_{50}$ drill cuttings concentration exposed *P. aurita* on day 16, which was subsequently followed by a fall on day 32.

Correlation between GST activity level and exposure time was positive for control *P. aurita* ($b = 0.707$ for control *P. aurita*), almost absent in *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration ($b = 0.008$) and weakly negative in 0.1 96hLC$_{50}$ drill cuttings concentration exposed *P. aurita* ($b = -0.286$). Pearson correlation revealed significant correlation ($P < 0.05$) between control and *P. aurita* exposed to 0.01 96hLC$_{50}$

drill cuttings concentration (r = -0.615, p-value = 0.015) but no significant (P > 0.05) was observed between control and 0.1 96hLC$_{50}$ drill cuttings concentration exposed *P. aurita* (r = -0.060, p-value = 0.833). Correlation (r = -0.259, p-value = 0.31) between the two exposed groups was also not significant (p > 0.05).

Analysis of variance showed that there was no significant difference (P > 0.05) in control *P. aurita*, while significant difference (P < 0.05) was observed in *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration. High significant difference was also observed in 0.1 96hLC$_{50}$ drill cuttings concentration exposed *P. aurita*. Post hoc analysis with Duncan revealed that in *P. aurita* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration, GST activity observed on day 0 was significantly different (P < 0.05) from the values obtained on other sampling days, while GST activity on day 4, 8, 16 and 32 was not significantly different (P > 0.05) from each other. In *P. aurita* exposed 0.1 96hLC$_{50}$ drill cuttings concentration background GST activity was not significantly different (P > 0.05) from the value obtained on day 32, but was significantly different (Duncan, P < 0.001) from the values obtained on other sampling days. The values obtained on day 4, day 8 and day 16, each was significantly different (P < 0.05) from the value observed on any other sampling day.

GST activity was significantly different (ANOVA, P < 0.001) among control *P. aurita* and *P. aurita* exposed to drill cuttings.

Comparison of GST activity between *T. fuscatus* and *P. aurita* revealed no significant difference (P > 0.05) in their response at all treatment (Unpaired t-test, P > 0.05).

Table 19: Glutathione *S*-Transferase Activity in the Test Species on Exposure to Sublethal Drill Cuttings Concentration Expressed as nmol/min/mg protein (mean ± SD)

GST Activity in T. fuscatus Activity expressed as nmol/min/mg protein			
Incubation Time (day)	**Control**	**1.02 ml/l**	**10.24 ml/l**
0	5.7 ± 0.1^{a}	5.7 ± 0.15^{a}	5.7 ± 0.15^{b}
4	5.7 ± 0.06^{a}	5.2 ± 0.25^{c}	8.57 ± 0.07^{a}
8	5.72 ± 0.01^{a}	5.22 ± 0.03^{c}	5.02 ± 0.03^{c}
16	5.72 ± 0.00^{a}	5.31 ± 0.02^{b}	5.0 ± 0.10^{c}
32	5.72 ± 0.00^{a}	5.49 ± 0.00^{b}	5.0 ± 0.10^{c}
GST Activity in P. aurita Activity expressed as nmol/min/mg protein			
Incubation Time (day)	**Control**	**1.22 ml/l**	**12.22 ml/l**
0	5.7 ± 0.06^{a}	5.7 ± 0.20^{a}	5.7 ± 0.20^{c}
4	5.71 ± 0.00^{a}	5.2 ± 0.25^{b}	8.52 ± 0.00^{a}
8	5.72 ± 0.01^{a}	5.21 ± 0.04^{b}	5.11 ± 0.00^{d}
16	5.72 ± 0.00^{a}	5.38 ± 0.10^{b}	6.02 ± 0.00^{b}
32	5.72 ± 0.00^{a}	5.47 ± 0.00^{b}	5.68 ± 0.02^{c}
GST Activity in Male *Sesarma huzardi* Activity expressed as nmol/min/mg protein			
Incubation Time (day)	**Control**	**8.15 ml/l**	**81.5 ml/l**
0	0.015 ± 0.00^{c}	0.015 ± 0.00^{d}	0.015 ± 0.00^{b}
4	0.025 ± 0.00^{b}	0.041 ± 0.00^{a}	0.027 ± 0.00^{a}
8	0.027 ± 0.00^{a}	0.03 ± 0.00^{b}	0.027 ± 0.00^{a}
16	0.027 ± 0.00^{a}	0.025 ± 0.00^{c}	0.027 ± 0.00^{a}
GST Activity Female *Sesarma huzardi* Activity expressed as nmol/min/mg protein			
Incubation Time (day)	**Control**	**8.15 ml/l**	**81.5 ml/l**
0	0.015 ± 0.00^{b}	0.015 ± 0.00^{d}	0.015 ± 0.00^{b}
4	0.022 ± 0.00^{a}	0.039 ± 0.00^{a}	0.026 ± 0.00^{a}
8	0.027 ± 0.00^{a}	0.03 ± 0.00^{b}	0.025 ± 0.00^{a}
16	0.027 ± 0.00^{a}	0.025 ± 0.00^{c}	0.025 ± 0.00^{a}

Mean ± SD; n =3 (mean and SD are calculated from three measurements, considering three pooled samples at each concentration. Means not sharing the same superscript (a, b, c or d) in each column are significantly different ($P < 0.05$, Duncan).

In crabs, the background 0.015 GST nmol/min/mg protein levels increased in all treatment groups including control by the end of the experimental period as shown in **Table 19**. In both male and female crabs, GST concentrations were higher than the background level in all groups during the study. For any treatment including controls, GST values were practically the same for male and female crabs, with exception of few cases where the value of the male was marginally higher than that of the female. The highest GST level was in the group exposed to 8.15 ml/l on day 4 **(Table 19)**. Also, GST concentration tend to stabilise in control organisms and in organisms exposed to 8.15 ml/l over the experimental period, but continued to fluctuate in 8.15 ml/l exposed organisms to the end of the exposure period.

The mean GST activity observed during the study was 0.0235 GST nmol/min/mg protein in male control, 0.0278 GST nmol/min/mg protein in male exposed to 8.15 ml/l drill cuttings exposure and 0.0240 in male crabs exposed to 81.5 ml/l. Pearson correlation revealed significant difference ($P < 0.05$) between male control crab and male crab exposed to 0.01 $96hLC_{50}$ drill cuttings concentration ($r = 0.666$, p-value = 0.018), while high significant correlation ($P < 0.01$) was observed between male control crabs and 0.1 $96hLC_{50}$ drill cuttings concentration exposed male crabs ($r = 0.952$, p-value = 0.000) as well as between the two exposed male groups ($r = 0.780$, p-value = 0.003).

In female crabs, the mean GST activity observed was 0.0242 GST nmol/min/mg protein in female control, 0.0273 GST nmol/min/mg protein in female crabs exposed to 8.15 ml/l and 0.0228 GST nmol/min/mg protein in female crabs exposed to 81.5 ml/l. Pearson

correlation was highly significant ($P < 0.01$) between control and each of the drill cuttings exposed female *S. huzardi* group ($r = 0.822$, p-value = 0.001 between female control crab and female crab exposed to 0.01 96hLC_{50} drill cuttings concentration, $r = 0.844$, p-value = 0.001 between female control crabs and 0.1 96hLC_{50} drill cuttings concentration exposed female crabs). The correlation ($r = 0.548$, p-value = 0.065) between the two exposed female groups was not significant ($P > 0.05$).

There was significant difference ($P < 0.01$) in all groups during the study in both male and female crabs. In male control crab, post hoc test using Duncan indicated that the background value as well as the value on day 4, each was significantly different ($P < 0.001$) from the value observed on other sampling days. In female control crabs, while the background value was significantly different ($P < 0.001$) from the value observed on subsequent sampling days, the values obtained on day 4, day 8 and day 16 were not significantly different ($P > 0.05$) from one another.

In both male and female crabs exposed to 0.01 96hLC_{50} drill cuttings concentration, the GST activity was significantly different on all sampling day ($P < 0.001$). In 0.1 96hLC_{50} drill cuttings concentration exposed crabs, the background value was significantly different ($P < 0.05$) from the values obtained on other sampling day, while the values obtained on day 4, day 8 and day 16 values were not significantly different from each other ($P > 0.05$) for both male and female crabs.

Analysis of variance showed that there was significant difference ($P < 0.01$) in GST activity among control and the drill cuttings treated group in both male and female crabs. However, post hoc test with Duncan revealed that the values obtained in control and crabs exposed to 0.1 $96hLC_{50}$ drill cuttings concentration were not significantly different ($P > 0.05$) during the study, but each was significantly different from the value obtained in 0.01 $96hLC_{50}$ drill cuttings concentration exposed crabs for both male and female crabs. Sex related difference in GST activity was not observed during the study at all exposure concentration between male and female *S. huzardi* (Unpaired t-test, $P > 0.05$).

4.7.4 Lipid Peroxidation Activity in Test Species exposed to Sublethal Concentrations of Drill Cuttings

Lipid peroxidation (LPO) was monitored in all test species over a period of 16 days on day 0, 4, 8 and 16. By the end of 16 days, lipid peroxidation values in the periwinkles were lower than background values in all groups. The values however were higher in the crab in all groups including control by the end of 16 day experimental period.

In *T. fuscatus*, Lipid peroxidation was lower than the background level in all treatment groups including control at the end of experimental 16 day period. Highest rate was observed on day 4 in the group exposed to 10.24 ml/l. Paradoxically, the lowest recorded value was also in the group exposed to 10.24 ml/l on day 16 (Table 20). In control, lipid peroxidation rate fell on day 4 and day 8 and thereafter stabilised. Correlation between lipid peroxidation activity level and exposure time was strongly negative for control *T. fuscatus* ($b = -0.877$ for control).

Both groups exposed to 1.024 ml/l and those exposed to 10.24 ml/l exhibited the same trend in the rate of lipid peroxidation. In both exposed organisms, an increase in the rate of oxidation was observed on day 4, which was followed by a reduction in the rate of peroxidation on subsequent sampling days. By the end of the 16 day exposure period, the rate of lipid peroxidation was lower than the background rate in all groups. In drill cuttings exposed groups, lipid peroxidation activity was also negatively correlated with time (b = -0.835 for *T. fuscatus* exposed to 0.01 96hLC_{50} drill cuttings concentration, and b = -0.586 for 0.1 96hLC_{50} drill cuttings concentration exposed *T. fuscatus*).

The background lipid peroxidation activity in *T. fuscatus* was 2.0 LPO nmol/min/mg protein. The mean observed during the study in *T. fuscatus* was 1.568 LPO nmol/min/mg protein in control, 1.592 in *T. fuscatus* exposed to 1.024 ml/l drill cuttings and 2.90 LPO nmol/min/mg protein in *T. fuscatus* exposed to 12.22 ml/l drill cuttings. Pearson correlation was highly significant ($P < 0.01$) between control *T. fuscatus* and *T. fuscatus* exposed to 0.01 96hLC_{50} drill cuttings concentration ($r = 0.917$, p-value = 0.000). Signification correlation ($P < 0.05$) was also observed between control *T. fuscatus* and 0.1 96hLC_{50} drill cuttings concentration exposed *T. fuscatus* ($r = 0.626$, p-value = 0.029), while high significant correlation ($P < 0.01$) existed between the two drill cuttings exposed *T. fuscatus* groups ($r = 0.876$, p-value = 0.000).

Analysis of variance revealed statistical difference ($P < 0.05$) in lipid peroxidation during the study in all treatment groups, while post hoc test with Duncan showed that in control *T. fuscatus* and *T. fuscatus* exposed to 0.01 96hLC_{50} drill cuttings concentration, the background LPO activity and LPO activity observed on day 4 were each significantly

different from the values obtained on other sampling days ($P < 0.001$), while LPO activity on day 16 and 8 were not significantly different ($P > 0.05$) from each other. In 0.1 $96hLC_{50}$ drill cuttings concentration exposed *T. fuscatus* on the other hand LPO activity was significant ($P < 0.001$) on all the sampling days.

