PRIMARILY CULTURED GILL EPITHELIA AS PROTOTYPES FOR ASSESSING FISH RESPONSE TO HEAVY METAL EXPOSURE

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Reference number in book of proceedings: 16/SCI/139
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ABSTRACT
The global call to reduce the number of fish used in toxicological evaluations has necessitated the need to develop *in vitro* systems as viable alternatives. This study investigated the use of primarily cultured gill cells to assess changes in gill physiology in response to heavy metal exposure. Rainbow trout (*Oncorhynchus mykiss*) gill epithelia were cultured on permeable filter supports using a Double Seeded Insert (DSI) primary culture technique. The cells, which are tolerant to freshwater application on the apical surface, were exposed to a range of concentrations of zinc (Zn) [1-100 µM], lead (Pb) [0.5-50 µM] and cadmium (Cd) [0.01-1.0 µM] for 24 h. The expression of heavy metal responsive genes metallothionein A (mtA) and B (mtB) were quantified using Reverse Transcription quantitative Polymerase Chain Reaction (RT qPCR). Results showed that Zn significantly ($P < 0.05$) enhanced the expression of mtA and mtB in the cultured gill epithelia while Pb significantly ($P < 0.05$) inhibited the expression of mtA. This study demonstrated that primarily cultured gill epithelia is capable of detecting bioavailable metals in water and thus shows promise as a surrogate for fish toxicity tests.

Key words: Cell Culture; Aquatic Pollution; Heavy Metals; Metallothionein

INTRODUCTION

Traditionally, *in vivo* toxicity testing studies are used to measure the toxic effect of pollutants (organic / inorganic) (Don Pedro, 2009). Bioassay involves the measurement of the toxic effect of a pollutant by the changes it causes in a batch of living organisms exposed to it over a pre-determined period (Don Pedro, 2009). *In vivo* bioassays have been used extensively to carry out studies to identify possible adverse effects that may occur as a result of exposure to a toxicant and also to obtain dose-response data that are used to establish toxicity criteria for acceptable levels of chemical contamination in the environment (Bat *et al.*, 2001). They have also been used to evaluate accumulation and elimination of toxicants in exposed organisms. Invertebrates and several fish species have been employed in bioassays evaluating toxic potentials of pollutants detected in polluted aquatic

ecosystems and early life stages have been reported to be the most sensitive to toxicants (Rand *et al*., 1995).

However, in line with the global campaign to reduce, refine and replace the use of animals in *in vivo* toxicity testing studies, the use of *in vitro* assays has been proposed as a viable alternative and is receiving increased attention from the government, industry and scientific community (Fent, 1996). *In vitro* assays are also widely accepted because they serve as a rapid screening system for chemicals and give a better understanding of the mechanism of chemical induced toxicity in animals and humans. *In vitro* assays include the use of perfused organ preparations, isolated tissue preparations, single-cell suspensions, and cell-culture systems such as primary cell cultures and immortal cell lines. The cell culture system has been reported to be preferable to researchers because they are reliable, reproducible, and relatively inexpensive to assess chemical toxicity at the cellular level of biological organisation (National Research Council (NRC), 2006).

The fish gill is a multifunctional organ that is constantly in contact with the fish environment (water) and performs the functions of gas exchange, osmotic and ionic regulation, acid–base regulation, and excretion of nitrogenous wastes (Evans *et al*., 2005). The primary fish gill cell culture assay has been employed as an *in vitro* model for the study of the branchial epithelial response to aquatic toxicants (Bury *et al*., 2014). Fish gill cells cultured on permeable filter supports as described by Fletcher *et al*. (2000); Walker *et al.* (2007) and recently by Schnell *et al*. (2016) develop a polarized tight epithelium with the formation of tight junctions which results in high Transepithelial Electrical Resistance (TEER) among the cells. Cells cultured in this way have been demonstrated to be able to tolerate water on the apical surface in laboratory toxicity testing studies (Fletcher *et al*., 2000) and would be appropriate in evaluating molecular response of gill cells to heavy metal exposures. Several studies have used the primary gill cell culture system in eco-toxicological studies (Bury *et al*., 2014). Walker *et al*. (2008) have also demonstrated that toxicity results from this *in vitro* system is comparable to results from *in vivo* animal studies, reporting that exposure to 0.076 µM silver resulted in a reduction in whole body Na^+ influx by 50% and induced comparable metallothionein (MT) expression, a metal responsive gene. This study aims to investigate the ability of cultured rainbow trout gill epithelium to induce the expression of genes coding for metallothionein when exposed to heavy metals commonly detected in polluted aquatic ecosystems.

