



## Research article

Cultured rainbow trout gill epithelium as an *in vitro* method for marine ecosystem toxicological studiesKafilat Adebola Bawa-Allah<sup>a,\*</sup>, Adebayo Otitoloju<sup>b</sup>, Christer Hogstrand<sup>a</sup><sup>a</sup> Department of Nutritional Sciences, Faculty of Life Sciences & Medicine, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK<sup>b</sup> Department of Zoology, Faculty of Science, University of Lagos, Akoka, Lagos, Nigeria

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## ABSTRACT

Accurate assessment of the toxic potential of waterborne chemicals is vital to pollution control and management in aquatic ecosystems. However, there is a global advocacy for the reduction, replacement, and refinement of the use of whole organisms in chemical screening studies. This has encouraged the development of alternative *in vitro* and computer-based techniques. In this study we investigated the possibility of optimising cultured rainbow trout gill epithelium to tolerate seawater and its use to assess toxicity of waterborne chemicals. Gill cells were obtained from rainbow trout acclimated to freshwater or to artificial seawater and were cultured in L-15 culture medium supplemented with or without cortisol. Intact gill epithelia were subjected to 20‰, 25‰ or 30‰ artificial seawater for 24 h and cell viability was assessed. The viability of gill cells obtained from freshwater or artificial seawater acclimated fish and grown without cortisol reduced to less than 80% compared to controls. The addition of cortisol to culture medium improved cell viability in seawater with 94%–95% viability compared to controls. The optimised gill cell epithelium was exposed to trace elements at concentrations previously reported as causing 50% response or mortality (EC/LC<sub>50</sub>) using other cell-based and *in vivo* studies. Viability of the gill cells were compared to the 50% response or survival reported. The gill cells were found to be more sensitive than other isolated primary seawater-fish cells, having 5%, 16% and 37% survival on exposure to arsenic, cadmium, and lead, respectively. Results from this study has shown that cultured rainbow trout gill epithelia can be optimised to tolerate seawater and can be used in toxicological evaluations of pollutants resuspended in seawater, mimicking marine ecosystem conditions. The optimised gill cell system can serve as a viable *in vitro* method for marine ecosystem toxicological studies which would facilitate effective pollution control and management.

## 1. Introduction

Experimental procedures pertaining to aquatic ecosystem toxicology, pollution monitoring and regulation are based largely on *in vivo* studies using fish. The favoured use of fish in aquatic ecotoxicology research can be attributed to their diversity, ecological relevance, adaptive capabilities, ease of culture and maintenance under laboratory conditions (Hassan et al., 2016). However, over the last few years, scientists are exploring a gradual shift to *in vitro* studies, this is in line with the global call to Reduce, Replace and Refine the use of vertebrate organisms in research. In a technical issue paper of the (Society of Environmental Toxicology and Chemistry SETAC, 2019) released in 2019, three additional Rs (Reproducible and reliable, Relevant, Regulatory accepted)

were incorporated into this mantra to enhance the acceptance of new *in vitro* protocols as regulatory standard methods. *In vitro* alternatives to the use of fish based *in vivo* studies include continuous cell lines and primary cell cultures among other non-living systems such as computer simulations (Schaeck et al., 2013). Cells are the basic unit of life, and they play a vital role in the manifestation of toxicity (Schirmer, 2006). Several continuous cell lines have been established from fish, most of them originating from salmonids and cyprinids (Castaño et al., 2003). These cell lines have been successfully used in ecotoxicology and regulatory testing studies and are being proposed as standard protocols for investigating environmental contaminants (Dayeh et al., 2013). Primary cultures have also been successfully propagated from various tissues in fish including the gills, liver, gonads, skin, and kidney. A major advantage of

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primary cultures over immortalised cell lines is that they express the structure and function of the originating tissue thus can be used to assess toxicant action on specific tissues.

The fish gill is a multifunctional organ which is vital to maintaining physiological balance and it is also the primary site of uptake of pollutants in fish (Schnell et al., 2016). The rainbow trout (*Oncorhynchus mykiss*) gill epithelial cells have been effectively propagated as primary cultures on plates (Pärt et al., 1993) and on permeable supports using the Single Seeded Insert (SSI) (Wood and Part, 1997) and the Double Seeded Insert (DSI) (Fletcher et al., 2000; Schnell et al., 2016) techniques. Gill epithelial cells cultured using the DSI technique develop a polarised epithelium and tight junctions enabling them to be able to tolerate direct exposures to freshwater (Schnell et al., 2016). Gill cells grown using this protocol also have many of the cell types found in the intact gill (e.g., pavement cells, mucous cells, and mitochondria rich cells). The advantage of these properties in aquatic toxicity testing is that the influx/efflux pathway of organic and inorganic pollutants dissolved in water as well as toxic effects in the fish gill can be assessed precisely, without the extraction phase or resuspension of toxicants in culture medium which is necessary when using immortalised cell lines. Gill cells cultured using the DSI technique has been successfully used to evaluate toxicity of pollutants in freshwater environments (Minghetti et al., 2014; Schnell et al., 2015) and they have also been used to characterise trace element toxicity (Walker et al., 2008) as well as pharmaceutical uptake and efflux (Stott et al., 2015; Chang et al., 2019) in freshwater.