Significant difference (ANOVA, $P < 0.001$) was also observed among control *T. fuscatus* and *T. fuscatus* exposed to drill cuttings. However, LPO activity in control was not significantly different ($P > 0.05$) from LPO activity observed in 0.01 $96hLC_{50}$ drill cuttings concentration exposed *T. fuscatus* during the study, while LPO activity observed in *T. fuscatus* exposed to 0.1 $96hLC_{50}$ drill cuttings concentration was significantly different (Duncan, $P < 0.001$) from LPO activity observed in other exposure concentrations.

The rates and trend of lipid peroxidation in *P. aurita* during the experimental period were similar to those of *T. fuscatus*. The values observed at the end of experimental period were lower than the 2.0 LPO nmol/min/mg protein observed background values in all treatment groups. Also, the highest rate was observed in the group exposed to 12.22 ml/l on day 4 **(Table 20)**.

In control the rate of lipid peroxidation was negatively correlated with time ($b = -0.994$ for control *P. aurita*). Their values were similar to those observed in the control group of *T. fuscatus*, but for day 8 when the observed lipid peroxidation activity was higher than the 1.24 LPO nmol/min/mg protein observed in *T. fuscatus*. As was observed in *T. fuscatus*, there was initially an increase in the rate of lipid peroxidation in drill cuttings

exposed organisms on day 4, followed by a fall on subsequent sampling days (b = -0.865 for *P. aurita* exposed to 0.01 96hLC_{50} drill cuttings concentrations , and -0.597 for 0.01 96hLC_{50} drill cuttings concentrations exposed *P. aurita*). By the end of exposure period, the rate of peroxidation was below the background level in both exposed groups.

The mean observed values in *P. aurita* was 1.685 LPO nmol/min/mg protein in control, 1.681 LPO nmol/min/mg protein in *P. aurita* exposed to 1.22 ml/l and 2.085 LPO nmol/min/mg protein in 12.22 ml/l drill cuttings exposed *P. aurita*. Pearson correlation was highly significant ($P < 0.01$) between control *P. aurita* and *P. aurita* exposed to 0.01 96hLC_{50} drill cuttings concentration ($r = 0.803$, p-value = 0.0003). No signification correlation ($P > 0.05$) was observed between control *T. fuscatus* and 0.1 96hLC_{50} drill cuttings concentration exposed *P. aurita* ($r = 0.533$, p-value = 0.074), while high significant correlation ($P < 0.01$) existed between the two drill cuttings exposed *P. aurita* groups ($r = 0.901$, p-value = 0.000).

ANOVA revealed significant difference ($P < 0.05$) in LPO activity in all the treatments for *P. aurita*. Post hoc tests with Duncan showed that in control *P. aurita*, LPO activity on day 0 and day 16 each was significantly different from LPO activity on other sampling days, while LPO activity on day 4 was not significantly different ($P > 0.05$) to that observed on day 8. In drill cuttings exposed *P. aurita*, LPO activity was significantly different on each sampling day (Duncan, $P < 0.001$).

Significant difference (ANOVA, $P < 0.001$) was also observed among control *P. aurita* and *P. aurita* exposed to drill cuttings. However, LPO activity in control was not

significantly different ($P > 0.05$) from LPO activity observed in 0.01 96hLC$_{50}$ drill cuttings concentration exposed *T. fuscatus* during the study, while LPO activity observed in *T. fuscatus* exposed to 0.1 96hLC$_{50}$ drill cuttings concentration was significantly different (Duncan, $P < 0.001$) from LPO activity observed in other exposure concentrations.

Table 20: Lipid Peroxidation Activity in Test Species expressed as nmol/min/mg protein (mean ± SD)

T. fuscatus			
Incubation Time (day)	**Control**	**1.02 ml/l**	**10.24 ml/l**
0	2.0 ± 0.20^{a}	2.0 ± 0.20^{b}	2.0 ± 0.20^{b}
4	1.79 ± 0.00^{b}	2.25 ± 0.7^{a}	3.92 ± 0.07^{a}
8	1.24 ± 0.05^{c}	1.14 ± 0.05^{c}	1.47 ± 0.05^{c}
16	1.24 ± 0.02^{c}	1.0 ± 0.05^{c}	0.98 ± 0.13^{d}
P. aurita			
Incubation Time (day)	**Control**	**1.22 ml/l**	**12.22 ml/l**
0	2.0 ± 0.15^{a}	2.0 ± 0.15^{b}	2.0 ± 0.15^{b}
4	1.79 ± 0.0^{b}	2.3 ± 0.15^{a}	3.78 ± 0.03^{a}
8	1.68 ± 0.00^{b}	1.35 ± 0.05^{c}	1.52 ± 0.04^{c}
16	1.24 ± 0.08^{c}	1.01 ± 0.00^{d}	1.01 ± 0.00^{d}
Male *Sesarma huzardi*			
Incubation Time (day)	**Control**	**8.15 ml/l**	**81.5 ml/l**
0	10.0 ± 0.00^{b}	10.0 ± 0.00^{c}	10.0 ± 0.00^{d}
4	15.0 ± 2.54^{a}	15.2 ± 2.65^{a}	11.1 ± 1.90^{c}
8	15.0 ± 2.17^{a}	15.0 ± 2.10^{a}	18.8 ± 2.26^{a}
16	15.0 ± 2.45^{a}	11.3 ± 2.06^{b}	15.4 ± 2.29^{b}
Female *Sesarma huzardi*			
Incubation Time (day)	**Control**	**8.15 ml/l**	**81.5 ml/l**
0	10.0 ± 0.00^{b}	10.0 ± 0.00^{d}	10.0 ± 0.00^{d}
4	15 ± 0.85^{a}	15.2 ± 0.35^{a}	11.6 ± 0.85^{c}
8	15 ± 0.11^{a}	12.9 ± 0.35^{b}	18.2 ± 0.15^{a}
16	15 ± 0.15^{a}	10.87 ± 0.22^{c}	15.5 ± 0.30^{b}

Mean ± SD; n =3 (mean and SD are calculated from three measurements, considering three pooled samples at each concentration. Means not sharing the same superscript (a, b, c or d) in each column are significantly different ($P < 0.05$, Duncan).

Lipid peroxidation activities in *S. huzardi* were similar in both male and female crabs for any treatment group. At the end of experimental period of 16 days, rate of lipid peroxidation in all groups were higher than background level. The highest value recorded was in the group exposed to 81.5 ml/l on day 8 **(Table 20)**. LPO increased above the background 10.0 LPO nmol/min/mg protein on day 4 in all groups, but the rate was lowest in organisms exposed to 81.5 ml/l on day 8 compared to other groups during this period.

No further change in LPO was observed in control organisms on subsequent sampling days. Correlation between lipid peroxidation rate and exposure period was positive for control (b = 0.683 for both male and female control *S. huzardi*). In organisms exposed to 8.15 ml/l, the initial rise in lipid peroxidation level was followed by reduction in the rate of lipid peroxidation on subsequent sampling days. By 16 day, the rate was close to the background rate.

Regression analysis revealed a weak correlation between lipid peroxidation activity level and exposure period for crabs exposed to 8.15 ml/l (b = 0.020 for male and -0.103 for female exposed to 0.01 96hLC_{50} drill cuttings concentration). Increase in the rate of LPO activity continued to day 8 in 81.5 ml/l exposed crabs, with a subsequent fall in activity on day 16 to a value comparable to that of the control for the same period. The rate of activity was similar in both male and female crabs in this treatment group and regression analysis show a positive correlation between LPO and exposure time (b = 0.651 for male and 0.694 for female exposed to 0.1 96hLC_{50} drill cuttings concentration).

The mean observed value in lipid peroxidation observed in male crab was 13.76 LPO nmol/min/mg protein in male control crab, 12.88 LPO nmol/min/mg protein in male crab exposed to 8.15 ml/l and 13.43 LPO nmol/min/mg protein in 81.5 ml/l drill cuttings exposed male crabs. The mean LPO activity observed for female crab included 13.74 LPO nmol/min/mg protein in female control crab, 12.21 LPO nmol/min/mg protein in female crab exposed to 8.15 ml/l and 13.80 LPO nmol/min/mg protein in 81.5 ml/l drill cuttings exposed crabs.

Pearson correlation revealed that in male *S. huzardi* a high significant correlation ($P < 0.01$) in LPO activity existed between male control crabs and male crabs exposed to 0.01 $96hLC_{50}$ drill cuttings concentration ($r = 0.718$, p-value = 0.009), while there was a $P < 0.05$ significant level between male control crabs and 0.1 $96hLC_{50}$ drill cuttings concentration exposed male crabs ($r = 0.618$, p-value = 0.032). However, no significant correlation ($P > 0.05$) existed between the two drill cuttings groups exposed male crabs ($r = 0.402$, p-value = 0.195). In female crabs, significant correlation ($P < 0.05$) in LPO activity was observed between control and each of the drill cuttings exposed groups ($r = 0.641$, p-value = 0.025 between control female crabs and female crab exposed to 0.01 $96hLC_{50}$ drill cuttings concentration, and $r = 0.702$, p-value = 0.011 between control female crabs and 0.1 $96hLC_{50}$ drill cuttings concentration exposed female crabs). No significant correlation in LPO activity ($P > 0.05$) existed between the two drill cuttings groups exposed female crabs ($r = 0.108$, p-value = 0.737).

Analysis of variance showed that there were significant differences ($P < 0.001$) in LPO in all the treatment during the study in both male and female *S. luzardi*. Post hoc test using Duncan revealed that in control of male and female crabs, LPO activity on day 0 was significantly different ($P < 0.001$) from the values observed on other sampling days. while LPO activity on day 4, day 8 and day 16 were not significantly different ($P > 0.05$) among each other.

In male crabs exposed to 0.01 96hLC_{50} drill cuttings concentration LPO activity on day 0 and day 16, each was significantly different (Duncan, $P < 0.001$) from LPO activity on other sampling days, while LPO activity 0n day 4 and day 8 were not significantly different ($P > 0.05$) from each other. In female crabs exposed to 0.01 96hLC_{50} drill cuttings concentration on the other hand LPO activity was significantly different (Duncan, $P < 0.001$) on all sampling days.

In both male and female crabs exposed to 0.1 96hLC_{50} drill cuttings concentration, Duncan test indicated that LPO activity was significantly different ($P < 0.05$) on all sampling days.

Analysis of variance also indicated that there was significant difference ($P < 0.01$) in LPO activity among control and drill cuttings exposed crabs both for male and female crabs. Post hoc Duncan test indicated that in male crab, LPO activity observed during the study in control male crabs and male crabs exposed to 0.1 96hLC_{50} drill cuttings was not significant ($P > 0.05$), while LPO activity observed during the study in male crabs

exposed to 0.01 96hLC_{50} drill cuttings concentration was significantly different from all other exposure concentrations. In female crabs on the other hand, LPO activity observed in control was significantly different from that observed in drill cuttings exposed treatments, while there was no significant different ($P > 0.05$) observed during the study in the two drill cuttings exposed treatments in female crabs.

Unpaired t-test showed no significant difference ($P > 0.05$) in LPO activity between control male crab and control female crab as well as between male and female crabs exposed t0 0.01 96hLC_{50} drill cuttings concentrations, while significant difference was observed between male and female crabs exposed to 0.1 96hLC_{50} drill cuttings concentrations.