MATERIALS AND METHODS

Test Organisms: Description, Source and Acclimatisation

Juvenile **Oncorhynchus mykiss** (weight; 120.0 ± 50.0 g) were purchased from a trout farm in Berkshuire, United Kingdom. The fish were transported to the animal unit at King's College, London and were acclimatised in 1000 L fiberglass aquaria and maintained at 13-14°C in re-circulating aerated city of London tap water (Na^+: 0.53 mM, Ca^{2+}: 0.92 mM, Mg^{2+}: 0.14 mM, K^+: 0.066 mM and NH_4^+: 0.027 mM), which was passed through carbon, mechanical and biological filters. Photoperiod was maintained at a constant 14 hour light, 10 hour dark cycle and fish were fed daily at 1 % (*w*/*w*) ration of fish chow.

Test Compound

Three heavy metals (Pb, Cd and Zn) were used as test compounds in this study. All metals were in form of salts, $Pb(NO_3)_2$ (J. T Baker), $CdSO_4.3H_2O$ (L.N.L Laboratories) and $ZnSO_4.7H_2O$ (J.T. Baker) respectively. All salts were of analytical grade.

Cell Culture: *Oncorhynchus mykiss* Gill Epithelial Cells

All procedures were carried out using sterile techniques in a laminar flow hood. Dissecting instruments were disinfected with 70% ethanol prior to use. Pipette tips and glass wares were sterilised by autoclave while other disposables were purchased sterile. Cell incubation media (with and without antibiotics) were kept at 4°C until needed while working solutions were freshly prepared in 50 ml falcon tubes at the start of each cell culture procedure and kept on ice until needed. All solutions were sterilised by filtration using 0.2 µm syringe filters unless otherwise stated.

Cell Isolation and Culture

Gill epithelial cells obtained from *O.mykiss* were cultured using the Double Seeded Insert (DSI) primary culture technique (Schnell *et al*., 2016). One *O.mykiss* was sacrificed following UK Home Office schedule 1 procedures (Animals Act, 1986). The head was blotted dry using clean tissue, the opercula were removed and the gills were carefully cut out. The gill filaments were isolated and washed using Phosphate Buffer Solution (PBS) and Antibiotics. The filaments were then subjected to tryptic digestion using 0.05% Trypsin-EDTA (ThermoFisher Scientific). Cells isolated from the filaments were seeded onto cell culture inserts (cyclopore polyethlylene terephthalate membrane, surface area 0.9 cm^2, pore size 0.4 µm, Falcon) at a cell density of 1.5 x 10^6 per insert, in Leibovitz (L-15) culture medium (ThermoFisher Scientific) supplemented with antibiotics (5% fetal bovine serum (FBS, Sigma-Aldrich); 2% penicillin and