Pollutants in marine (seawater) ecosystems undergo chemical speciation which affects bioavailability and toxicity (Bielmyer et al., 2004). A gill cell system in which pollutants can be tested resuspended in seawater will be a vital tool for marine ecotoxicology research and a viable alternative to vertebrate toxicity tests, providing a platform to accurately assess the bioavailability, bioactivity, and toxic effects of chemical pollutants in these ecosystems. The objective of this study, therefore, was to assess the possibility of optimising primary cultures of rainbow trout gill epithelial cells to be able to tolerate seawater. In addition to the fact that rainbow trout gill epithelium has been successfully propagated as primary cultures and has also been shown to be able to withstand freshwater, the species is also natively anadromous and can survive in both freshwater and seawater as part of its normal life cycle, hence the choice for use in this study. In the present study, the sensitivity of the conceivably optimised cells, to pollutant toxicity was also assessed in comparison with other cell-based methods and *in vivo* studies. This was done using a simple cell viability screening assay. The optimised cells were exposed to selected trace elements resuspended in artificial seawater to mimic marine ecosystem conditions. Trace elements are inorganic pollutants that are commonly detected in aquatic ecosystems. These elements occur naturally in the Earth's crust, but increased concentrations have been reported in the environment over the last several decades because of anthropogenic activities, majorly industrial operations, and mining activities (Ansari et al., 2004; Tchounwou et al., 2012). Several studies have been carried out to assess trace element toxicity to aquatic organisms especially fish and most of these studies have been done using *in vivo* and cell-based *in vitro* methods.

## 2. Materials and method

### 2.1. Chemicals

Leibovitz's (L-15) culture medium, Penicillin Streptomycin (PEST), Gentamicin and Trypsin-EDTA (0.5%) were purchased from Gibco, (Life Technologies, USA). Fetal Bovine Serum (FBS), Hydrocortisone, Arsenic (III) oxide ( $As_2O_3$ ), Cadmium Sulfate Hydrate ( $3CdSO_4 \cdot 8H_2O$ ), Copper (II) Sulfate pentahydrate ( $CuSO_4 \cdot 5H_2O$ ), Nickel Chloride hexahydrate ( $NiCl_2 \cdot 6H_2O$ ), and Lead (II) Nitrate [ $Pb(NO_3)_2$ ] were purchased from Sigma-Aldrich (USA). Phosphate Saline Buffer (PBS, Dulbecco A) was purchased from Oxoid Ltd (England). Instant Ocean Sea salt was purchased from Aquarium Systems (France).

### 2.2. Animal husbandry

Fish were purchased from Padworth Fish farm in Reading (UK) and were housed at the Biological Support Unit (BSU) at King's College London. They were acclimatised in 1000 L fiberglass aquaria for two weeks. Aquarium water during acclimation was 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) maintained at 13 °C, aerated, and passed through carbon and mechanical filters. Photoperiod was maintained at 14 h light, 10 h dark cycle and fish were fed daily with fish chow at 1% body weight.

### 2.3. Cell culture

Gill epithelial cells were obtained from Rainbow trout (*Oncorhynchus mykiss*, weight:  $120.0 \pm 50.0$  g). After acclimation, gill epithelial cells were isolated from fish and cultured according to the DSI technique described by Schnell et al. (2016). Briefly, fish was euthanised according to UK Home Office schedule 1 protocol. Gills were isolated and the gill filaments cleaned and digested using Trypsin-EDTA (0.05%). Gill cells extracted after tryptic digestion were seeded onto cell culture inserts [permeable cyclopore polyethylene membrane (PET), 0.4  $\mu$ m pore size and 0.9  $cm^2$  surface area- Falcon] fitted in companion well plates. The cells were incubated for 24 h at 18 °C in an air atmosphere cooled incubator (Panasonic MIR 254). After this period, the cells were washed twice with PBS and cells obtained from a second fish were seeded on the previously seeded cells at the same density in L-15 medium. The cells were grown at 18 °C, and culture medium was changed every 48 h. The development of a tight gill epithelium was monitored daily from 96 h after the second seed onwards by measuring the Transepithelial Electrical Resistance (TER) of the cells using a modified epithelial tissue voltohmmeter (EVOM<sup>2</sup>, World Precision Instruments, Hertfordshire) fitted with chopstick electrodes (STX2). Inserts with TER >3000  $\Omega$   $cm^2$  were used for subsequent experiments. A TER >3000  $\Omega$   $cm^2$  was usually recorded between 9 to 12 days after second seeding of cells on inserts.