5.0 DISCUSSION

5.1 ACUTE TOXICITY TEST

In this study, the LC_{50} value based on the 96hrs value of drill cuttings was 102.43 when tested against *T. fuscatus*. The drill cuttings was significantly more toxic to each of the test species when exposed for 96hr than at each other exposure periods (24hr, 48hr, 72hr) as was indicated by the increase in toxicity factor which increased with exposure time. For instance it was found that the cuttings used was 37.2 times more toxic to *T. fuscatus* at 96hrs ($96hLC_{50}$ = 102.43 ml/l) of exposure than 24hrs ($24hLC_{50}$ 3808.80 ml/l); 16.8 times more toxic to *P. aurita* when acting at 96h ($96hLC_{50}$ = 122.15 ml/l) of exposure than 24hrs ($24hLC_{50}$ 205.93 ml/l) and 1.07 times more toxic to *S. huzardi* when acting at 96hours ($96hLC_{50}$ = 814.72 ml/l) of exposure than at 48 hours ($48hLC_{50}$ = 871.42) of exposure. No mortality was recorded at 24 hours in *S. huzardi*.

Results of toxicity tests are of interest to environmental scientists because of their use as basis for ecological prediction, though emphasis has shifted to the validation of laboratory results in field studies (Reid and MacFarlane, 2003). They also provide information on which are derived water quality guidelines for the large numbers of chemicals currently in use. Consequently different investigators have employed different sentinel species in investigating the toxicity of different chemicals and pollutants including heavy metals (Ferrer *et al.*, 2006; Tsiridis *et al.*, 2006; Otitoloju, 2002; 2003; Udoidiong and Akpan 1991) and organic compounds (Chukwu, 2006a; 2006b; Chukwu and Okeowo 2006; Chukwu and Ogunmodede 2005; Chindah *et al.*, 2004; Reid and MacFarlane (2003); Teh *et al.*, 2003; Udoidiong and Akpan (1991)). Chapman (2002)

has discussed considerations for choosing test species for both laboratory and field studies.

Acute and sublethal toxicity measure the effect on individual organisms, and when performed in laboratory lacks ecological realism because of the entirely different conditions that obtain in the field, hence the development of micro- and mesocosms (Millward *et al.* (2001) by researchers in an attempt to simulate the natural environment. Even here, it is suggested that data obtained should be applied with caution (Deplegde and Hopkin (1995). Interactions between pollutants (Chukwu 2006c; Chukwu and Odunzeh 2006; Otitoloju, 2002; 2003; Amiard *et al.*, 2004; Tsiridis *et al*, 2006) and organisms (Chapman *et al.*, 1982, Mitchelmore *et al.*, 2003) are very critical, as the actual exposures of organisms may be quite more complicated than are indicated by total concentrations of pollutants present in their environment (Tsiridis *et al*, 2006). However investigators have continued to study acute toxicities because they often provide information which cannot be obtained from the field. It has also been pointed out that dose is a more important consideration than concentration as the predictor of effect (Moriarty, 1988; Chapman, 2002), as this reflects the amount of the pollutant that is bioavailable to the organisms, especially since the rate of intake does not necessarily follow exposure to pollutants. Environmental factors such as salinity may also act to modify the toxicity of chemicals to organisms (Enajekpo, 2000).

The present study used a composite toxicant, and there is no previous local data to compare results with. Direct comparisons of work done using organisms elsewhere

however cannot be made due to different units of measurements. Newton and Douglas McKenzie (1998) reported an LC_{50} value of 52,800 ppm total hydrocarbons in test sediment for the brittlestars (*Amphiura chiajei and A. filiformis*) exposed to drill cuttings containing oil-based muds. 96hLC_{50} values ranging from 30740 and 78271 ppm suspended particulate phase (SPP) has also been reported for the toger prawn *Penaeus monodon* exposed to used drilling mud (Soegianto *et al.*, 2008).

Nguyen (1998, 1999) as reviewed by Roddie *et al.* (1999) had reported LC_{50} of drill cuttings to the marine amphipod *Corophium* of 0.1 % - 0.33 % in sediment, and 0.03 % - 3 % in the water overlying the sediment. The author suggested that the toxicity can be attributed by the total hydrocarbon concentrations and that adverse effects were observed at THC concentrations of 43-62 mgg^{-1}. This THC concentration is much higher than the value (25.14 μgg^{-1}) observed in the cuttings used in the present study. Grant and Briggs (2002) had also suggested that hydrocarbon was more important than other chemical constituents as the cause of toxicity of OBM contaminated sediments. According to the authors, 3 % contaminated sediment was acutely toxic to *Corophium volutator*, while feeding was almost completely inhibited on exposure of the polychaete *Arenicola marina* to a 10 % contaminated sediment. Though sediments did not inhibit Microtox light output, organics extracted using dichloromethane were very toxic, with 15minEC50 values of 0.25 mg/ml.

On the other hand Neustadt *et al.* (1995) have suggested that toxicity of drill cuttings can also be attributed to other organic constituents more soluble than hydrocarbon. The

authors demonstrated using Microtox test that while extraction with sea water gave only 1 % depression in luminescence of the bacterium *Photobacterium phosphoreum*, extraction with 1% dimethylsulphoxide in sea water and with 1% ethanol in sea water gave a depression in luminescence that was more than 50 %.

The present study did not set out to investigate the chemical constituent that can be attributed the cause of the toxicity of the drill cuttings, but the toxicity of the whole cuttings to the test species, which is expected to be a sum total of the various interactions of the drill cuttings constituents.

The evaluation of toxicological response and the relative sensitivity of the tropical estuarine macroinvertebrates test organisms based on 96hLC_{50} values showed sensitivity to drill cuttings as follows: *T. fuscatus* > *P. aurita* > *S. huzardi*, with ratio of 1:1.19:7.95 respectively. Earlier study by Otitoloju (2003) had shown that *T. fuscatus* was more sensitive to mixtures of heavy metals than *S. huzardi*.

Sensitivity to pollutants are affected by factors relating to the permeability of body membranes or cuticles, metabolism, excretory capacity, sex, age, body size, site of action and behaviour (Don Pedro, 1996). Ability of organisms to metabolise and excrete exogenous compounds is also a factor that affects susceptibility/tolerance to toxicant exposure. Teh *et al.* (2003) reported that age is a factor that affects the sensitivity of white sturgeon *Acipenser transmontanus* to didecyldimethylammonium chloride. Ferrer *et al* (2006) reported that first zoeae stages of the crab *C. granulata* were more sensitive

than young adult of the same species to acute exposure of heavy metals. The present study employed adult stages of the test species. Factors relating to mobility may have played a role in the higher tolerance of the crab compared to the periwinkles. Retraction into shell is a common response of molluscs in the presence of external irritant or stress. For instance, Reid and MacFarlane (2003) reported that adults of the intertidal gastropod *Austrocochlea porcata* are able to avoid contamination by retraction into the shell and sealing of the operculum on exposure to petroleum constituents. Observation of the retraction behaviour of the periwinkles showed that *P. aurita* retracts into its shell to a higher degree than *T. fuscatus*, a factor that probably partly accounted for its higher tolerance to the toxicant.

5.2 BIOACCUMULATION OF HYDROCARBONS AND HEAVY METALS

In this study bioaccumulation of hydrocarbon and heavy metals in the digestive glands of the tests species followed neither a dose- nor a time-dependent pattern. Organisms usually do not respond to surrounding chemicals in their environment but only to those which are associated with the organisms. With organic compounds, it is possible for the organisms to metabolise these if absorbed such that their total concentration decreases or become totally absent in the organism. Metals on the other hand are not degraded but may become bound to various molecules in the organism (Mason and Jenkins, 1995) Route of exposure is very critical, and in large organisms body surfaces become increasingly unimportant as exposure route (Fowler, 1982). In this study, THC was almost absent in the digestive glands of the test organisms. It is possible to attribute the low accumulation of hydrocarbons either to metabolism of hydrocarbon by the organism

and/or to storage of hydrocarbon in some other tissues in the organisms- only the digestive gland was studied in the present study. Stagg and McIntosh (1996) also reported low accumulation of hydrocarbon in the liver and muscles of the dab *Limanda limanda* exposed to drill cuttings, and possible metabolism of hydrocarbon by the fish. Sex and lipid contents are factors which have been reported to affect accumulation of PAH in the copepod *Microarthridion littorale* (Klosterhaus *et al.*, 2002). Previous studies by Ideriah *et al.* (2006) showed a THC concentrations ranging from 764.31 µg/g THC to 1242.19 µg/g THC in the soft tissues of field collected periwinkles.

The present study investigated the accumulation of heavy metals in the digestive glands of the test species, and revealed higher levels of some heavy metals in control organisms compared to drill cuttings exposed organisms in some cases. Chapman (2002) has earlier pointed out that measurable responses to toxicant do not necessarily follow a linear dose dependent manner, and that time and dose dependent responses may actually be the exception rather the rule. Uptake of metal in the digestive glands of the test species was low as indicated by the calculated low bioaccumulation factors. Stagg and McIntosh (1996) had previously reported that metals associated with drill cuttings were not bioavailable to dab *Limanda limanda* as no difference in heavy metal concentration in the liver and muscles of the fish was observed on exposure of the fish to drill cuttings. Metals present in drill cuttings are known to be practically non bioavailabe owing to the barite matrix covering them (Neff *et al.*, 2005), as metal bioaccumulation is greatly influenced by the nature of the metal in the experimental medium (Amiard *et al.*, 2004). The level of heavy metals in the drill cuttings used during this study was low thus, it is logical to

suggest that even the measurable accumulated heavy metals during the study may have been taken up from experimental media owing to the decrease in heavy metal concentrations observed in the sediment. Also, metal partitioning in the different organs may also be a factor for the low concentration of heavy metals in the digestive gland, for instance Leung and Furness (1999) reported that the Lieblein gland contain highest concentration of Cadmium than any of the other organs investigated in the dogwhelk *Nucella lapillus*. Cadmium has also been shown to preferentially accumulate in the kidney of the sea bass *Dicentrarchus labrax*, which correlated with increased Zinc level while Copper uptake was more pronounced in the liver (Roméo *et al.* 2000) than either the kidney or muscles.

Another factor to consider is the different interactions that occur between metals present in a given medium. It is known that metals affect each other in different ways when present according to the particular pairs of metals (Ahsanullah *et al.*, 1981), and this can be antagonistic, additive or synergistic (Otitoloju, 2002). The particular combination of the metals present would therefore, affect uptake of metals from the experimental medium.

Langston and Spence (1995) had pointed out that organisms are able to regulate essential metals but not non-essential ones. Hence the low accumulation of heavy metals recorded in the digestive gland of the test organisms may also be related to the nature of the metals studied namely Iron, Copper and Zinc. These are transitive metals which are biologically important in aquatic organisms and therefore are bio-regulated. Since the test species

were exposed to low levels of these metals, it is possible that these metals were actively regulated by the test organisms.

In the present study, it was found that concentration of the metals Fe, Cu and Zn was non monotonic in the digestive gland of *T. fuscatus* suggesting metal regulation. Similar observation for whole body metal concentration of heavy metals in *T. fuscatus* was made by Daka *et al.* (2006), who also showed non-linear accumulation of Zn and Cu in *T. fuscatus* exposed to singly and binary mixtures of heavy metals. The authors reported that binary mixtures of Cd and Cu showed interaction ranging from enhanced to depressed accumulation depending on the ratio of mixture of the metals, synergistic effect is favoured at low Copper concentration, while antagonism is favoured at higher Cu concentration. Otitoloju and Don-Pedro (2004) studied the accumulation of heavy metals by *T. fuscatus* in field and laboratory experiments and concluded that under both laboratory and field conditions, *T. fuscatus* exhibited similar accumulation pattern with respect to each of the metals studied. The animal was able to concentrate both Zinc and lead several times over relative to control, in a concentration and time dependent manner. Though the concentration of Copper was higher in the exposed organism compared to control, the level of Copper was found to fluctuate throughout the experimental period, a situation the authors attributed to the ability of the organism to regulate its internal Copper concentration.