streptomycin (PEST) and 2% gentamicin, ThermoFisher Scientific). The cells were incubated at 18°C for 24hrs after which they were washed using PBS to remove debris. Another fish was dissected, and cells extracted using procedures previously described. The cells were seeded on previously seeded cells at 1 x 10^6 per insert, in supplemented L-15 culture medium. The double seeded cells were incubated at 18°C for 24hrs after which they were washed using PBS, and the culture medium was replaced at a volume of 1.5 ml in the apical chamber of the insert and 2.0 ml in the basolateral chamber. The cell cultures were grown at 18°C. After 96hrs, the cells were grown in culture medium supplemented with 5% FBS only, without antibiotics and the medium was changed once every 48hrs. The Transepithelial Electrical Resistance (TEER) of the cells were measured and recorded from 96hours (day 4) of initial seeding using a custom-modified epithelial tissue voltohmeter (EVOMX; World Precision Instruments) fitted with chopstick electrodes (STX-2). The TEER of the cells was measured to monitor the development of an intact gill epithelium and a TEER > 2000 kΩ cm^2 was used as a criterion for formation of a tight epithelium. Inserts with TEER of 2000 kΩ cm^2 and above were used in heavy metal exposures.

Exposure of Cultured *Oncorhynchus mykiss* Gill Epithelial Cells to Heavy Metals.

The cultured cells (with TER ≥ 2000 kΩ cm^2) were exposed to sublethal concentrations of Pb, Cd and Zn (Walker *et al*., 2007). Stock solutions with known strength (1 mM Pb and Zn; 10 µM Cd) were made up to desired volume with autoclaved Moderately Hard Synthetic Water (MHSW) and sterile filtered before use. Working concentrations of the heavy metals were made by serially diluting stock solutions of the respective heavy metal with sterile filtered MHSW to final concentrations of 1.0, 10.0, 25.0, 50.0, and 100.0 µM for Zn; 0.5, 2.5, 10.0, 25.0, and 50.0 µM for Pb; and 0.01, 0.10, 0.25, 0.50, 1.00 µM for Cd. The exposure medium was added to the apical side of the inserts at 1.5 ml volume while 2 ml of plain L-15 medium (without FBS and antibiotics) was added to the basolateral side of the inserts. MHSW containing no metals was designated as control. The cells were exposed for 24hrs and in quadruplicates (4 inserts) for each concentration after which total Ribonucleic Acid (RNA) was extracted from the cells and used for further analysis. The TEER of the cells was measured before and after the exposure period to determine the effect of media change on the cells.

RNA Extraction from Cultured Cells and Conversion to Complementary DNA (cDNA)

Total RNA was extracted from the cells using TRizol reagent and phase separation was done using Phase Lock heavy tubes (Minghetti *et al*., 2014). Extracted RNA was DNase treated using Turbo-DNA free kit (Ambion® The RNA Company®, Cambridgeshire, UK) according to manufacturer's instruction. After DNA treatment, the samples were precipitated to remove salt impurities using Sodium Acetate and ethanol. The RNA samples were converted to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to manufacturer's instruction.

Reverse -Transcription Quantitative Polymerase Chain Reaction (RT qPCR)

RT qPCR was performed using the cDNA as templates to quantify the expression of two metal responsive genes Metallothionein A (mtA) and Metallothionein B (mtB) (Walker *et al.*, 2007). Primers for target genes and reference genes were obtained from Integrated DNA Technologies (Table 1) and the qPCR reaction mix was made using SYBR premix Ex Taq II (Takara) according the manufacturer's instructions. The qPCR was run on a 7500 fast Real-Time PCR system (Applied Biosystems) and thermal cycling conditions followed that suggested by Takara for reference genes (ARP and Eef1b) and the three-step cycling program for target genes (MtA and MtB) as suggested by Minghetti *et al*. (2014).

