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# Differential expression of metallothionein-1 and cytochrome p450-2a5 (cyp2a5) in mice in response to lead acetate exposure and industrial effluents in Ibadan, Nigeria

**Ndudi Cyril Chidinma[1], Adebayo Adewale[2] and Anumudu Chiaka[2]**

## Abstract

Metallothionein-1 (*MT-1*), cytochrome P450-2A (*CYP2a*) and other genes are involved in the detoxification of xenobiotics such as heavy metals and toxins. Changes in their expression precede overt toxic effects and can serve as a marker for exposure to pollutants. We used a mouse experimental system and quantitative reverse transcription polymerase chain reaction to determine changes in gene expression and the direction of change, in response to exposure to lead acetate (LA) and waste water (WW) from an industrial area in Ibadan. *MT-1* and *CYP2a5* genes were quickly and highly induced at different exposure periods and concentrations. *MT-1* was mostly downregulated by the LA exposure, but upregulated by several folds on exposure to WW. *CYP2a5* expression was mostly downregulated with LA exposure. The optimum expression of *MT-1* and *CYP2a5* genes induced by both LA and WW was at 48 h. We conclude that rapid assays to determine the direction of change in the expression of *MT-1* and *CYP2a5* could be a fast and reliable method in developing countries for screening humans exposed to pollutants from industrial waste.

## Keywords

gene expression, heavy metals, industrial pollution, RT-PCR, toxicogenomics

## Introduction

Industrial waste water (WW) and effluents are potential threats to the quality of surface and groundwater, aquatic ecosystem and human health. It is estimated that about 2 million tonnes of sewage and other effluents are discharged into the world's waters everyday. In developing countries, the situation is worse because over 90% of raw sewage and 70% of untreated industrial wastes are dumped into surface water sources (Azizullah et al., 2011). In order to confirm whether industrial WWs are treated according to guidelines or not, the presence and quantity of inorganic chemicals (such as heavy metals), organic chemicals and volatile compounds (such as benzene and toluene) may be determined. Heavy metals occur naturally in the environment and are present in rocks, soil, plants and animals; however, following industrial development, there are fears that local waters are now heavily contaminated with them.

Heavy metal pollution in water bodies may damage marine organisms at the cellular level, affect the ecological balance and cause deleterious effects on biota and human health. These effects could be due to their high densities, which are at least five times that of water (Faweya and Babalola, 2010). Among these heavy metals, lead (Pb) compounds such as lead acetate (LA) are soluble and are often used in industrial processes for the production of cosmetics and dyes; whilst they may also be by-products of

[1]Department of Zoology, Ecology and Environmental Biology Unit, University Of Ibadan, Ibadan, Nigeria
[2]Department of Zoology, Cell Biology and Genetics Unit, University Of Ibadan, Ibadan, Nigeria

**Corresponding author:**
Anumudu Chiaka, Department of Zoology, Cell Biology and Genetics Unit, University Of Ibadan, Ibadan 200284, Nigeria.
Email: cianumudu@yahoo.com

other processes. Since these metals are non-biodegradable and have the ability to bioaccumulate in the environment, the danger they pose has attracted serious public concern. Water bodies in Nigerian cities including Ibadan have been reported to be polluted, principally due to the discharge of untreated wastes and heavy metals, including Pb, into rivers by industries (Daso and Osibanjo, 2012). The threat is further compounded by the frequent use of these water bodies for domestic and economic activities in many local communities.

The activities of antioxidant enzymes in animals are influenced by the presence of various environmental factors, which increase the formation of reactive oxygen species (Puppel et al., 2014). Such antioxidants include metallothionein (MT), cytochrome P450-2A (CYP2a), superoxide dismutase, catalase and heme-oxygenase (Wang et al., 2011; Yu et al., 2009). MT is a heat stable, low-molecular weight (7 kDa), cysteine-rich protein, which has high affinity for metals. Besides detoxification of toxic metals, MT is involved in the homeostasis of essential trace elements and scavenging of free radicals (Maines, 2009). Its expression is influenced by heavy metals, alkylating agents, ultraviolet irradiation and hormones (Yu et al., 2009).