In *P. aurita*, the concentration of the metals Fe, Cu and Zn also followed a non-linear pattern in the digestive gland of this organism. The organisms increased its level of Iron

and Cu in all groups by the end of 16 days experimental period though in a non-concentration dependent manner. Iron levels of the control group were consistently above the level of the exposed group, but for day 16 when the level was the same as that of the $^{1}/_{10}^{th}$ 96-h LC_{50} exposed group. Igwegbe (2006) has also reported increased level of Iron in whole body tissue of *P. aurita* on exposure to drill cuttings. It is instructive that the pattern of accumulation of Cu in this species followed the same pattern in all treatment groups including control in this study. However, the group exposed to 10.24 ml/l showed a greater level of Copper accumulation on day 4, while the 1 02 ml/l exposed group has greatest concentration on day 8 and 16, with the least values being found in the control group on all sampling day. These differences are however due to chance as shown by statistical analysis. Previously, Roméo *et al.* (2000) had reported that injection of 1000 ng g^{-1} Cu to *D. labrax* resulted in increased concentration of Cu in the liver of the fish, and that Zinc concentration in the kidney was enhanced in the kidney of the fish by injection of high dose of Cu. In the present study, Zinc levels were found to increase above the basal level on all sampling days in the control group, and also in the exposed groups but for day 16.

Bioaccumulation of heavy metals in the digestive gland of *S. Sesarma* also revealed fluctuations in the concentration in the digestive gland during the study period. It was observed that the background level of Iron and Copper was higher in the male compared to that of the female crab, but the background level of Zinc was higher in female than in male but by the end of the experiment, Zinc levels in the male was found to be higher than in the female. The concentration of Iron was found to fluctuate during the exposure

period in this crab with female having lower concentration than the male. Ibanga (2006) also observed similar finding in the fluctuation of Iron in the whole body tissue of *S. huzardi* exposed to drill cuttings.

Zinc levels in the control female was however found to be less than the background level on each sampling day, but the exposed groups actually exhibited a slight increase in the value of Zinc by the end of the exposure period. In the male on the other hand, but for a marginal fall on day 8, Zinc level was always higher than the background level in the digestive gland of the control group. There was increase in the concentration of Zinc of the groups exposed to drill cuttings. Ibanga (2006) reported positive correlation for Zinc present in the whole body tissue of *S. huzardi* and the sediment.

Comparing the trends in Copper and Zinc levels, it was found that in the control group male *S. huzardi*, Copper and Zinc levels fluctuated in opposite directions. The female control also showed a dissimilar pattern in Copper and Zinc accumulation, where a steady-state in Copper levels was seen to be achieved on day 4, 8 and 16, Zinc levels continued to reduce on day 4 and 8 before some increase was observed on day 16, but the levels was still below the observed background concentration. In male crabs exposed to 0.01 96hLC_{50} drill cuttings concentration, the concentrations of Zinc and Copper in the digestive gland followed opposing trend on day 4 and 8, , but both metals were found to increase on day 16 respectively. In the similarly exposed female group, Copper level was maintained at the same concentration on all sampling but for the marginal increase observed on day 16, but Zinc levels fluctuated, first an increase on day 4, followed by a

decrease below the background level on day before a rise to background value on day 16. This trend tends to suggest active regulation of these essential metals by *S. huzardi*.

The male group exposed to $^{1}/_{10}^{th}$ 96-h LC_{50} drill cuttings exhibited similar trend in the concentration of both Copper and Zinc, but the female showed different trend. For instance, Copper levels were found to increase on both day4 and 8 before a fall on day 16, Zinc levels on the other hand fell marginally on day 4 before increasing on day 8 and 16.

In general, in the group exposed to 8.15 ml/l, the female more or less maintained its Copper level over time but fluctuations were more apparent in the male. The female also seemed able to maintain a steady state in Copper concentration in the control group from day 4, which was not apparent in the male. Ibanga (2006) reported a time and concentration dependent pattern in the accumulation of Copper by *S. huzardi*, and suggested that this crab probably does not regulate its Copper concentration, but the author did take into consideration the influence of sex on the bioaccumulation of metals by *S. huzardi*. While the accumulation pattern of the female may seem to partially support the idea of non-regulation of Copper by *S. Sesarma* at very low concentrations, the fluctuations in accumulation in the digestive gland of the male and in the higher exposed female group do not support the positing on non-regulation of Copper by the crab on exposure to drill cuttings.

5.3 BIOMARKER RESPONSES

Metallothionein Induction

The presence of metallothionein in the test organisms was not unexpected as the protein has been found in different animal taxa (Margoshes and Vallee, 1957; Casterline and Yip, 1975; Olafson *et al*, 1979, Rainbow and Scott, 1979; Bebianno *et al.*, 1992; Bordin *et al.*, 1994; Noel-Lambot, 1976; Bebianno and Langston 1992). The order of the basal level of MT observed in the test species was as follows: *P. aurita* > *T. fuscatus* > *S. huzardi*. This order of concentration of MT was observed to the end of exposure period in control groups of all the test animals, but not in drill cuttings exoposed groups. For instance, it was observed that the concentration of MT was higher in *T. fuscatus* than in *P. aurita* on day 4, 8 16 and 32 for each drill cuttings exposed group. Also MT concentration was higher on day 4 and 8 in *S. huzardi* exposed to 0.01 96hLC_{50} drill cuttings concentration compared to the similarly exposed groups of *P. aurita* and *T. fuscatus*. In the same manner, MT concentration was also higher on day 4, 8 and 16 in *S. huzardi* exposed to 0.1 96hLC_{50} drill cuttings concentration compared to the similarly exposed groups of *P. aurita* and *T. fuscatus*.

The function of metallothionein includes metal detoxification and homeostasis (Roesijadi, 1994). The protein has also been implicated in protection against oxidative stress (Viarengo *et al.*, 1999, 2000), though it is poorly induced by organic pollutants (Viarengo *et al.*, 2000a). The present study shows that induction of MT was influenced by the presence of drill cuttings as was indicated by the fluctuation pattern of this protein

during the experimental period. For each species, MT was affected more on exposure to 0.1 96hLC_{50} drill cuttings concentration than at 0.01 96hLC_{50} drill cuttings concentration.

In *T. fuscatus*, MT level increased above the background value at the end of 32 day exposure period. In control, there was drop in MT production on day 4 and day 8 indicated by the lower value of cysteine during the period. Production of other proteins also dropped on day 4, but the organism increased its production of proteins thereafter, while increases in cysteine residues were observed only on day 16 and 32. Comparing value of MT to total protein in the organism shows that the ratio of MT to other protein was decreasing after day 8 because the organism synthesised other proteins at a rate that was greater than that of its production of MT as calculated from cysteine residue values.

In organisms exposed to 0.01 96hLC_{50} drill cuttings concentration, exposure to drill cuttings initially depressed the production of protein on day 4 and day 8, while at the same time enhanced the induction of MT as indicated by the increase in cysteine residue over the basal level during the period. On continued exposure at the same concentration, the organism was able to significantly increase production of protein including MT on day 16 and 32. The increase in cysteine and protein values on day 16 and 32 were by almost equivalent factors hence the gradual decline in the ratio of MT to total protein during this time. In 0.1 96hLC_{50} drill cuttings concentration exposed organism, exposure to drill cuttings enhanced the production of protein in *T. fuscatus*. However, the induction of MT was somewhat favoured relative to other proteins as indicated by the increasing ratio of MT to other proteins during the study period.

In summary on exposure to drill cuttings in *T. fuscatus*, the organism responded by mobilization of endogenous metallothionein. At low (0.01 96hLC_{50}) exposure concentration, the organism initially favoured MT production to other proteins but with time, the production of enzymes and proteins was enhanced and the ability to produce more MT increased. Over time the ability of low exposed organisms to synthesise other proteins was comparable to that of the control, however a much higher percentage of the protein synthesised was metallothionein indicating that the organism was stressed and production of MT was favoured as defense mechanism. Organisms exposed to higher (0.1 96hLC_{50}) concentration immediately mobilized protein synthesis including MT in response. The increase in the rate of protein production including MT was however gradual such that by the end of the experiment the amount of proteins produced was lower than those of control organisms and low exposed organisms. It may therefore be said that on exposure to higher concentration of drill cuttings, the organism responded by mobilisation of enzymes (proteins) necessary for MT induction, but the ability to produce proteins was inhibited with the result that the organism production of MT was suppressed. The higher MT level and the lower concentration of protein observed in this group compared to the levels in control organisms indicated that the organisms were under stressed condition.

In *P. aurita*, exposure to drill cuttings also elicited different responses in each treatment group during the study. In control, though the change in MT levels during the study was significant, the difference in the average mean value observed on any sampling was very

low. In drill cuttings exposed *P. aurita* on the other hand the organism actively synthesised proteins during the period. MT production was however suppressed during the period. Consequently, the ratio of MT to total protein decreased at the end of the study period.

In organisms exposed to 0.01 96hLC_{50} drill cuttings concentration, production of total protein continued to the end of the study period. However, after the initial suppression of MT production on day 4, its production was thereafter elevated gradually at first and rapidly later such that by the end of exposure the amount of MT was greater than the background value. Consequently, the ratio of MT to the total protein in this group was found to increase after the initial drop on day 4. In 0.1 96hLC_{50} drill cuttings concentration exposed organisms, after the initial increase in total protein on day 4, the organism thereafter suppressed the production of protein, while at the same time increased the production of MT. The result was that the ratio of MT to total protein increased after day 4 however, the amount of MT produced was still significantly lower than the background or control value at the end of the study period.

In summary, exposure to drill cuttings suppressed the production of MT, while at the same time enhanced the production of other proteins in *P. aurita*. At very low (0.01 96hLC_{50}) concentration exposure, the organism tended to recover with time and normal MT production rate resumed. It may therefore be said that exposure of *P. aurita* to very low concentration of drill cuttings resulted in the inhibition of endogenous MT, and the organism in response increased protein synthesis of enzymes required for metallothionein

production as was observed as time of exposure increased. At higher exposure concentration (0.1 96hLC_{50}), MT production was severely suppressed. The organism in response greatly elevated protein production necessary for metallothionein production. Over time the synthesis of MT increased but at a rate lower than that observed in low concentration exposed organism as the amount of other proteins decreased. It may therefore be said that exposure of *P. aurita* to higher concentration of drill cuttings resulted in the inhibition of MT production. The organism tried to compensate by increasing the production of enzymes and proteins for production of MT, but this compensatory mechanism was severely inadequate as the organism's ability to produce endogenous MT remained suppressed and the organism remained stressed.

In crab, MT level remained relatively stable over the experimental period in control groups of both male and female with the absolute value being higher in male than in female, as indicated by the value of cysteine residue during the study. However, the organisms actively synthesised other proteins during the period, with values that were higher in male than in female on any sampling day. The result was that the ratio of MT to total protein was decreasing as exposure time increased in both male and female *S. huzardi*. Sex related differences was observed in control and in 0.1 96hLC_{50} drill cuttings concentration exposed crabs, but not in crabs exposed 0.01 96hLC_{50} drill cuttings concentration.

Crabs exposed to drill cuttings responded by increasing the production of proteins including MT, but the increase in the rate of production of MT was greatly higher than

that of other proteins. In 0.01 96hLC$_{50}$ drill cuttings concentration exposed organism, enhanced MT synthesis continued but the rate of its production relative to other proteins dropped on day 16 in both male and female *S. huzardi*. The result meant that the ratio of MT relative to other proteins increased greatly above the background value on day 4 but dropped on day 16 though the values were still much higher than observed background value. This response pattern was true for both male and female crabs, though the absolute value for each parameter was higher for male than female on each sampling day.