Table 1: Primers for target and reference genes (Minghetti *et al*., 2014)

Gene name	Forward primer 5' – 3'	Reverse primer 5'-3'	Repository ID
Metallothionein A	ACACCCAGACAAACTACTAC	GGTACAAAAGCTATGCTCAA	M18103[b]
Metallothionein B	GCTCTAAAACTGGCTCTTGC	GTCTAGGCTCAAGATGGTAC	M18104[b]
ARP[a]	GCCCTGGCCAGCGTAGACATTG	GACCGAAGCCCATGTCGTCATCG	TC205875[c]
Eef1b[a]	TTGGCGGCATAGGCTGCGATTC	TGGGCCAGTATGGTCCTTCCGG	FP321654[b]

[a]ARP, Acidic Ribosomal Protein; eef1b, eukaryotic translation factor 1 beta
[b]GenBank (http://www.ncbi.nim.nih.gov/)
[c]Rainbow trout gene index (http://compbio.dfci.harvard.edu/tgi/)

Data Presentation and Analysis

Microsoft Excel and SigmaPlot (Version 13.0) were used to design bar charts and line graphs. Microsoft word was used to design tables. The Statistical Package for Social Sciences (SPSS Version 16) was used to carry out One-way analysis of

variance (ANOVA) which was set at 0.05 level of significance to test significant differences in groups of data.

RESULTS

Effects of Media change on Transepithelial Electrical Resistance (TEER) of Cultured Gill Epithelia

There was significant ($P < 0.05$) reduction in the TEER of cultured cells after 24hrs of exposure during toxicological studies. The decrease in TEER was recorded in the control cells and in those exposed to the heavy metals (Zn, Pb and Cd) respectively (Fig. 1 - 3).

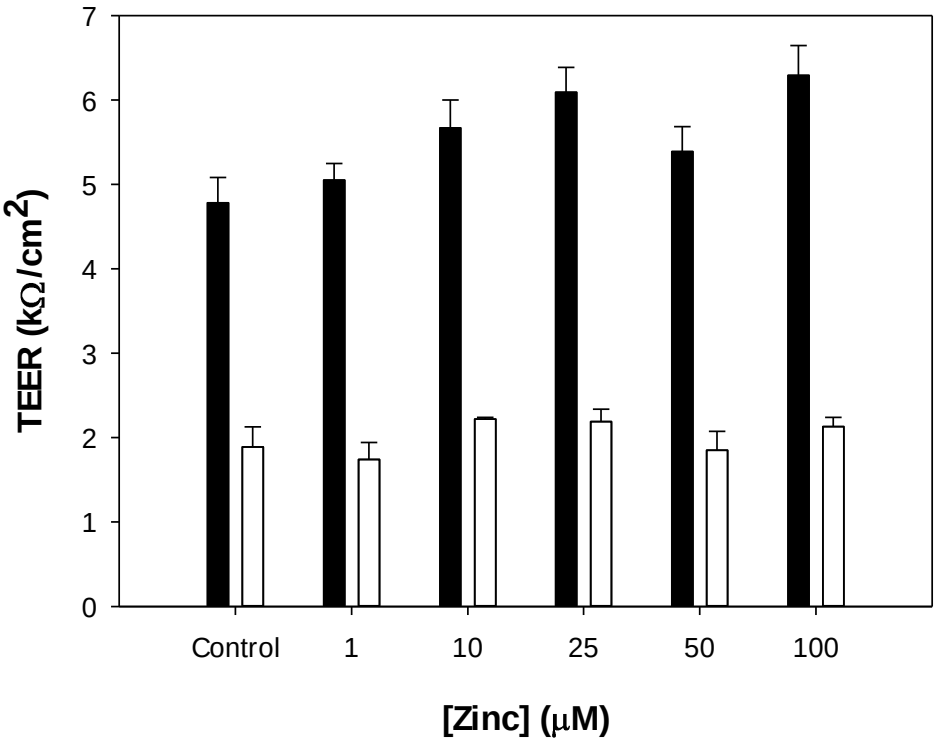

Fig. 1: Transepithelial Epithelial Electrical Resistance (TEER) (kΩ cm^2) of primarily cultured gill cells prior (black bars) and following exposure (white bars) to zinc. Values represent mean ± sem, n = 4 inserts