### 2.4. Preparation of artificial seawater

Artificial seawater was prepared using Instant Ocean Sea salt (Aquarium Systems, France). The composition of the sea salt as reported by Atkinson and Bingman (1996) is  $Na^+$ , 462 mmol/kg;  $K^+$ , 9.4 mmol/kg;  $Mg^{2+}$ , 52 mmol/kg;  $Ca^{2+}$ , 9.4 mmol/kg;  $Cl^-$ , 521 mmol/kg;  $SO_4^{2-}$ , 23 mmol/kg and  $TCO_2$ , 1.90 mmol/kg. To prepare seawater, 33 g of the sea salt was resuspended in 1 L of freshwater (33 g/l) which gave a 29.5‰ seawater solution at room temperature. Seawater of desired salinity was subsequently prepared based on this proportion (20‰–22.37 g/l; 25‰ - 27.97 g/l and 30‰–33.56 g/l) and sterile filtered (0.2  $\mu$ m, Sartorius) before use in experiments involving cells. The salinity of the solutions was confirmed with a seawater refractometer (Red Sea) to the nearest 0.1‰.

### 2.5. Optimisation of cells to tolerate seawater

Modifications were made to Double-seeded Insert (DSI) culture technique (Schnell et al., 2016) briefly described above. These modified protocols (described below) were tested, and viability of cultured cells were assessed to arrive at the best protocol to optimise the cultured fish gill cells to be able to tolerate artificial seawater.

#### 2.5.1. Freshwater acclimated trout (FW)

Gill epithelial cells were obtained from fish acclimated under freshwater conditions as described in animal husbandry and were cultured using DSI cell culture procedures (Schnell et al., 2016). Cells with TER >3000  $\Omega$   $cm^2$  were subjected to artificial seawater with a salinity of 20‰, 25‰ or 30‰ respectively on the apical compartment, for 24 h. L-15 culture medium without antibiotics was maintained on the basolateral compartment. Cells maintained in L-15 culture medium without

antibiotics on the apical and basolateral compartment served as control. The TER of the cells was measured at 0 h and after 24 h, and viability of the cells was assessed after the exposure period.

### 2.5.2. Artificial seawater acclimated trout (SW)

Gill epithelial cells were obtained from fish maintained in 20% artificial seawater in a 1,000-L circular fiberglass tank. The artificial seawater was aerated and passed through aquarium external filter (EFX+, All Pond Solutions, UK). Fish were maintained in seawater for 48 h to avoid osmotic shock, before gill cells were extracted and cultured according to DSI cell culture protocols (Schnell et al., 2016). Cells with TER >3000  $\Omega$  cm<sup>2</sup> were subjected to artificial seawater with a salinity of 20‰ and 25‰ respectively on the apical compartment, for 24 h. L-15 culture medium without antibiotics was maintained on the basolateral compartment. Cells maintained in L-15 culture medium without antibiotics on the apical and basolateral compartment served as control. The TER of the cells was measured at 0 h and after 24 h, and viability of the cells was assessed after the exposure period.

### 2.5.3. Culture medium supplemented with cortisol (FW + C)

Gill epithelial cells were obtained from fish acclimated under freshwater conditions as described in animal husbandry and were cultured using DSI cell culture procedures (Schnell et al., 2016). The culture medium in the basolateral compartment of cells with TER >3000  $\Omega$  cm<sup>2</sup> was then replaced with L-15 culture medium supplemented with 1  $\mu$ M cortisol and cells were grown in this medium for 48 h. L-15 culture medium without cortisol was maintained on the apical compartment during this period. After 48 h incubation with cortisol, the cells were subjected to artificial seawater with a salinity of 20‰, 25‰ or 30‰ respectively on the apical compartment, for 24 h. Culture medium supplemented with cortisol was maintained on the basolateral compartment during the exposure period. Cells maintained in L-15 culture medium without antibiotics on the apical compartment served as control. The TER of the cells was measured at 0 h and after 24 h, and viability of the cells was assessed after the exposure period.