*CYP* is a multigene superfamily encoding a considerable number of isoforms, all of which are involved in the metabolism of numerous exogenous and endogenous compounds (Wang et al., 2003). These CYP enzymes are expressed in a tissue-selective manner, and the main site of detoxification is the liver. A typical feature of xenobiotic-metabolizing P450s is their inducibility by a large number of different agents, each showing a typical pattern of response in terms of the specific isoenzymes and activities induced (Alexandre et al., 2012; Kirby et al., 2011).The *CYP2a* family represented by the *CYP2a5* gene in mouse and the orthologous *CYP2a6* in humans are involved in liver metabolism of toxins and response to toxic insults (AbuBakar et al., 2013). There is evidence for circadian accumulation of CYP2a5 in liver microsomes, and they can also be present in non-hepatic tissues such as the olfactory mucosa (Alexandre et al., 2012; Genter et al., 2006).

There is keen interest in the human molecular response to conditions of oxidative stress brought about by pollution and heavy metal contamination, through the study of gene expression. Animal models such as mice are very useful in this regard because they offer a physiological system close to that of humans. It is from this point of view that we aim to investigate the level of expression of *MT-1* and *CYP2a5* genes in response to exposure to WW and LA from industrial effluents in a local river.

## Materials and methods

### Study area

Ibadan in Oyo state, Southwestern Nigeria, is regarded as the largest city in West Africa covering an area of 240 $km^3$. The city is located on longitude 3°5′E and latitude 7°20′N. The Ona River is a major water body in Ibadan, and it flows through the city and nearby rural localities and receives water from smaller streams within the city (Adeyemo, 2008). A large proportion of manufacturing industries in the city are cited in the Oluyole Industrial Estate, through which the river passes. Hence, the Ona River serves as a means of disposal for wastes and effluents from several industries, where waste treatment procedures cannot be vouched for. Previous studies on the Ona River have reported that there is moderate to high pollution, as a result of the introduction of WW and heavy metals (especially zinc (Zn), Pb, cadmium (Cd) and copper (Cu)) in industrial effluents, above permissible levels (Ogedengbe and Akinbile, 2011).

### Sampling and exposure

Male albino mice (10–12 weeks old) were housed in plastic cages and allowed to acclimatize to the conditions in the animal house for 14 days, in 12-h light/12-h dark condition. They were fed with standard mice feed pellets (Livestock Feeds, UAC Plc, Nigeria) and tap water. The average weight of the mice was 27 g. All procedures were in accordance with ethical guidelines of the University of Ibadan Guide for Care of Laboratory Animals in Experimental Investigations. After acclimatization, three groups of 8 mice totaling 24 were exposed to LA, and a similar group was exposed to WW. Six mice served as control.

WW was collected from the study site. This was administered intraperitoneally to the mice (of the WW treatment subgroup), after serial dilutions to give concentrations of 50, 75 and 100%, and the control subgroup was given tap water. LA treatment subgroups were exposed to 100, 200 and 300 µmol/kg concentrations of LA (BDH Reagents, BDH-Merck, Poole Dorset, UK) via intraperitoneal injection, while the control subgroup was given normal saline.

The three treatment subgroups for both LA and WW had exposure periods of either 24, 48 or 72 h. There was no mortality or strange behavioural changes during the treatment period.

### Heavy metal analysis

From the main stock of pooled WW, 5 ml was digested with 10 ml of acid mixture (nitric acid/perchloric acid at 100°C for 1 h). After cooling, the beaker was rinsed with distilled water into a 25-ml volumetric flask. Then, 25 ml of the digested WW sample was transferred to a Buck Atomic Absorption Spectrophotometer (model 210 VGP American specifications; Norwalk, Connecticut, USA) for Zn, Cu, Pb, Cd and cobalt analysis. A standard solution that had known concentrations of Cu, Zn, Cd, cobalt and Pb was used to calibrate the spectrophotometer at the following wavelengths for the metals: 325, 214, 228.9, 240.7 and 283 nm, respectively. The concentrations of the heavy metals were calculated using the following formula:

$$\text{Concentration (mg/l)} = \frac{\text{mg/l of machine reading} \times \text{volume of digest}}{\text{weight of sample}}.$$

Uniformly, volume of digest was 25 ml and weight of sample was 5 g = 5 ml of sample.

### RNA isolation

At the end of the exposure to WW and LA, mice were killed via cervical dislocation. The livers were quickly removed and fixed in RNAlater™ (Life Technologies, Chesire, UK) RNA stabilization reagent, followed by storage at −80°C until required. Pooled RNA from each treatment subgroup and control (total RNA) was extracted from the frozen liver tissues after homogenization, by using RNeasy Protect Minikit (QIAGEN, Valencia, California, USA) and following the manufacturer's protocol.