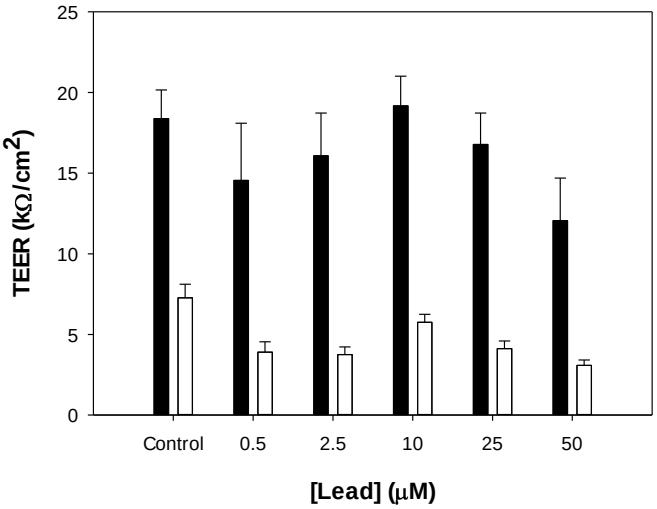

Fig. 2: Transepithelial Epithelial Electrical Resistance (TEER) (kΩ cm^2) of primarily cultured gill cells prior (black bars) and following exposure (white bars) to lead. Values represent mean ± sem, n = 4 inserts

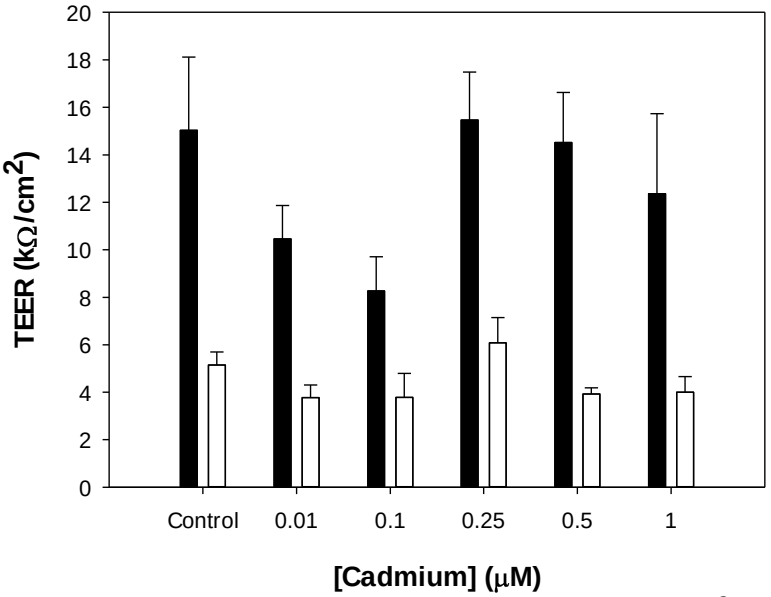

Fig. 3: Transepithelial Epithelial Electrical Resistance (TEER) (kΩ cm^2) of primarily cultured gill cells prior (black bars) and following exposure (white bars) to cadmium. Values represent mean ± sem, n = 4 inserts

Effect of Heavy Metals on Metallothionein A and B Expression in Cultured Gill Epithelia

Metallothionein A and B Cycle Threshold (CT) expression values were normalised with eff1b reference genes and log transformed to obtain final expression values and then expressed as a percentage of the control values (water exposure only cells). Final fold increase showed a significant ($P < 0.05$) dose response increase in the expressions of mtA and mtB on exposure to Zn reaching a maximum fold induction of 5.14 for mtA and 5.92 for mtB at 100.00 µM Zn (Fig. 4). Lead significantly ($P < 0.05$) reduced the expression of mtA with increasing Pb concentrations, with a maximum decrease in expression of 0.34-fold at 50.0 µM. Lead also reduced the expression of mtB but this was not significant at $P < 0.05$ (Fig. 5). Cadmium increased the expression of mtA and mtB with increasing concentrations up to 0.25 µM but this was not significant ($P > 0.05$), beyond which (0.50 and 1.0 µM) the expression of the genes was reduced ($P > 0.05$) (Fig. 6).