In crabs exposed to 0.1 96hLC$_{50}$ drill cuttings concentration, elevation of MT level continued to day 16, but the rate of increase decreased with exposure time in both male and female crab. In both male and female crabs, the amount of total protein produced during the study was significantly different from that produced in crabs exposed to 0.01 96hLC$_{50}$ drill cuttings concentration. Cysteine values increased consistent during the study, which reflected in the significant increase in MT observed during the study. On day 4, the rate of increase observed for cysteine was much higher than the increase in protein during the same period such that MT relative to total protein was very high compared to the background value. Subsequently however, rate of production of cysteine fell below that of protein on day 8 and 16 with a concomitant fall in the value of MT per total protein in the cytosol. This pattern was true for both male and female. However sex related differences in MT relative to total protein was observed in control and in crabs exposed to 96hLC$_{50}$ drill cuttings concentration, but not in crab exposed to 0.01 96hLC$_{50}$.

In summary on exposure of *S. huzardi* to drill cuttings, the organism responded by increasing production of enzymes and proteins for the synthesis of metallothionein. In very low exposure increased production of protein continued, but synthesis of new MT synthesis reached a plateau and then decreased. In control, increased protein production over the study period did not coincide with increase in MT induction, thus the enhanced induction of MT in exposed crab is a measure of response to stress condition. This conclusion holds true for both male and female *S. huzardi*. In the higher exposed organisms, exposure to drill cuttings resulted in enhanced production of metallothionein in the crab. In male crabs, the organism responded by increasing the rate of protein synthesis and enzymes necessary for MT production. In female crabs, the rate of production of new proteins was inhibited over time compared to control, but the organisms still favoured the synthesis of metallothionein as a compensatory response. The suppressed protein production over time and the enhanced production of MT revealed that female crabs were more stressed than male at higher exposure to drill cuttings.

This study demonstrated the presence of MT in the test organisms, and the altered induction of the protein when the organisms are exposed to drill cuttings. Background MT level was higher in the periwinkles compared to the crabs. Between the periwinkles, basal level of MT *in P. aurita* was more than 4 times the value in *T. fuscatus*. However, the induction of the protein in exposed organisms was severally depressed in *P. aurita*, but the organism tended to recover with time. On the other hand exposure of *T. fuscatus* to drill cuttings enhanced the production of the metallothionein in this organism.

The initial response of the crabs was similar to that of *U. fuscatus* since MT production in crabs was enhanced on exposure to drill cuttings. However it was observed that in the groups exposed 0.01 96hLC_{50} drill cuttings concentration that MT levels fell on day 16 in both male and female though the amount of MT was over 16 and 18 times that of control in male and female respectively during the period. Such reduction in MT concentration was absent in organisms exposed to 0.1 96hLC_{50} drill cuttings concentration. In these latter exposed groups, MT concentration was 20 and 19 times the value found in control groups of male and female crabs respectively. Compared to that of female crabs, male crabs had higher basal level of MT. Induction of MT was also more affected in male crab relative to female crab as the fluctuation in MT level was more marked in male than in female crab.

MT is strongly induced by non essential metals such as Cadmium and Silver. These two metals were below the detection level in the digestive gland of the test species during the study. Butler and Roesijadi (2001) demonstrated that MT protects against Cadmium toxicity in molluscs and that Zinc was a poor inducer of MT in the bivalve *Crassostrea virginica*. Viarengo *et al.* (1999; 2000a) also reported that MT is poorly induced by organic contaminants. It is therefore reasonable to suggest that the induction of MT in the present study was in response to fluctuating levels of essential metals such as Copper and Zinc. However, this assumption does not preclude other functions of MT such as defence against oxidative stress (Viarengo *et al.*, 1999; 2000a; Coucelo *et al.*, 2000).

The induction of metallothionein is usually a primary response to heavy metal pollution both in fish (Killie *et al.*, 1992; Hogstrand *et al.*, 1996) and in invertebrates (Viarengo and Nott, 1993; Roesijadi, 1994), and has also been associated with acclimation of fish (Dixon and Sprague, 1981) and invertebrate (Sathyanathan, 1996) to metal. MT induction has been linked to reduced growth in laboratory experiments in fish (Roch and McCarter, 1984) and in field experiment in invertebrates (Leung *et al.*, 2001), while allowing the organisms to withstand higher levels of exposure to heavy metals.

In the present study, test organisms were exposed to a composite toxicant, and the organisms metallothionein levels were clearly affected as the organisms battled to cope with the imposed stress condition. The response pattern was clearly different in the two species of periwinkles. In *T. fuscatus*, metallothionein production was enhanced on introduction of the toxicant, with induction higher in organisms exposed to 0.01 $96hLC_{50}$ drill cuttings concentration compared to 0.1 $96hLC_{50}$ drill cuttings concentration exposed group. In *P. aurita* on the other hand, MT was initially depressed following introduction of drill cuttings to the test media. Overtime however, the organisms tended to increase the production of MT such that by the end of the exposure period organisms exposure to 0.01 $96hLC_{50}$ drill cuttings concentration seemed to have fully recovered the normal production MT. Metallothionein in organisms exposed to 0.1 $96hLC_{50}$ drill cuttings concentration was still below the background level by the end of the 32 day exposure though some amount of recovery was evident during this time. This finding is not totally alien, for instance, Roméo *et al* (1997) had reported that when 500 ng g^{-1} Copper were intra peritoneally injected into *D. labrax* for 24 hours, MT levels in the liver of injected

fish were lower than those of control fish. The authors suggested that the Copper level was toxic to the fish and acted by inhibiting the synthesis of metallothionein.

Clearly this study has demonstrated the induction of MT in these organisms and its potential use in biomonitoring programmes. However various biotic and abiotic (e.g. Mouneyrac *et al.*, 1998; Leung and Furness, 1999) factors affecting its induction should be taken into consideration for proper interpretation.

Lysosomal membrane stability test

Lysosomal membrane stability test was very clear in demonstrating the health of the organisms during the study period. Comparing the background NRR time of the test species shows the background labialisation period to be as follows: *S. huzardi* >*T. fuscatus* > *P. aurita*. Decrease in NRR time was observed with increase in concentration of the toxicant and time of exposure in all the species studied. Sex related difference in NRR times was observed in the 0.01 96hLC_{50} exposed groups the crabs, but not in control and the groups exposed to 0.1 96hLC_{50} drill cuttings concentration. For the periwinkles on the other hand, NRR time for any treatment varied for each species, but validated the finding that *T. fuscatus* was more vulnerable than *P. aurita* on exposure to drill cuttings. Though background NRR time was higher for *T. fuscatus* compared to *P. aurita*, the values were lower in *T. fuscatus* by the end of the experimental period in all drill cuttings exposed groups. Also for any exposure group, lysosomal integrity was more affected in the periwinkles than in the crab.

Using the neutral red retention assay, it was possible to demonstrate that exposure to drill cuttings was causing harm to the organisms. In control, there was no significant change in retention time of the neutral red dye in the cells of the haemolymph of *T. fuscatus*, while significant difference was observed on day 8 and day 32 in *P. aurita*. On the other hand, ability to retain neutral red dye in exposed species decreased with increase with toxicant concentration and time of exposure in drill cuttings exposed groups. The decrease in neutral red retention time in drill cuttings exposed organism is indicative of the uptake of the pollutant, or its metabolic products, into the lysosomes, and its effects on the lysosomal membrane (Moore, 1988) by the test organisms. This is because lipophilic organic xenobiotics are readily taken up into the tissues of *Mytilus* sp. and other molluscs and concentrated in lysosomes of digestive gland and other tissues causing deleterious effect (Livingstone, 1991). Köhler *et al.* (2002) demonstrated a good dose-response relationship between the severity of lysosomes alterations in liver cells of the flatfish flounder (*Pleuronectes flessus*) and the degree of liver degeneration (from cell injury through to hepatocellular carcinoma) as a result of PAH and organochlorine exposure.

Lysosomal stability has also been used in the direct relationship between lysosomal integrity in hepatopancreatic cells of oysters (*Crassostrea virginica*) and larval viability (Ringwood *et al.*, 2004). Ringwood *et al.* (2004) were able to show that membrane destabilisation >35 % have significant effect in reproductive success and larval survival in oysters. Brown *et al* (2004) exposed three marine invertebrate species, the shore crab *Carcinus maenas*, the common limpet *Patella vulgata* and the blue *Mytilus edulis* to

Copper concentrations over 7 days, and were able to show that the neutral red retention time was significantly affected in all three species.

Domouhtsidou *et al.* (2004) showed that lysosomal membrane stability test performed on the digestive cells of *Mytilus galloprovincialis* was able to detect a pollution gradient among the different sampling stations investigated, with cells from the reference station showing statistically the greatest lysosomal membrane labilization period values. Nolde *et al.* (2006) recorded rapid lysosomal response of the hepatopancreatic cells of the terrestrial isopod *Porcellio* scaber exposed to elevated concentration of mercury. The authors also reported that laboratory animals exposed to mercury in their food had lower lysosomal membrane stability than animals collected from a site that is chronically exposed to much higher environmental mercury concentrations, suggesting tolerance to mercury in animals from the contaminated site or a protective role of autophagy to animals chronically exposed to pollution.

Roméo *et al.* (2000) observed that the lysosomal membrane labialisation period of kidney cells of *Dicentrarchus labrax* are significantly reduced compared to control, and that Copper was more toxic than Cadmium in the kidney of the fish; the time of labialisation was also negatively correlated to the damage to the cell membranes. Labialisation was defined as the length of time of preincubation of a tissue section at pH 4.5 that is required to give maximum staining intensity for the lysosomal hydrolases such as *N*-acetyl-β-hexosaminidase. The authors also reported that based on the result of the lysosomal

membrane stability, that Copper was more toxic than Cadmium in the kidney of *D. labrax*.

The present study therefore is in conformity with the findings of other workers and emphasis the use of lysosomal membrane stability as an indicator of general stress (Taleb *et al.*, 2007).

Antioxidant Responses

Organisms are known to mobilise antioxidant defence mechanism when confronted with oxidative stress (Coucelo *et al.*, 2000; Römeo *et al.*, 2000; Keller *et al.*, 2004). The present study indicated that the organisms experienced oxidative stress, not necessarily from exposure to the toxicant, but which may also partly be related to stress from imposed experimental condition such as hunger and substrate condition or from normal metabolic processes (Abele, 2002). Increase in certain antioxidant enzymes partly accounted for the increase in total proteins observed for all species and at all treatment.

In *T. fuscatus*, the results pointed that the organism experienced oxidative stress and responded by mobilisation of antioxidant defence system. Exposure to drill cuttings also increased oxidative stress in this periwinkle species as was indicated by the initial concentration dependent lipid increase in lipid peroxidation on day 4. No such increase in lipid peroxidation was observed in control on any sampling day.

In control, catalase activity increased on day 4 and thereafter remained unchanged. GST levels on the other were stable throughout the study period, while lipid peroxidation decreased as exposure period increased. In organisms exposed to 0.01 96hLC_{50} drill cuttings concentration, catalase activity increased on day 4 and day 8 and then fell on day 16. GST level was significantly lowered during the study period. Lipid peroxidation though elevated initially, significantly reduced by the end of the study. Its value on day 16 was half the background level and lower than the level observed in control organisms.

