Metallothionein induction in edible mangrove periwinkles, *Tympanotonus fuscatus var radula* and *Pachymelania aurita* exposed to Oily Drill Cuttings

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Abstract: Metallothionein levels were monitored over 32 days in two gastropod species *Tympanotonus fuscatus* and *Pachymelania aurita* exposed to oil coated drill cuttings. In *T. fuscatus*, metallothionein levels were enhanced in all treatment groups during the study, with highest level concentrations being observed in organisms exposed 0.01 96h LC₅₀ drill cutting concentration. Metallothionein levels in *Pachymelania aurita* on the other hand was reduced in test animals exposed to the drill cuttings in comparison to their background level. The implications of the finding and possible inclusion of metallothionein in biomonitoring programmes involving the evaluation of impact of drill cuttings disposal on aquatic ecosystems are discussed. [Journal of American Science 2010;6(2):89-97]. (ISSN: 1545-1003).

Key words: Metallothionein, induction, mangrove periwinkles, Drill Cuttings

1. Introduction

Several tools are available for measuring impacts from environmental stressors at different levels of biological organisation (Depledge and Hopkin, 1995). Conventionally pollution has been monitored by measurement of levels contaminants in environmental receptors including measurement of bioaccumulation of toxicant in the body tissues of organisms. Currently, emphasis has shifted to measurement of biological responses to complement traditional methods in biomonitoring programmes (Damiens et al., 2004). Biological responses otherwise known as biomarkers are sensitive indicators but need to be prognostic to be relevant (Galloway et al., 2002). There proper application according to Handy et al., (2003) is in building a weight of evidence case that pollution is affecting the health of organisms and that certain classes of chemical are responsible. Some commonly measured biomarkers include metallothionein, a common indicator of heavy metal exposure; lysosomal stability, a general indicator of stress; EROD activity, which commonly indicates oxidative stress and acetylcholinesterase inhibition, a marker of organophosphorus and carbamate pesticide exposure.

Biomarker responses are affected by environmental factors (English and Storey, 2003). Times taken to induce the biomarker as well as the persistence of the biomarker are issues to consider in incorporating biomarkers in biomonitoring studies (Howard et al., 2002). Biomarker based approaches has gained immense support and research in developed world (Brown et al., 2004; Ringwood et al., 2004; Handy et al., 2003; Galloway et al., 2002; Viarengo et al., 1999; Moore et al., 1999) but their use in developing world especially in Nigeria is non-existent possible due to lack of local literature and expertise.

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. Metallothioneins are believed to be important in homeostasis of heavy metals such as copper and zinc, and detoxification of heavy metal (Butler and Roesijadi, 2001), thus they are commonly associated with heavy metal pollution. Their induction confers metal tolerance to organisms (Klaasseen et al., 1999) due to their ability to bind some heavy metals such as cadmium and copper (Coucelo et al., 2000). This concentration of heavy metals has raised questions on possible trophic transfer especially in edible species (Wang, 2002). In addition accumulation of heavy metals may disturb essential metal homeostasis by causing changes in cellular metal binding and speciation patterns. Because they are additionally synthesis following heavy metals exposure, metallothioneins have been proposed as a potential biomarker for heavy metal exposure (De Smet et al., 2001; Roesijadi, 1994). Evidence of protection against oxidative stress by metallothionein has been
were able to bide 14C from 14C-CCl4 in the MT-induced animals than in controls, with a concomitant reduction of covalent binding of 14C-CCl4 to cellular protein and lipid. Furthermore, induction of metallothionein is affected by various biotic and abiotic factors (English and Storey, 2003; Leung et al., 2001; Ghoshal et al., 1998). 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 objective of this study was to study the metallothionein responses of two common gastropod species of Lagos lagoon on exposure to drill cuttings. Drill cuttings are produced during exploitation of crude oil, and in Nigeria their discharges are allowed after treatment in offshore water according to guidelines of the Department of Petroleum Resources (DPR, 2002). Accidental discharges in inland waters and swamps areas are not ruled out and may affect the natural biota in the influence of exploitation areas. This study is therefore a step in studying a common biomarker response of local organisms exposed to sublethal concentration of drill cuttings.