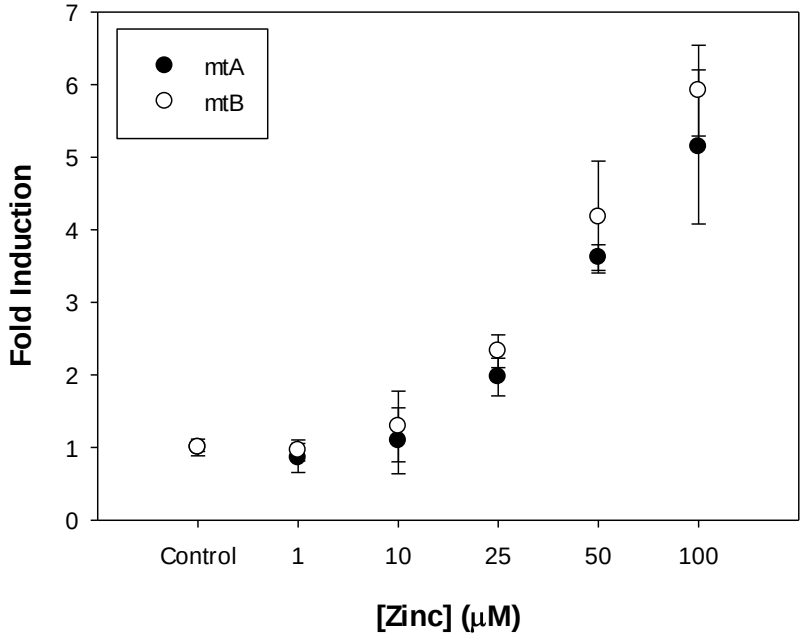

Fig. 4: Metallothionein A (black circles) and B (white circles) expression in primarily cultured gill cells exposed to zinc. Values represent mean ± sem, n = 4 inserts.

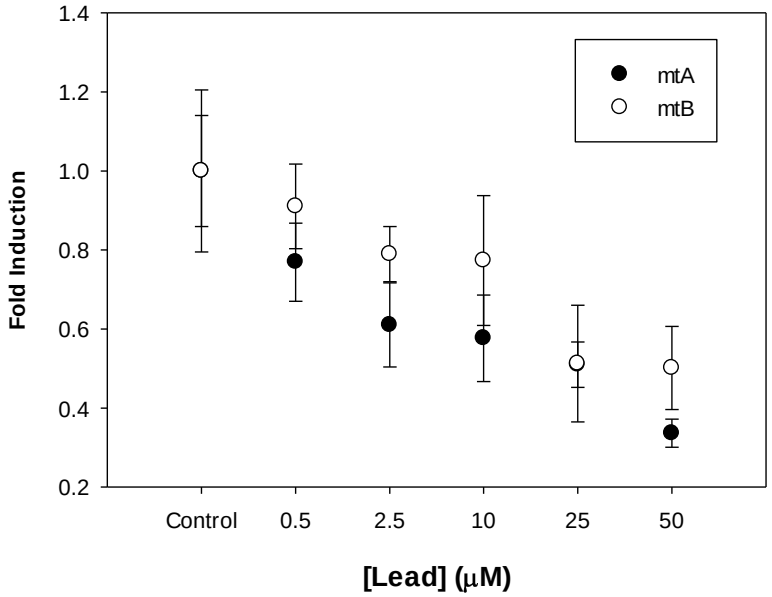

Fig. 5: Metallothionein A (black circles) and B (white circles) expression in primarily cultured gill cells exposed to lead. Values represent mean ± sem, n =4 inserts.