### Preparation of cDNA

Complementary DNA (cDNA) for each time point and exposure level was made from TaqMan gold reverse transcription PCR (RT-PCR) reaction mix, consisting of RT buffer (10×) (2.5 µl), 25 mM magnesium chloride ($MgCl_2$; 5.5 µl), dNTP mix (5.0 µl), Oligo(dT) (1.25 µl), RNase inhibitor (0.50 µl), multisense reverse transcriptase (1.60 µl) and total RNA (8.65 µl). A NanoDrop spectrophotometer (ThermoScientific; Wilmington, Delaware, USA) was used to quantify whole cDNA. Before quantifying gene expressions, the extracted total RNA from the samples was confirmed intact prior to cDNA synthesis, with wide bright bands for the internal control 23S and 5S ribosomal RNA on agarose gel. The cDNA was also intact with visible bands on gel lanes.

### Real time-quantitative PCR

RT-PCR analysis was carried out on a 7500 Applied Biosystems real-time PCR machine (Carlsbad, California, USA). The reaction mix was made up of SYBR Green buffer (10×, 2.0 µl; Sigma Aldrich (St Louis, Missouri, USA), 25 mM $MgCl_2$ (2.4 µl), dNTP mix (1.6 µl), amplitaq gold (0.1 µl), Amperase uracil *N*-glycosylase, which prevents cross contamination (0.2 µl), forward primer (1.5 µl), reverse primer (1.5 µl), template (cDNA; 1.5 µl) and water (7.8 µl). The PCR conditions were as follows: a hold time of 10 min at 95°C, denaturation at 95°C for 15 s, annealing at 60°C for 1 min and looping for 40 times. The primers used for the respective genes were as follows: *MT-1* F5′-CACCAGATCTCGGAATGGAC-3′; *MT-1* R5′-GGAGGTGCACTTGCAGTTCTTG-3′; *β-actin* F5′ sense: 5′-CATGGATGACGATATCGCT-3′; *β-actin* R3′ antisense: 5′-ATGAGGTAGTCTGTCAGGT-3′; *CYP2a5* F 5′-GAGATTTCCTCCTCCCCAAG-3′; and *CYP2a5* R 5′-TAGCCAGTCCTTCTCCGAAA-3′. Actin was included as a housekeeping or normalization gene, and relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

After determination of the mean cycle threshold and $\Delta C_t$ (delta threshold cycle) values, the relative quantity (RQ) was calculated as $2^{-\Delta C_t}$. RQ values less than 1 were equivalent to downregulation, and the number of folds of such regulation was determined by the ratio of RQ value of control to the RQ value of sample treatment. RQ values above 1 (or log RQ above zero) were equivalent to upregulation, and the number of folds of such regulation was determined by the ratio of RQ value of sample treatment to the RQ value of control. For all control samples, RQ value was 1 and log RQ values 0. Log RQ values were determined in samples and plotted in graphs using Tableau™ software (version 8.1.2, 2013) (Figure 1).

## Results and discussion

### Heavy metal content

The mean concentrations of Cu, Zn and Cd from WW samples at the study site were above the World