*Tympanotonus fuscatus* and *Pachymelania aurita* were chosen for the study because of their abundance in the Niger Delta region and around the mangrove swamps and sandy shores of the Lagos lagoon complex. Two subspecies of *T. fuscatus* are found in West Africa, *T. fuscatus fuscatus* (Linne) with tubercular spines and *T. fuscatus* (var radula) with smooth or granular shell ornamentations. Both types are found at 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). *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 m2/yr and a production: biomass ratio that varies between 0.02 and 0.12 (Brown, 1991). Both gastropod species are edible and very rich in protein (Egonmwan, 1980, Adebayo-tayo et al., 2005), and thus serve as important source of protein to local inhabitants, but their high microorganisms content is a health issue which raises concern over their consumption (Adebayo-tayo et al., 2005).

2. Materials and Methods

**Animals**

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

1. *Tympanotonus fuscatus* var *radula L* (periwinkle) (Mollusca, Gastropoda, Melanidae), (length 32-35 mm , diameter of aperture 0.7 – 9.0 mm)

2. *Pachymelania aurita* (periwinkle) (Mollusca, Gastropoda, Potamidae), (length 35 – 40 mm, diameter of aperture 0.8 – 1.0 mm).

*P. aurita* and *T. fuscatus* species were collected from field by hand picking into a holding bucket from the edge of the Lagos Lagoon and adjacent mangrove swamps at low tide. They were taken to the laboratory and left in holding tanks with a thin layer of sediment serving as substrate and food source to organism, and some water to allow them to acclimatise to laboratory conditions. Lagoon water and sediments were also simultaneously collected for use in the bioassay procedures.

**Test Chemicals**

The toxicant used for the bioassays is drill cuttings which were collected from Shell Development Petroleum Corporation. The cuttings were coated with the oil based mud used during the drilling process.

**General Bioassay procedure**

Biomarker studies were carried out using plastic tanks, which measured 30 cm x 25 cm x 16 cm. The collected sediments were sun dried to standardise moisture content, and thereafter passed through a sieve (0.25mm) to obtain uniform substrate particles. 100 g of sediment was introduced into the experimental chamber and lagoon water was used as the diluents for the entire bioassay test conducted.. 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.

**Sublethal Toxicity Test**

Static renewal method was adopted during exposure period. Test solutions and sediment were renewed on day 4, 8, 16 and 32. For the series of bioassays, one hundred and twenty (120) specimens of each test species were exposed to drill cuttings including control in two replicates (20 animals per treatment per replicate). The concentrations of drill cuttings used were derived from the 96hLC50 value.
obtained for the toxicant in a previous study with the organisms, and are as follows:
Drill cuttings was tested against *T. fuscatus* as follows:
- 10.24 ml (i.e. 0.01 of the 96hLC₅₀) plus 989.76 ml lagoon water,
- 1.024 ml (i.e. 0.1 of the 96hLC₅₀) plus 998.976 ml lagoon water, and
- Untreated control.
The test concentrations for *P. aurita* were as follows:
- 12.2 ml/l (i.e. 0.01 of the 96hLC₅₀) plus 987.78 ml lagoon water,
- 1.22 ml/l (i.e. 0.1 of the 96hLC₅₀) plus 998.78 ml lagoon water, and
- Untreated control.

**Tissue preparation**
For each treatment, specimens of each species were randomly collected from bioassay chambers on day 4, 8, 16 and 32 for analysis. Samples from different individuals of the same species were pooled to minimise individual variability. The digestive glands of the organisms were chosen for metallothionein studies. In removing the digestive gland of the periwinkles, the anterior shell was gently broken and the digestive gland removed.

**Metallothionein Determination**
Metallothionein levels were assessed on the digestive gland homogenates content of the samples by evaluating the sulphydryl (-SH) residue according to Ellman (1959); Viarengo *et al* (1997). 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 (nmol MT).g⁻¹. Chemicals were obtained from Ikzus Environment, Italy.

**Reagent and working solutions preparation:**
Sulphydryl reference (reduced glutathione) standard was prepared by adding 0.725 Sol C₂ (resuspension buffer Component 2) to a microtube. The reaction buffer (sol D) was obtained by mixing 200 ml stabilised Ellman’s buffer and 3.4 ml concentrated Ellman’s reagent and equilibrated at 23°C. Absolute ethanol was also equilibrated at −20°C. The homogenising 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 C₁ (resuspension buffer, Component 1) and sol C₂ (resuspension buffer, Component 2). The blank solution was obtained by mixing 50 μl 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 20 min. The protein content was then determined from the supernatant by means of Bradford assay. The supernatant was collected in a 2 ml tube. 1.5 ml of cold absolute ethanol was added to the tube and incubated at −20°C for 30-60 min. The mixture was then centrifuged at 12-16,000g at 4°C for 5 minutes. 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 room temperature and then centrifuged at 12-16,000g. Absorbance was thereafter read at 412 nm 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 ABS₄₁₂ value of sample was interpolated over the standard curve to obtain the concentration (nmol) of sulphhydryl groups, i.e. cysteine residues (nmol Cys⁻¹), due to metallothionein present in the sample. To obtain the concentration of metallothionein (nmol MT) per gram of tissue, the following was applied:

\[
\text{nmol MT} \cdot g^{-1} = \frac{\text{n} \cdot \text{Cys}^{-1}}{0.1g} \cdot \text{ABS} \cdot 412
\]