In 0.1 96hLC_{50} drill cuttings concentration exposed organisms, catalase activity increased on day 4 and 8 and then fell on day 16. The levels of increase were however lower than was observed in organisms exposed to 0.01 96hLC_{50} drill cuttings concentration, but higher than those observed for organisms in control. Significant increase in GST levels on day 4 was responsible for the statistical difference observed in this group during the study; though test of homogeneity revealed equal variances for the group. Lipid peroxidation was also significantly enhanced on day 4, and then decreased over time such that by the end of exposure the value was marginally lower than was observed in 0.01 96hLC_{50} drill cuttings concentration exposed organisms and in control organisms.

In conclusion, it seemed that the organism mobilised compensatory mechanisms when faced with oxidative stress condition. In control, it is possible that the organism may have experienced some degree of oxidative stress as indicated by the elevation of catalase activity during the study. Catalase functions by removing degrading hydrogen peroxide in the cytosol, which may result from physiological condition related to metabolism since

the organisms were starved during the study, and used stored energy for normal respiratory processes (Abele, 2002). Reactive oxygen species have been implicated in the induction of lipid peroxidation process (Ayres *et al.*, 1998). The organisms therefore mobilised some antioxidant defense mechanisms including production of metallothionein induction to compensate for this condition. Viarengo *et al.* (1999); Coucelo *et al.* (2000); Viarengo *et al.* (2000a) have implicated MT as an antioxidant protein. This inference relies partly on the relative constant activity level of GST observed during the study. GST catalyses glutathione conjugation, i.e. the addition of an electrophilic site on a substrate during phase II metabolism. Its relative stability especially at very low exposure during the study period suggests that the enzyme is secondarily induced behind catalase in *T. fuscatus* during oxidative stress. This conclusion that induction of catalase was more important than GST in fighting oxidative stress in *T. fuscatus* was supported by the reduction in lipid peroxidation activity in control organisms with time, and also by the persistent elevated increase above background value observed for catalase activity but not for GST during the study. Also, GST activity was not significantly altered during the study in control.

Organisms exposed to 0.01 96hLC_{50} drill cuttings concentration clearly experienced oxidative stress as was indicated by the increase in lipid peroxidation level. The organisms responded by increased induction of catalase activity and metallothionein level. These compensatory mechanisms seem to be adequate as the level of lipid peroxidation fell overtime and catalase activity level reduced to that observed in control organism. GST induction during this period was initially slightly lowered on exposure to

the toxicant. It is possible that the concentration of the toxicant was too low to enhance glutathione conjugation, and in fact may have acted to inhibit the activity. Over time GST activity seem to increase but the level was still below the background activity level.

In organisms exposed to 0.1 96hLC$_{50}$ drill cuttings concentration, oxidative stress in the group was clearly indicated by the elevated GST activity level as well as by the significant level of lipid peroxidation. The organism galvanised antioxidant defense mechanism by increasing catalase activity and production of metallothionein, which resulted in reduction of lipid peroxidation and the lowered GST activity. It is also possible that the organism probable used part of its energy in detoxification of toxicant from its cells as was indicated by the initial elevation GST activity observed.

P. aurita responded in similar manner to oxidative stress as *T. fuscatus* with comparable values. In control, catalase activity level increased and stabilised thereafter. GST activity level was unchanged during the period. Lipid peroxidation decreased with exposure time.

In 0.01 96hLC$_{50}$ drill cuttings concentration exposed organisms, catalase activity significantly increased on day 8 and 16 compared to control and fell to background level on day 16. GST activity was lowered during the period and lipid peroxidation initially increased above background value and subsequently fell.

In summary, *P. aurita* experienced oxidation in the face of drill cuttings exposure and stressful condition. In control, the stressful condition may have been occasioned by physiological changes relating to metabolism as the organisms were fasted during the

study. The organism responded by increasing the level of production of catalase as a compensatory mechanism to cope with the state of oxidation. This proved adequate as lipid peroxidation level actually fall over time as the experiment continued.

In both organisms exposed to 0.01 96hLC$_{50}$ and 0.1 96hLC$_{50}$ drill cuttings concentration organisms, oxidative stress resulted in initial increase in the rate of lipid peroxidation in a concentration dependent manner. In response, the organism increased the production of catalase as a protective mechanism, as well as GST in the higher drill cuttings exposed organisms, which resulted in reduction in lipid peroxidation. In the lower exposed *P. aurita* on the other hand, it is possible that the concentration of drill cuttings was not enough to enhance GST activity, and may have in fact acted to suppress it. GST activity was however, found to increase as exposure time increased, but the level of activity was still below the background value.

Oxidative stress was more pronounced in organisms exposed to 0.1 96hLC$_{50}$ drill cuttings concentration as indicated by the level of lipid peroxidation on day 4. In response, *P. aurita* responded by increasing the production of the antioxidant enzyme catalase. GST was also statistically enhanced during the study. These protective activities seemed to be enough as lipid peroxidation level fell below the background level over time. It is also possible that the organism used part of its energy in xenobiotic biotransformation shown by the varying level of GST observed.

The changes in the antioxidant enzymes in the crabs suggest that the crabs must have undergone some degree of oxidative stress during study. There was no significant difference in lipid peroxidation activity response between male and female crabs. In control, catalase activity initially fell on day 4, significantly increased above background activity level on day 8, and thereafter remained stable. SOD levels consistently reduced during the study period and by the end of 16 day exposure the value was less than half of the background level. The background GST activity observed for the crab was low when compared to the levels observed for the periwinkles, however GST activity seems to be important during oxidative stress as the its activity was statistically affected in all treatment during the study. There was an initial rise in GST activity which subsequently stabilised after day 8 but at a significant level above the background value. The rate of lipid peroxidation stabilised after the initial rise on day 4.

In organisms exposed to 0.01 $96LC_{50}$ drill cuttings concentration, catalase activity level fell on exposure to drill cuttings and thereafter rose significantly above the background level on day 16. SOD levels consistently fell during the study period and by the end of exposure period, the level was significantly below the background value. GST level was significantly elevated on day 4, and subsequently fell but at the end of the study, its level was still significantly above the background level. Lipid peroxidation level rose on exposure, and later fell as exposure time lengthened.

Organisms exposed to 0.1 $96hLC_{50}$ drill cuttings concentration also responded by mobilising antioxidant defense system in the face of oxidative stress. On exposure,

catalase activity level was significantly enhanced, but the level fell on day 16 to a value comparable to the background value. Just as in other treatment groups, SOD levels consistently reduced with time of exposure. GST activity stabilised after the initial rise on exposure. Increase in lipid peroxidation activity was slow at first, but peaked on day 8 and thereafter fell at the end of the experiment, but the value was still significantly higher than the background value.

As was observed for *T. fuscatus*, the induction of catalase seemed to be primarily induced to GST in response to oxidative stress. The reasons for this suggestion are the same as those given for *T. fuscatus*.

Oxidative stress was also evident in the crab *S. huzardi* during the study. Physiological factors relating to metabolism may have contributed to stress since the crabs were not fed during the study period. The organisms responded by mobilising antioxidant defense system to combat oxidative effects. GST seemed to be an active antioxidant defense enzyme in the crab as its level was the first to be enhanced before catalase in all treatment groups during the study. In control, the organism increased its catalase and GST activity levels. These simple compensatory actions seemed adequate as lipid peroxidation activity stabilised and SOD levels decreased. Metallothionein seemed not to be required by *S. huzardi* to fight oxidative stress as the protein level was stable in control organisms during the experimental period.

Evidence of oxidative stress in *S. huzardi* exposed to 0.01 96hLC$_{50}$ drill cuttings concentration was the increased level in lipid peroxidation. The organism responded by increasing its antioxidant defense system primarily by increasing the activity of GST. At the end of the study, lipid peroxidation was statistically reduced compared to control in this group. Catalase seemed to be secondarily preferred in fighting oxidation as the increase in its activity was observed much later during exposure, at which time GST level was falling. Metallothionein may also have played a role in antioxidant defense, but its induction primarily in this species is probably not related to oxidative defense. This inference relied principally on the observation that induction of metallothionein was not enhanced in control organism in the face of oxidative stress. SOD was not a major factor in fighting oxidative stress in the crab as the level of this enzyme decreased with exposure time. Over all, the organism was able to cope with oxidative stress as the rate of lipid peroxidation fell below background level.

In organisms exposed to 0.1 96hLC$_{50}$ drill cuttings concentration, increased lipid peroxidation level was observed on exposure but at a rate lower than that observed in control and in 0.01 96hLC$_{50}$ drill cuttings concentration possibly due to the increased catalase activity level during this period in the crab. As exposure time increased, lipid peroxidation continued to increase to day 8 before falling on day 16 to comparable values observed in control during the same period. GST remained elevated and stable during the study, while reduction in catalase was observed only on day 16. It is possible therefore that the organism preferred the induction of GST to catalase to fight oxidative stress as exposure continued. Metallothionein may also have played a role in combating oxidative

stress in *S. huzardi* though its induction primarily may not be related to oxidative response. SOD level decreased throughout exposure as the enzyme was not required to cope with oxidative stress during the period.

Another explanation may be that the organisms' enhanced GST and catalase activity levels delayed increase in lipid peroxidation level, and that failure in sustaining the catalase activity at a higher level was responsible for the relatively high peroxidation level relative to background level observed in the crab at the end of the experiment.

In this study, various antioxidant enzymes as well as lipid peroxidation were utilised to indicate the degree of oxidative stress experienced by the test species. Catalase activity was elevated in all treatment groups in all the species studied. Catalase is induced in the cytosol to protect biological systems against ROS. ROS are produced during normal metabolic processes (Abele, 2002) as well as by toxicant induced interactions (Livingstone, 2001). SOD is another cytosolic antioxidant enzyme that protects against oxidative stress by mopping up the dismutase radical. Its induction was monitored only in the crabs and the levels reduced with time in all treatment groups. In periwinkles GST activity was unchanged in control, but was significantly elevated initially and then returned to comparable background value in drill cuttings exposed organisms. In the crabs, GST activity was significantly elevated among all treatment groups. However GST values was more stable after the initial rise in control organisms and 0.1 $96hLC_{50}$ drill cuttings concentration exposed organisms. Fluctuations were more apparent in the 0.01 $96hLC_{50}$ drill cuttings concentration exposed organisms. Pérez-López *et al.* (2002) had

earlier pointed out that total GST activity measurement may not reveal differences in the induction of this enzyme and advocated the characterisation of the different isoforms in order to distinguish enzymatic induction.

A comparison of the background values of antioxidant parameters shows that both catalase and GST levels were several folds higher in the two periwinkle species compared to the crabs. Lee (1988) also found GST activity higher in the hepatopancreas cells of *Littorina littorea* compared to the digestive cells of *Mytilus edulis* and *Carcinus meanas*. The author suggested that these could be related to the presence of secondary metabolite in the macroalgae diet of the gastropod. Comparison could not be made for the background SOD activity for the crabs and periwinkles since this enzyme was monitored only in the crabs. In summary, it is plausible to attribute the lower lipid peroxidation level in the periwinkles compared to the crabs the level of antioxidant enzymes present in their cytosol.

Differences in responses with respect to new antioxidant enzymes have been described by previous researchers. Coucelo *et al* (2000) reported that catalase and SOD were efficient in protecting *Halobatrachus didactylus* from lipid peroxidation processes after acute exposure to sub-lethal Cadmium concentration. According to Roméo *et al* (2000), catalase activity was significantly reduced during in vitro exposure of kidney cells of *Dicentrarchus labrax* to cadmium, while there was no significant difference in the response of Copper. The authors suggested that the results were not unexpected since metabolic processes regulating enzyme induction do not occur in vitro and Cadmium may

have bound to –SH residues of the enzyme system. Fluoride exposure has also been known to significantly increase catalase activity of the 4th instar larva of the silkworm *Bombyx mori* while lowering glutamate dehydrogenase (GDH) activity and the levels of protein and free amino acids in haemolymph (Miao *et al.*, 2004). Parasitic infection of the decapod *Palaemonetes argentinus* by *Probopyrus ringueleti* has been reported to reduce the level of SOD in the shrimp rending the organisms vulnerable to ROS effects (Neves *et al.*, 2000).