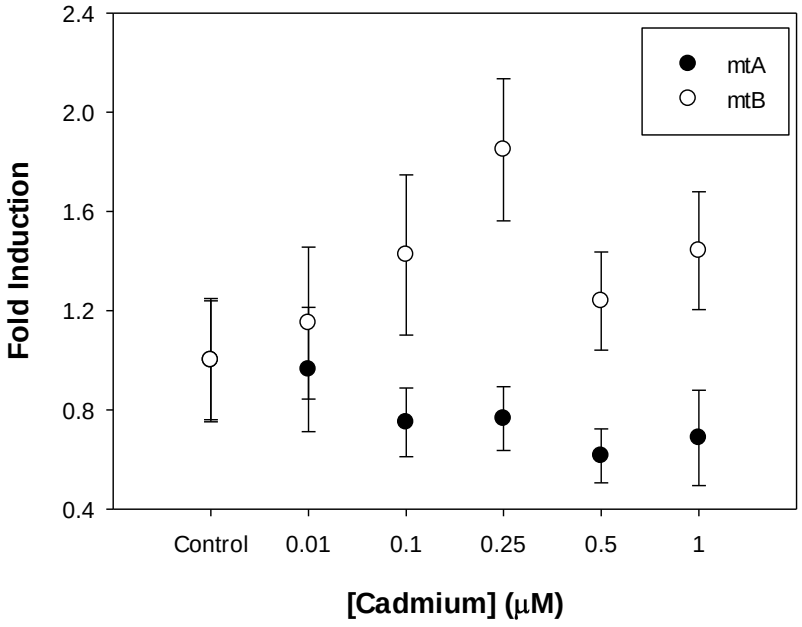

Fig. 6: Metallothionein A (black circles) and B (white circles) expression in primarily cultured gill cells exposed to cadmium. Values represent mean ± sem, n =4 inserts

DISCUSSION

The decrease in TEER of cultured cells observed in this study can be attributed to stress on the cells due to application of fresh water on the apical surface, this agrees with the study carried out by Schnell *et al*. (2015). The lack of nutrient for the 24hr period of exposure may have resulted in death of some of the cells during the exposure period hence the subsequent decrease in TEER. However, the TEER of the cells did not fall below 2 kΩ cm^2 which is the minimum recommended TEER for cultured cells to be used for toxicological studies (Walker *et al*., 2007) and similar drops in TEER were observed in water only exposure conditions, suggesting this drop is not a consequence of exposure to the metals.

Studies, including those of Haylland *et al.* (1992), Park *et al.* (2001), Roy *et al.* (2011) have documented the induction of metallothionein in invertebrate and vertebrate species exposed to heavy metals in *in vivo* studies. In this study, the effects of heavy metals on the expression of genes coding for metallothionein was assessed *in vitro* using primarily cultured fish gill cells. This was done to further substantiate the use of primary fish gill cell culture system as a potential replacement for *in vivo* studies on heavy metal toxicity to aquatic organisms. Metallothioneins are low molecular weight cysteine-rich proteins which are capable of binding metals. The cellular role of metallothioneins is complex and partially unknown; it is believed to protect cells against free heavy metal ion-induced damage. Zinc and Cd increased the expression of metallothionein A and B genes in the cells. The expression of the genes increased linearly with increasing exposure concentrations of the heavy metals. However, Pb did not increase the expressions of the genes. These results corroborate earlier reports from *in vivo* studies that only Cu, Zn, Cd or Hg induces expression of metallothionein in fishes (Hylland *et al.,* 1992). These results have also shown that gill cells respond to heavy metal pollution in a similar way to fish organs *in vivo.* Hence, the fish gill cell culture system is a viable *in vitro* system that can be used to replace *in vivo* studies on heavy metal toxicity in aquatic organisms.

ACKNOWLEDGEMENTS

This research was supported by Staff Doctoral Grant from the University of Lagos, Nigeria.

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