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

\[\text{n} \cdot \text{Cys}^{-1}\]

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:

\[
\text{nmol MT} \cdot mg^{-1} = \frac{\text{n} \cdot \text{Cys}^{-1}}{\text{mg} \cdot \text{n} \cdot \text{Cys}^{-1}}
\]

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

**Statistical Analysis**
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 to test for significant difference between means of biomarker results obtained between *T. fuscatus* and *P. aurita*. 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.

### 3. Results
For *T. fuscatus* MT values 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 1). Metallothionein level was enhanced in all treatment groups including control during the experiment. The concentrations were significant for all treatment groups (ANOVA, P < 0.05). Post hoc test with Duncan indicated that for all treatment groups there was significant difference (P < 0.01) in MT concentration on all sampling days. MT was however greater induced in organisms exposed to drill cuttings than in control organisms, with higher values in T. fuscatus exposed to 0.01 96h LC₅₀ than those exposed to 0.1 96h LC₅₀ drill cuttings concentration. Concurrently with the observed enhanced production of MT was increase in total protein in the organism during the study (Table 1). The observed increase in protein production was higher in control and in 0.01 96h LC₅₀ drill cutting concentration exposed T. fuscatus than in T. fuscatus exposed to 0.1 96h LC₅₀ drill cutting concentration, but a higher percentage of the new protein was converted to MT in 0.01 96h LC₅₀ drill cutting concentration exposed T. fuscatus, but not in control and in 0.1 96h LC₅₀ drill cutting exposed T. fuscatus (Figure 1).

In P. aurita, the observed background MT level was higher than that of T. fuscatus. The values of MT recorded during the experiment ranged from 3.4430 nmolMT g⁻¹ to 3.8865 nmolMT g⁻¹ in control organisms (mean = 3.4998 nmolMT g⁻¹), 0.1902 nmolMT g⁻¹ to 3.8807 nmolMT g⁻¹ (mean = 2.2543 nmolMT g⁻¹) in organisms exposed to 1.22 ml/l, and 0.3301 nmolMT g⁻¹ (mean = 1.4554 nmolMT g⁻¹) to 3.3865 nmolMT g⁻¹ in 12.22 ml/l exposed organisms (Table 2). In the control, the concentration of MT was comparatively unchanged with time, 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 drill cuttings 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 2, Figure 2). Correlation between MT concentration in the digestive gland of P. aurita and exposure time was positive for control (b = 0.965) and for P. aurita exposed to 0.01 96hLC₅₀ drill cuttings (b = 0.523) and weakly negative for P. aurita exposed to 0.1 96hLC₅₀ drill cuttings concentration (b = -0.096).

<table>
<thead>
<tr>
<th>Table 1: Protein and metallothionein concentrations in T. fuscatus on sublethal exposure to drill cuttings (mean ± SD)</th>
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<tbody>
<tr>
<td>Incubation Time (Day)</td>
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<td>------------------------</td>
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<tr>
<td>0</td>
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<tr>
<td>4</td>
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<tr>
<td>8</td>
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<td>16</td>
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<tr>
<td>32</td>
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<tr>
<td>Incubation Time (Day)</td>
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<td>0</td>
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<td>Incubation Time (Day)</td>
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<td>16</td>
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<tr>
<td>32</td>
</tr>
</tbody>
</table>

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).
Table 2: Induction of metallothionein in *P. aurita* on sublethal exposure to drill cuttings (mean ± SD)