GST catalyses glutathione conjugation during phase II biotransformation of electrophilic xenobiotics making them amenable for excretion from the organism's body. No significant difference in GST activity was observed in *Mytilus edulis* and *Littorina littorea* collected in polluted sites relative to those collected from control sites as reported by Lee (1988), however significant difference was observed in the hepatopancreas cells of the crab *Carcinus maenas* collected from polluted sites as opposed to those collected from reference sites during the same study. The author suggested that the high GST activity observed in the crab could be probably be due to present of some substance not monitored during the study. Lee (1988) also reported that the results of the mesocosm experiment revealed no differences in GST activity of all treatment groups including control of *M. edulis*, *C. maenas* and in the low and medium exposed groups of *L. littorea*. Significant difference was observed only in the *L. littorea* exposed to high concentration of diesel oil and Copper mixture.

Lipid peroxidation results in damage to polyunsaturated fatty acids located in cell membranes. This damage can decrease fluidity, increase leakiness, and inactivate membrane-bound enzymes. An ultimate result may be cell death and tissue damage (Halliwell and Gutterirdge, 1985; Wills, 1985). Thus, lipid peroxidation is considered a first step of cellular membrane damage by xenobiotics (Viarengo, 1989). Lipid peroxidation is most often induced by dismutase oxide (O_2^-) and hydrogen peroxide (H_2O_2) and Ayres *et al.* (1998) have demonstrated in vitro protection by estradiol-17b in terms of lipid peroxidation to be due to the ability of the estrogen to inhibit generation of superoxide radical and prevent further chain propagation. Increased lipid peroxidation has been reported to be induced in the digestive gland of *P. corneus* exposed to 450 and 800 µg l–1 of pentachlorophenol (PCP) (Klobučar *et al.* 1997), which is probably related to increased oxygen uptake by the PCP exposed organisms (Roszell and Anderson, 1996).

In summary, this work was able to demonstrate

1. Enhanced induction of antioxidant enzymes of the test species on exposure to drill cuttings,
2. The diminished cumulative effect of oxidative stress brought about by the antioxidant defense system, as indicated by reduction in lipid peroxidation process over time in the periwinkles
3. That the antioxidant system was overwhelmed in the crab as shown by the increased level of lipid peroxidation during the study.

6.0 CONCLUSION

Organisms in the environment are faced with multiple stressors both naturally and anthropogenically induced. The effects of these stressors usually occur at the molecular and subcellular levels before being manifested at higher biological levels. In order to cope, organisms may synthesis different biological molecules such as proteins and enzymes, and may also biotransform xenobiotics. A steady state may be reached at which point compensatory mechanism is equal to stress. If the stressor is removed, the organism may recover, but if the stressor persists and compensatory mechanisms overwhelmed, the organism begins to deteriorate and become diseased. Death is an irreversible phenomenon, and may affect whole populations and communities.

The present study investigated the biomarker responses of estuarine benthic macroinvertebrates species of Lagos lagoon on exposure to drill cuttings, and demonstrated that exposure to drill cuttings was causing biological harm to the test species. In response, the organisms increased the production of proteins and enzymes to fight the stressor.

In *T. fuscatus* and *S. luzardi*, increased production of metallothionein suggested that energy for growth and reproduction was concentrated to increase the production of this protein. The implication is that organisms chronically exposed to low levels of drill cuttings are more likely to have stunted growth and lower reproductive rate and population density than reference organisms. In *P. aurita*, exposure to drill cuttings suppressed metallothionein production in a concentration dependent manner. The

implication was that the organism's ability to cope with stress may have been compromised, making the organism more vulnerable to external stressors.

All three species seemed to have been coped against oxidative stress as indicated by the reduction in lipid peroxidation activity over time. Catalase was an important antioxidant enzyme in the three organisms. GST seemed to be preferred in the crabs though it occurred at very low levels in the organism. SOD was not important to fight oxidative stress due to exposure to drill cuttings at least in the crabs. This enzyme was not monitored in the periwinkles.

Lysosomal integrity test was able to show unequivocally that exposure to drill cuttings was causing harm to the organisms, and that effect increases with concentration and time of exposure.

The importance of this study is not just to demonstrate that drill cuttings is causing harm to the organisms, but that the findings may be taken into management consideration. While the use of biomarkers in biomonitoring programmes have gained immense support in developed economies, inclusion of biomarkers in any remote manner during biomonitoring programmes or EIAs in developing countries especially in Nigeria seems nonexistent. This could be due to the lack of specialists in this field in most developing countries, and the practically nonexistent local literature and local examples in this area. It is therefore hoped that this work will serve as a reference material and a pilot finding that will encourage other researchers for work in this field and thus encourage the

incorporation of biomarker studies in routine biomonitoring and environmental assessment programmes.

The following conclusions are reached based on the laboratory studies that were carried out:

1. The order of sensitivity of the test species to drill cuttings based on the computed toxicity factors were as follows:

T. fuscatus > *P. aurita* > *S. huzardi.*

2. The concentration of total hydrocarbons in the digestive gland of the test animal species was low and may be related to factors such as high n-octanol/water partition coefficient, metabolism of hydrocarbon and/or possible accumulation in other tissues other than the digestive gland.

3. The levels of heavy metal in the digestive gland of the test animal species was low, which may be due to non-availability of metals in drill cuttings.

4. Uptake of metals from test solution was neither concentration nor time dependent indicating regulation of essential metals such as Copper and Zinc by the test species.

5. The presence of metallothionein in the 3 species studied was demonstrated.

6. The study demonstrated the presence of antioxidant enzymes namely catalase and GST in the periwinkles, while SOD was determined only in the crabs.

7. The study demonstrated that antioxidant enzymes were induced in the face of oxidative stress in the organisms and were effective in diminishing oxidative related injury to the cells.

8. This study also suggested phase II biotransformation process in the crabs as GST seemed to be preferentially induced during exposure to drill cuttings in all treatment groups.

9. Increased lipid peroxidation observed in the control organisms of crabs suggested metabolic related oxidative stress, which may be linked to breakdown of stored energy since the organisms were starved.

10. Lysosomal membrane stability test was able to integrate the results above and clearly demonstrated toxicity to the organisms on exposure to drill cuttings to sublethal concentrations of drill cuttings to be concentration and time dependent.

7.0 CONTRIBUTIONS TO KNOWLEDGE

1. The study has established that biomarkers responses of estuarine macro-invertebrates can be incorporated in routine environmental monitoring and risk assessment programmes of the coastal marine environments.

2. Lysosomal membrane stability test is a good indicator of general anthropogenic induced stress in estuarine macroinvertebrates and in its application in combination with other biomarkers help to understand the pathway of toxicant effect in estuarine macroinvertebrates.

3. The study has elucidated the toxicokinetic mechanisms of phase II biotransformation process and metabolic related oxidative stress in littoral macrobenthic invertebrates.

4. The study also demonstrated that uptake of heavy metals from drill cuttings using estuarine macroinvertebrates cannot be used as a bioremediation method of drill cuttings.

5. Chronic exposure to drill cuttings is toxic to estuarine macroinvertebrates and the relative sensitivity is *T. fuscatus* > *P. aurita* > *S. huzardi*

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9.0 APPENDICES

APPENDIX I: Results of Chemical Characteristics of Water Used in the Bioassay

Chemical Parameter	Concentration
Ph	7.32
Total Organic Carbon	0.04 mg/l
Total Hydrocarbon	ND
Salinity	8.4 ‰
Iron (Fe)	1.42 mg/l
Copper (Cu)	0.65 mg/l
Zinc (Zn)	11.26
Cadmium (Cd)	0.005
Silver (Ag)	ND

APPENDIX II: Metallothionein determination

Colorimetric determination of Metallothionein Content in the tissue of mollusks and other organisms.

Reagents provided: Sol A 125ml; tissue extraction buffer

Sol B 1.25ml; Protease inhibitor, 100X Concentrate

Sol C_1 5ml; resuspension buffer, Component 1

Sol C_2 5ml; resuspension buffer Component 2

Sol D_1 200ml; Stabilized Ellman's buffer

Sol D_2 3.4ml; concentrated Ellman's Reagent

Sol E 2x 1.45ml; 4mM sulfhydryl reference standard solution

Reagent and working solutions preparation:

1. Add 0.725 Sol C_2 to one microtube containing the component E to obtain a 4mM sulfhydryl reference standard solution (reduced glutathione). Label properly and store at 2-8°C in the dark for 2-3 months.
2. Mix all the content of solution D_2 with solD_1 to obtain the functional sol D, reaction buffer. Equilibrate sol D at 23°C.
3. Equilibrate absolute ethanol at –20°c.
4. Add 1 part of sol B protease inhibitor to 99 parts of sol A extraction buffer to obtain sol A+B, homogenating buffer. Utilize sol A+B within 1hr and discard after each use.
5. Mix in equal amount part of sol C_1 with a part of sol C_2 to obtain sol C, resuspension buffer. One (1)ml of buffer C is sufficient for 10 analyses plus standards.

Procedure for the extraction and evaluation of metallothionein content.

1. Homogenize, at 0-4°C, 0.5g of tissue with the addition of 1.5ml sol A+B
2. Transfer the homogenate into a 2ml microtube and centrifuge at 30,000g at 4°c for 20min. calculate the total protein content from the supernatant for further normalization of data dy means of Bradford assay.
3. Withdraw 0.315ml of cold absolute ethanol at –20°c.
4. Mix and centrifuge at 12-16,000g at 4°c for 5min.
5. Collect accurately all the supernatant and transfer it into a clean 2ml tube.

6. Add 1.5ml of –20°c absolute ethanol and incubate at –20°c for 30-60min.
7. Centrifuge at 12-16,000g at 4°c for 5mions.
8. Discard all the supernatant.
9. Add 1.95ml of sol D equilibrated at RT. Mix briefly and incubate for 2mins at room temperature and then centrifuge at 12-16,000g.
10. Read absorbance at 412nm against a blank (50µl Sol C +1.950 Sol D).
11. Calibrate the photometer using the reconstituted sol E. Add 1.950µl of RT. Sol D
12. Record absorbance at 412nm against the blank and draw a standarad curve by plotting absorbance against the concentration.

Calculation of metallothionein concentration

The (ABS^{MT}_{412}) value was interpolated over the standard curve to obtain the concentration (nmol) of sulfhydryl groups, i.e. cysteine residues, due to metallothionein present in the sample: (nmol Cys^{MT}).

To obtain the concentration of metallothionein (nmol MT) per gram of tissue, the following was applied:

$$(\text{nmol MT})g^{-1} = \frac{(\text{nmol } Cys^{MT})}{0.1\text{g. } n^{ocys}}$$

where: 0.1g is the amount of tissue equivalent to 0.3ml of supernatant subjected to precipitation.

n^{ocys} is the number of cysteine residue present in the investigated metallothionein.

To express metallothionein concentration per mg of the total protein present in the supernatant, the following was applied:

$$(\text{nmolMT}).mg^{-1} = \frac{(\text{nmol } Cys^{MT})}{mg.n^{ocys}}$$

where: mg is the amount of protein present in the 0.3ml supernatant subjected to ethanol precipitation.