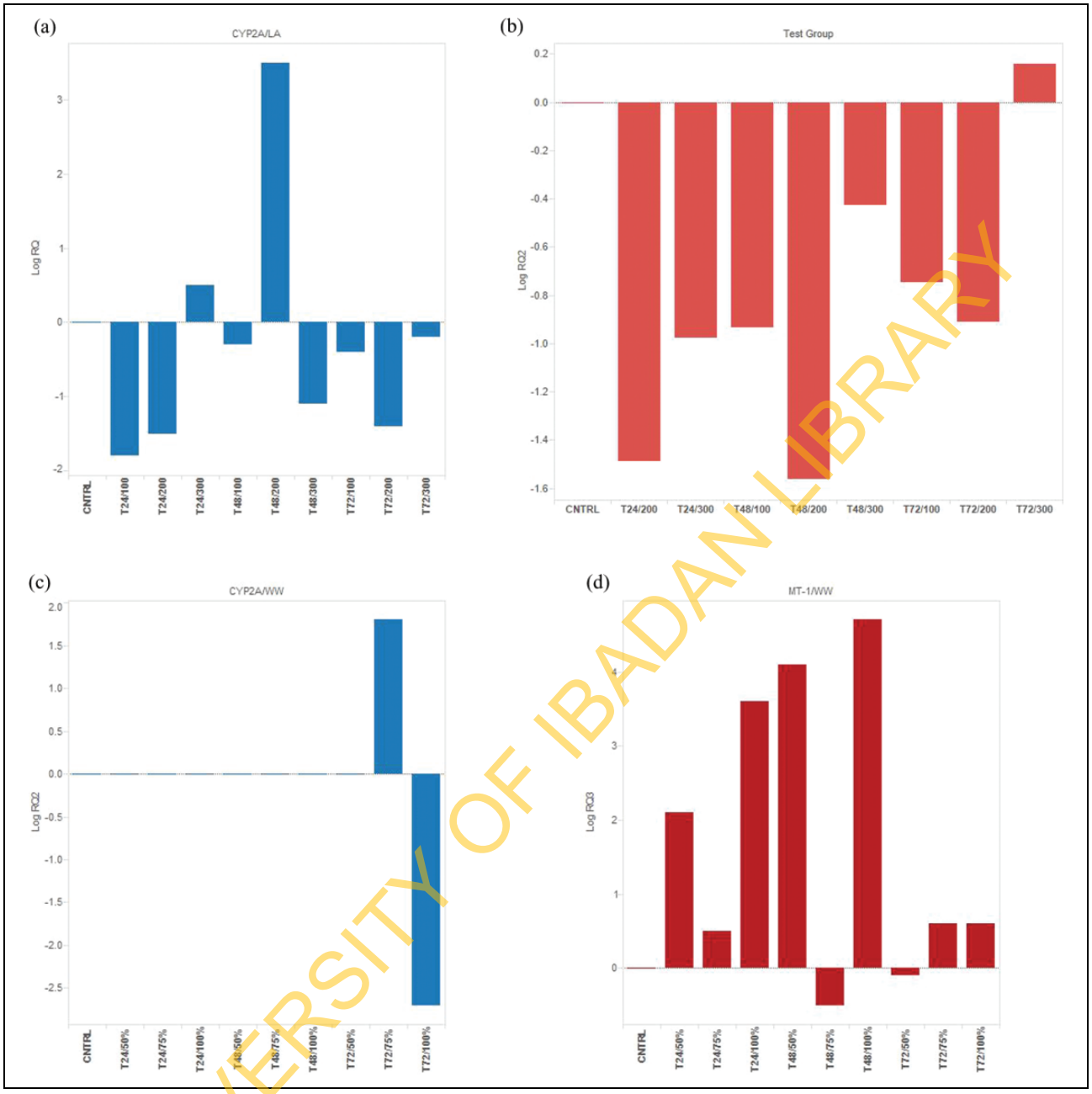


**Figure 1.** Graphs showing changes in gene expression (CYP2a5 in (a) and (c) and MT-1 in (b) and (d)) due to LA exposure (a and b) and WW exposure (c and d) from industrial effluents in Ibadan. CYP2a5: cytochrome P450-2A; MT-1: metallothionein-1; WW: waste water.



Health Organization standard for freshwater habitats (Table 1). The heavy metal analysis suggests that effluents were not properly treated before being discharged into the Ona River, and this may have a negative impact on aquatic life. The findings corroborate related studies (Ogedengbe and Akinbile, 2011).

*LA exposure.* In treatment samples, *CYP2a5* expression was downregulated (between 2- and 26-fold) at all concentrations, for the 72-h exposure group (T72; Figure 1(a)). The 24-h (T24) and 48-h exposure groups (T48) did not show uniform down- or upregulation. However, with higher concentration in the T48, *CYP2a5* expression was increased. Also, there was consistent *CYP2a5* downregulation with higher exposure periods (from T48/300 µmol to T72/300 µmol), though the number of folds by which the gene was downregulated did not increase progressively

**Table 1.** Heavy metal content of WW from Ona River, Ibadan, Nigeria.

| Heavy metal type | Sample concentration (mg/l) | WHO standard (mg/l) |
|---|---|---|
| Cu | 0.074 | 0.050 |
| Zn | 0.460 | – |
| Cobalt | 0.000 | – |
| Cd | 0.290 | 0.003 |
| Pb | 0.000 | 0.01 |

Cu: copper; Zn: zinc; Pb: lead; LA: lead acetate; WW: waste water; WHO: World Health Organization.

with increasing concentration. It was also observed that there was continuous shift in trends, that is, downregulation for almost all 24-h exposure, upregulation for T24/300 µmol and T48/200 µmol and eventual downregulation for T72 group. Overall, the induction of *CYP2a5* expression by LA was in the order of T48 > T24 > T72. Previous studies, such as those by AbuBakar et al. (2013) and Kirby et al. (2011), have reported *CYP2a5* gene induction by liver stress. The results from this study suggest that *CYP2a5* downregulation in response to LA may not be immediate; and that longer assays of up to 72 h or more should provide more confidence in the measurement of the response. The dynamics of *CYP2a* expression (in liver tissues) in response to Pb and heavy metal exposure periods need to be further investigated.