<table>
<thead>
<tr>
<th>Incubation Time (Day)</th>
<th>no Cys</th>
<th>Wt of tissue (g)</th>
<th>Protein (mg/l)</th>
<th>nmol Cys\text{MT}</th>
<th>nmol MT g\text{^{-1}}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23</td>
<td>0.1</td>
<td>11.14 ± 0.025^d</td>
<td>7.789 ± 0.00^b</td>
<td>3.3865 ± 0.00^a</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>0.1</td>
<td>10.915 ± 0.013^e</td>
<td>7.809 ± 0.00^d</td>
<td>3.3952 ± 0.00^b</td>
</tr>
<tr>
<td>8</td>
<td>23</td>
<td>0.1</td>
<td>20.906 ± 0.012^e</td>
<td>7.919 ± 0.00^c</td>
<td>3.443 ± 0.00^c</td>
</tr>
<tr>
<td>16</td>
<td>23</td>
<td>0.1</td>
<td>38.688 ± 0.028^b</td>
<td>8.01 ± 0.00^b</td>
<td>3.482 ± 0.00^d</td>
</tr>
<tr>
<td>32</td>
<td>23</td>
<td>0.1</td>
<td>42.666 ± 0.145^a</td>
<td>8.72 ± 0.00^a</td>
<td>3.7914 ± 0.00^e</td>
</tr>
</tbody>
</table>

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).
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.

As was observed in T. fuscatus the average protein concentration increased above the background level during the study. The observed increase in protein concentration was higher in control organisms relative to drill cuttings exposed groups. A further examination of the results show that in control, but for the observed increase on day 4, the relative concentration of MT relative to total proteins steadily reduced on all sampling days during the study. In drill cuttings exposed organisms, there was a fall in the concentration of MT relative to total protein on day 4, after which the ratio started to increase, but the basal ratio was not achieved in both groups during the study.

4. Discussion

In this study, metallothionein level in T. fuscatus increased in all treatment groups including control. However, over time MT synthesis was greatest in species exposed to 0.01 96h LC50 drill cutting concentration compared to control and species exposed to 0.1 96h LC50 rill cutting concentration. At low 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. At 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. Increase in MT production was observed only after day 16 following substantial increase in protein production during this period. A study of relative concentration of MT to total protein showed that in T. fuscatus, low exposure to drill cuttings promoted proteins synthesis including metallothionein. On the other hand enhanced protein synthesis was not proportionate to MT production in control and at higher exposure to drill cuttings, hence MT compared to total proteins concentration was reducing in these groups. The findings suggest that on exposure of T. fuscatus to sublethal concentration of drill cuttings, the organisms channelled energy for growth and reproduction into production of enzymes and metallothionein to fight the stressed environmental condition.

In P. aurita, exposure to drill cuttings 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 with high levels of protein synthesis. In drill cuttings exposed P. aurita on the other hand MT production was suppressed though increased concentration of proteins was observed during the
that the organism’s ability to cope with stress may have reference organisms. In lower reproductive rate and population density than cuttings are more likely to have stunted growth and organisms chronically exposed to low levels of drill production of this protein. The implication is that reproduction was concentrated to increase the chemical and suggested that energy for growth and physiological response of the organism on exposure to synthesis of MT by

(1994) It has also been associated with acclimation of invertebrates (Viarengo and Nott, (1993); Roesijadi (Sathyanathan, 1996) to metal. Induction of MT induction in response to restraint stress. Its induction to heavy metal pollution both in fish (Killie et al., (1992); Hogstrand et al., (1996)) and in invertebrates (Viarengo and Nott, (1993); Roesijadi (1994)) It has also been associated with acclimation of fish (Dixon and Sprague, 1981) and invertebrate (Sathyanaathan, 1996) to metal. Induction of MT however is not restricted by heavy metals alone. For instance, Ghoshal et al (1998) have demonstrated MT induction in response to restraint stress. Its induction has also been associated with non-metallic elements as well with endogenous biological processes. MT induction has been linked to reduced growth in laboratory experiments in fish (Roch and McCarter, 1984) and in field experiment with invertebrates (Leung et al., 2001). In the present study, additional synthesis of MT by T. fuscatus could be attributed to a physiological response of the organism on exposure to the chemical and 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. Our findings demonstrated the presence of MT in the two gastropod species studied and the variations in their induction on exposure to sublethal concentrations of the toxicant.

Ours is a preliminary study on the response of MT in these gastropod species and our findings lend credence to the inclusion of metallothionein as a general biomarker during biomonitoring programmes during evaluation of impacts of drill cuttings on aquatic lives. However, its application in this regard demand further study on characterization and quantification of the isoforms, confounding factors and most importantly the responses of the organisms in their natural environments.

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References


23. Leung KMY, Morgan JI, Wu RSS, Lau JS and Furness RW. Growth rate as a factor confounding the use of the dogwhelk Nucella lapillus as biomonitor of heavy metal contamination. Marine Ecology Progress Series 2001; 221:145-159.