APPENDIX III: In vivo determination of lysosomal membrane stability by the Neutral Red kinetic retention assay

Reagents provided: Sol A 0.100 ml; 20 mg/ml Neutral Red concentrate 200 X
Sol B 1.50 ml; 0.01% poly Lysine
Sol C 30 ml; 0.5 M di-Sodium EDTA
Sol D 1.0 L; Hanks balanced salt solution (HBSS) w/o Ca and Mg
Siliconised collection tube 50 eea

Reagent and working solutions preparation

A. Sol D HBSS preparation 1L

6. Equilibrate vial D, HBSS powdered medium to room temperature
7. Measure out 900 ml of distilled water. While gently stirring the water add powdered medium. Stir until dissolved.
8. Add 0.35 g sodium bicarbonate or 4.7 ml of 7.5 % bicarbonate solution. Stir til dissolved.
9. Adjust the pH to 0.1-0.3 pH unit below the desired pH since it may rise during filtration. The use of 1 N HCl or 1 N NaOH is recommended.
10. Adjust volume to 1 L.
11. Sterilise immediately by filtration using a membrane with a porosity of 0.22 micron.
12. Aseptically dispense medium into sterile container.

B 1X Neutral Red solution

To prepare 1.0 ml of 1X Neutral Red solution suitable for 25 determinations:

- Add 5 µl Neutral Red solution concentrate 200 X to 995 µl Sol D. Mix and store in the dark at 4°C.

C Microscopic slide treatment with Poly-Lysine to ensure adherence

- Put 2 µl Sol B, poly-Lysine on a microscopic slide, spread out using the edge of a cover slip and allow air dry. Poly-lysinated slides can be stored in a dry place for several weeks.

D Sample preparation: Haemolymph cells

1. Add 40 µl of Sol C, 0.5 M di-sodium EDTA to a 1.5 ml (siliconised) microcentrifuge tube
2. Fill a hypodermic syringe with 0.5 ml of salt adjust Sol D
3. Collect 0.5 ml of haemolymph, then transfer the whole content of the syringe to the normalised microtube.

E Procedure for the extraction and evaluation of metallothionein content.

13. Dispense 40 µl of cells onto the poly-lysinated slide and incubate for 30 minutes at 16-20°C in a humidity chamber to allow cell attachment. Gently tap excess solution turning the slide on a side.
14. Add 40 µl of 1X Neutral Red solution and incubate in a humidity chamber for 15 minutes at 16-20°C.
15. Examine the sample for maximum 2-3 minutes. Thereafter, transfer the slide to a humidity chamber to avoid excessive evaporation. If necessary a drop of physiological saline solution may be added prevent drying.
16. During the first examination (t_0) lysosomes should retain the Neutral Red dye. Observe different microscopic fields.
17. Repeat the observation every other 15 minute.

APPENDIX IV: MORTALITY RESPONSE OF TYMPANOTONUS FUSCATUS DURING ACUTE EXPOSURE TO DRILL CUTTINGS

Concentration (m/l)	Log Concentration	Replicate			Total No of organisms	Total Mortality	% Mortality	Probit
		1	2	3				
24 Hours								
Control	-	0	0	0	60	0	0	-
50	1.699	1	1	0	60	2	6.7	3.5015
100	2	1	1	1	60	3	10	3.7184
150	2.1761	1	1	2	60	4	13.3	3.8877
200	2.301	1	1	2	60	4	13.3	3.8877
250	2.3979	1	2	2	60	5	16.7	4.0399
300	2.4771	2	2	2	60	6	20	4.1584
48 Hours								
Control	-	0	0	0	60	0	0	-
50	1.699	2	2	2	60	6	20	4.1584
100	2	2	2	3	60	7	23.3	4.271
150	2.1761	3	2	3	60	8	26.7	4.3781
200	2.301	3	3	3	60	9	30	4.4756
250	2.3979	3	4	4	60	11	36.7	4.6602
300	2.4771	4	4	5	60	13	43.3	4.8313
72 Hours								
Control	-	0	0	0	60	0	0	-
50	1.699	3	2	3	60	8	26.7	4.3781
100	2	3	3	3	60	9	30	4.4756
150	2.1761	3	3	4	60	10	33.3	4.5084
200	2.301	4	4	4	60	12	40	4.7467
250	2.3979	4	5	5	60	14	46.7	4.9172
300	2.4771	5	6	6	60	17	56.7	5.1687
96 Hours								
Control	-	0	0	0	60	0	0	-
50	1.699	4	3	4	60	11	36.7	4.6602
100	2	5	4	5	60	14	46.7	4.9172
150	2.1761	5	5	6	60	16	53.3	5.0828
200	2.301	6	6	6	60	18	60	5.2533
250	2.3979	7	7	8	60	22	73.3	5.6219
300	2.4771	9	8	9	60	27	90	6.2816

APPENDIX V: PROBIT AND LOG DOSE OF DRILL CUTTINGS AGAINST TYMPANOTONUS FUSCATUS

24 Hours (Y = 2.11 + 0.81x)					
X	1	2	3	4	5
+ 0.81x	0.81	1.62	2.43	3.24	4.05
2.11	2.11	2.11	2.11	2.11	2.11
Y =	2.92	3.73	4.54	5.35	6.16
48 Hours (Y = 2.70 + 0.83x)					
X	1	2	3	4	5
+0.83x	0.83	1.66	2.49	3.32	4.17
2.7	2.7	2.7	2.7	2.7	2.7
Y =	3.53	4.36	5.19	6.02	6.87
72 Hours (Y = 2.63 + 0.96x)					
X	1	2	3	4	5
+6.96x	0.96	1.92	2.88	3.84	4.8
2.63	2.63	2.63	2.63	2.63	2.63
Y =	3.59	4.55	5.51	6.47	7.43
96 Hours (Y = 1.67 + 1.65x)					
X	1	2	3	4	5
+1.65x	1.65	3.3	4.95	6.6	8.25
1.67	1.67	1.67	1.67	1.67	1.67
Y =	3.32		6.62	8.27	9.92

APPENDIX VI: MORTALITY RESPONSE OF PACHYMELANIA AURITA DURING ACUTE EXPOSURE TO DRILL CUTTINGS

Concentration (m/L)	Log Concentration	Replicate			Total No of	Total Mortality	% Mortality	Probit
		1	2	3				
24 Hours								
Control	-	0	0	0	60	0	0	-
100	2	0	1	1	60	2	6.7	3.5015
150	2.1761	1	1	1	60	3	10	3.7184
200	2.301	1	1	2	60	4	13.3	3.8877
250	2.3979	1	1	2	60	4	13.3	3.8877
300	2.4771	2	1	2	60	5	16.7	4.0399
350	2.5441	2	2	2	60	6	20	4.1584
48 Hours								
Control	-	0	0	0	60	0	0	0
100	2	1	1	2	60	4	13.3	3.8877
150	2.1761	2	2	3	60	7	23.3	4.271
200	2.301	2	3	3	60	8	26.7	4.3781
250	2.3979	3	3	4	60	10	33.3	4.5084
300	2.4771	4	4	5	60	13	43.3	48313
350	2.5441	4	5	5	60	14	46.7	4.9172
72 Hours								
Control	-	0	0	0	60	0	0	-
100	2	2	2	2	60	6	20	4.1584
150	2.1761	2	3	3	60	8	26.7	4.3781
200	2.301	3	3	4	60	10	33.3	4.5084
250	2.3979	4	4	4	60	12	40	4.7467
300	2.4771	5	4	5	60	14	46.7	4.9172
350	2.5441	6	5	6	60	17	56.7	5.1687
96 Hours								
Control	-	0	0	0	60	0	0	-
100	2	5	4	5	60	14	46.7	4.9172
150	2.1761	6	5	6	60	17	56.7	5.1687
200	2.301	6	6	6	60	18	60	5.2533
250	2.3979	6	7	7	60	20	66.7	5.4316
300	2.4771	7	8	7	60	22	73.3	5.6219
350	2.5441	8	8	9	60	25	83.3	5.9661

APPENDIX VII: PROBIT AND LOG DOSE OF DRILL CUTTINGS AGAINST PACHYMELANIA AURITA

24 Hours (Y = 1.23 + 1.14x)					
X	1	2	3	4	5
+ 1.14x	1.14	2.28	3.42	4.56	5.7
1.23	1.23	1.23	1.23	1.23	1.23
Y =	2.37	6.51	4.65	5.79	6.93
48 Hours (Y = 0.13 + 1.88x)					
X	1	2	3	4	5
+1.88x	1.88	3.76	5.64	7.52	9.4
0.13	0.13	0.13	0.13	0.13	0.13
Y =	2.01	3.89	5.77	7.65	9.53
72 Hours (Y = 0.47 + 1.81x)					
X	1	2	3	4	5
+1.81x	1.81	3.62	5.43	7.24	9.05
0.47	0.47	0.47	0.47	0.47	0.47
Y =	2.28	4.09	5.9	7.71	9.52
96 Hours (Y = 1.47 + 1.69x)					
X	1	2	3	4	5
+1.69x	1.69	3.38	5.07	6.76	8.45
1.47	1.47	1.47	1.47	1.47	1.47
Y =	3.16		6.54	8.23	9.92

APPENDIX VIII: ACUTE TOXICITY RESPONSE OF SESARMA HUZARDI TO DRILL CUTTINGS EXPOSURE

Concentration (ml\l)	Log Concentration	Replicate			Total No of Organisms	Total Mortality	% Mortality	Probit
		1	2	3				
24 Hours								
Control	-	0	0	0	30	0	0	-
820	2.9138	0	0	0	30	0	0	-
840	2.9243	0	0	0	30	0	0	-
860	2.9345	0	0	0	30	0	0	-
880	2.9445	0	0	0	30	0	0	-
900	2.9542	0	0	0	30	0	0	-
48 Hours								
Control	-	0	0	0	30	0	0	-
820	2.9138	2	2	3	30	7	23.3	4.271
840	2.9243	3	2	3	30	8	26.7	4.3781
860	2.9345	5	4	4	30	13	43.3	4.8313
880	2.9445	6	5	6	30	17	56.7	5.1687
900	2.9542	7	6	7	30	20	66.7	5.4316
72 Hours								
Control	-	0	0	0	30	0	0	-
820	2.9138	4	4	5	30	14	46.7	4.9172
840	2.9243	5	6	6	30	17	56.7	5.1687
860	2.9345	6	7	6	30	19	63.3	5.3398
880	2.9445	6	8	7	30	21	70	5.5244
900	2.9542	8	8	8	30	24	80	5.8416
96 Hours								
Control	-	0	0	0	30	0	0	-
820	2.9138	6	5	6	30	17	56.7	5.1687
840	2.9243	7	6	7	30	20	66.7	5.4316
860	2.9345	8	8	8	30	24	80	5.8416
880	2.9445	8	9	9	30	26	86.7	6.1123
900	2.9542	9	10	10	30	29	96.7	6.8522

APPENDIX IX: PROBIT AND LOG DOSE OF DRILL CUTTINGS AGAINST SESARMA HUZARDI

48Hours (Y = -85.90 + 30.92X)					
X	1	2	3	4	5
30.92X	30.92	61.84	92.76	123.68	154.6
-85.9	-85.9	-85.9	-85.9	-85.9	-85.9
Y	-54.98	-24.06	6.86	37.78	68.7
72Hours (Y = -58.17 + 21.65X)					
X	1	2	3	4	5
21.65X	21.65	43.3	64.95	86.6	108.25
-58.17	-58.17	-58.17	-58.17	-58.17	-58.17
Y	-36.52	-14.87	6.78	28.43	50.08
96Hours (Y = -101.90 + 36.72X)					
X	1	2	3	4	5
36.72X	36.72	73.44	110.16	146.88	183.6
-101.9	-101.9	-101.9	-101.9	-101.9	-101.9
Y	-65.18	-28.46	8.26	44.98	81.7