*MT-1* gene in the same LA samples was downregulated (between 3- and 36-fold) across all exposure periods and all concentrations except for the T72/300 µmol group, in which the gene was upregulated by only 1.4-fold (Figure 1(b)). With increased exposure periods or concentration, there were fluctuations in the extent of the downregulation of *MT-1*. This indicated that generally, LA exposure induced the downregulation of *MT-1* at different doses within 24–72 h. The induction was in the order of T24 < T48 < T72, and it decreased with exposure time. These findings may align with the established protective function of MT-1 against heavy metal toxicity in previous studies and yet differ in some respects. Yu et al. (2009) corroborated observations that *MT* expression is induced by LA at both at the messenger RNA (mRNA) and protein levels in mice. But their data suggested that Pb exerted a dual effect on *MT* expression: the enhancement of *MT* gene transcription both in the liver and kidney and the suppression of *MT* mRNA translation in the kidney. Yasutake and Nakamura (2011) also found that there was induced *MT* expression on exposure to mercuric compounds, but the induction process was different among tissues. According to the authors, the induction would occur directly through the accumulation of mercury in brain and kidney but would occur in the hepatic tissues secondarily after elevation in plasma cytokines. Nevertheless, the results above suggested a rapid induction of *MT-1* by downregulation.

*WW exposure.* For WW samples, there was no amplification of *CYP2a5* in the controls, T24/WW and T48/WW. This resulted in zero $C_t$ values and consequently, affected the determination of the direction of regulation in the sample treatment groups of T24/WW and T48/WW. However, for the T72 group, *CYP2a5* expression fluctuated. It was nil at 50%, upregulated at 75% and downregulated at 100% (Figure 1(c)).

Also in the WW samples, *MT-1* was upregulated by several folds in most treatment groups, but notably, the gene was downregulated in the T48/75% and T72/50% groups in smaller measures. Upregulation in WW samples reached the peak at T48/100% (Figure 1(d)). The induction on *MT-1* expression was generally in the order of T48 > T24 > T72.

The results from this study showed that mice exposed to WW generally upregulated *MT-1* expression. This could be linked with the fact that MT helps in detoxification, maintenance of homeostasis of essential metals such as Zn and Cu, and in preventing oxidative stress resulting from the introduction of xenobiotics into a biological system. Thus, heavy metals were considered to be the main inducer of *MT* expression in the present study. However, there is also the possibility of other components of WW bringing about *MT* expression, since previous reports have indicated that cellular and genetic toxicity could be induced by WWs (Bakare et al., 2012; Singh et al., 2007). Indeed, the present study suggests *MT-1* upregulation, in particular, could be a quick indicator of acute toxicity and system pollution.

Studies have previously reaffirmed the important roles of *MT* and *CYP* genes in response to environmental stress. *CYP2a* is known to be induced by phenobarbital and various hepatotoxic agents such as cocaine, pyrazole, solvents, heavy metals, hexachlorobutadiene and carbon tetrachloride (Alexandre et al., 2012; Kirby et al., 2011). Animal studies have also shown that the expression of

hepatic *CYP2a* is upregulated by various types of biological insults such as infection and integration of hepatitis B virus into hepatocyte DNA. In addition, the expression of *CYP2a* is influenced by immunity, and tumours in mice can be activated through elevated levels of CYP2a5 (Zhou et al., 2012). Compared with previous studies, this study showed a bidirectional, several-fold induction of *MT-1* and *CYP2a5* in mice due to acute exposure of LA and industrial effluents from an important water body in Ibadan, Nigeria.

## Conclusion

Heavy metals and other environmental toxins from industrial effluents in the Ona River and LA induced *MT-1* and *CYP2a* expression in mice, since they are only expressed when the system is perturbed by toxicants. Particularly, MT-1 proved to be sensitive, it was sharply upregulated by WW administration and downregulated by LA. Therefore, use of these genes as definitive molecular bioindicator of acute toxicity and human system pollution from Nigerian water bodies should be fully explored since human communities are at risk. It has been previously established that the protective role of these genes helps to detoxify the biological system of the mice model against heavy metal toxicity. There must also be serious societal efforts to ensure that there is good water quality in all communities as well as to ensure strict and continuous monitoring of industrial compliance with environmental laws.

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