## 2.6. Trace element toxicity studies to assess sensitivity of optimised gill epithelial cells

After testing the modified cell culture protocols described above, the sensitivity of the gill cells, that were able to tolerate seawater using one of the described protocols (section 2.5.3), to trace element toxicity was assessed. To assess the sensitivity of the optimised primary fish gill cells to trace element toxicity, the cells were exposed to effective/lethal concentrations (EC/LC<sub>50</sub>) of five trace elements previously reported in literature, using other cell-based or *in vivo* methods. The percentage cell viability of the optimised gill cells was then compared to the 50% cell viability or organism survival reported at the exposure concentration for these other cell-based methods or *in vivo* studies, to evaluate the sensitivity and the effectiveness of the optimised gill cells for marine toxicity testing studies.

### 2.6.1. Trace elements: preparation of stock solutions and exposure concentrations

Gill epithelial cells were exposed to Arsenic (As), Cadmium (Cd), Copper (Cu), Nickel (Ni) and Lead (Pb) at predetermined concentrations (Table 1). Stock solutions of As, Cd, Cu, and Ni, were prepared by dissolving computed amounts of the trace element salts, based on the molar concentration of the trace elements, in artificial seawater (30‰) to give a 10 mM solution. Arsenic (III) oxide was dissolved in 1M NaOH before resuspension in artificial seawater. Lead nitrate 100 mM stock solution was prepared in freshwater; however, exposure concentrations were resuspended in artificial seawater (30‰). The stock solutions were sterile filtered (0.2  $\mu$ m, Sartorius) before use in preparing solutions containing predetermined exposure concentrations. The cells were exposed to three different concentrations (excluding control) of each trace element

**Table 1.** Nominal exposure concentrations of trace elements selected from documented studies.

Trace elements	Exposure concentrations (mM)			
Arsenic	0.000	0.017 <sup>a</sup>	0.119 <sup>b</sup>	3.800 <sup>c</sup>
Cadmium	0.000	0.00057 <sup>d</sup>	0.011 <sup>e</sup>	2.600 <sup>f</sup>
Copper	0.000	0.00076 <sup>f</sup>	0.00142 <sup>f</sup>	0.550 <sup>g</sup>
Nickel	0.000	0.0336 <sup>h</sup>	0.329 <sup>i</sup>	2.000 <sup>g</sup>
Lead	0.000	0.00062 <sup>h</sup>	0.007 <sup>j</sup>	29.000 <sup>c</sup>

<sup>a</sup> Lian and Wu (2017).

<sup>b</sup> Bhavani and Karuppasamy (2014).

<sup>c</sup> Morcillo et al. (2016).

<sup>d</sup> Diamond et al. (1990).

<sup>e</sup> Hutchinson et al. (1994).

<sup>f</sup> Wang et al. (2013).

<sup>g</sup> Babich et al. (1986).

<sup>h</sup> PAN Pesticide database.

<sup>i</sup> Svecovicus (2010).

<sup>j</sup> Davies et al. (1976).

(Table 1) selected from literature, representing the EC/LC<sub>50</sub> concentrations of the elements from cell-based and *in vivo* studies. The exposure concentrations were prepared by resuspending computed volumes taken from stock solutions in artificial seawater (30‰).

### 2.6.2. Exposure of optimised cells to trace elements

Gill epithelial cells were obtained from fish acclimated under freshwater conditions as described in animal husbandry and were cultured using DSI cell culture procedures (Schnell et al., 2016) as described in cell culture. Cells with TER >3000  $\Omega$  cm<sup>2</sup> were then supplemented with 1  $\mu$ M cortisol in the basolateral compartment, for 48 h. After this period, the cells were washed twice with PBS, then exposed to the predetermined concentrations of the respective trace elements resuspended in artificial seawater (30‰) in the apical compartment, for 24 h. L-15 culture medium supplemented with 1  $\mu$ M cortisol was maintained in the basolateral compartment during exposure to trace elements. Cells subjected to artificial seawater without trace elements in the apical compartment served as control. Cell viability was assessed after the 24 h exposure period.

### 2.6.3. Bioavailability of trace elements in artificial seawater

To determine the concentrations of the trace elements bioavailable to cells in the seawater toxicity tests, the speciation of the trace elements was determined using the Visual MINTEQ software (Version 3.1).

## 2.7. Cell viability assay

Cell viability was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; Sigma) assay. In this assay, yellow MTT is reduced to a purple formazan product in the mitochondria of living cells (Walker et al., 2008). Reduced mitochondrial activity was taken as a measure of reduced cell viability. After exposure of cells to artificial seawater and trace elements in separate experiments, the cells were washed with PBS and 800  $\mu$ l of culture medium without antibiotics containing 0.05 mg/ml MTT was added to the apical compartment of the cells. Culture medium without MTT was maintained on the basolateral compartment. Cells were incubated for 4 h at 18 °C and then air-dried at room temperature for 30 min. The formazan product was solubilised in 500  $\mu$ l of Dimethyl Sulfoxide (DMSO, Honeywell) and absorbance was read at 570 nm. Blank without cells were used to determine background absorbance.

## 2.8. Data analysis

Each experiment (seawater optimisation and trace element toxicity studies) was repeated three times with a total of six replicates for each

exposure and control, respectively. The viability of the cells was determined as a percentage of viability in control. All data for TER and cell viability measurements are expressed as mean  $\pm$  standard deviation (SD). Statistical differences between groups of TER data were determined by Two-way analysis of variance (ANOVA, GraphPad Prism 8.0). Statistical significance was set at  $p < 0.05$  and Sidak's multiple comparison test was used to identify source of difference.

### 3. Results

#### 3.1. Optimisation of gill epithelial cells to tolerate seawater

##### 3.1.1. Change in TER of gill epithelial cells

There was a reduction in the TER of the gill epithelium after being subjected to artificial seawater for 24 h compared to TER at 0 h. At 20‰ artificial seawater, reduction in TER was not significant ( $P > 0.05$ ) in cells harvested from freshwater (FW) or seawater (SW) acclimated fish as well as in cells harvested from freshwater acclimated fish and grown in culture medium supplemented with cortisol (FW + C) respectively. At 25‰ artificial seawater, reduction in TER was significant ( $P < 0.05$ ) in cells harvested from FW and SW groups of fish but was not significant ( $P > 0.05$ ) in cells harvested from FW + C group of fish (Figure 1).

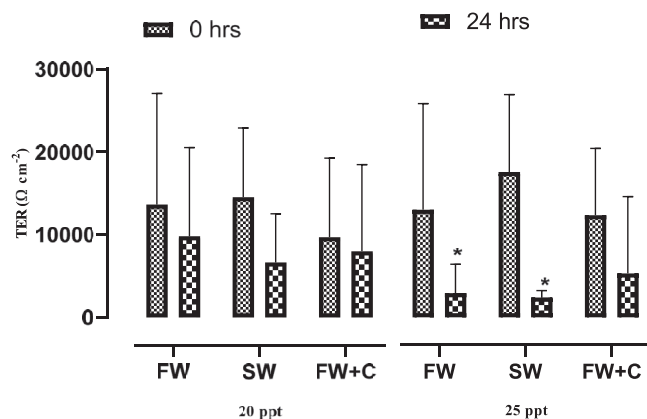
##### 3.1.2. Viability of gill epithelial cells

Cells harvested from fish acclimated in freshwater and grown in culture medium supplemented with cortisol (FW + C) had consistently a viability above 90% after 24 h of being subjected to artificial seawater with little variation among replicates. The average viability of the cells was 94% after culture in 20‰ artificial seawater and 95% after culture in 25‰ and 30‰ artificial seawater, respectively. The viability of cells from FW and SW groups of fish grown in culture medium without cortisol ranged between 74% - 118% and 77%-119% respectively after 24 h in artificial seawater (Figure 2).

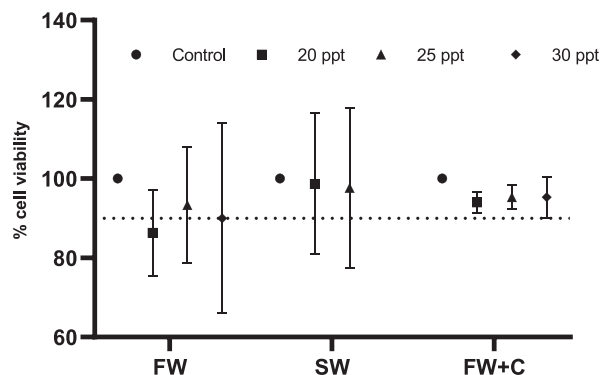
#### 3.2. Trace element toxicity studies to assess sensitivity of optimised gill epithelial cells

##### 3.2.1. Speciation of trace elements in artificial seawater

Speciation analysis showed that 87.6% of As, added as  $As_2O_3$ , was present in solution as arsenous acid at all three exposure concentrations. Only about 4% of Cd, added as  $3CdSO_4 \cdot 8H_2O$ , was present in solution as



**Figure 1.** Change in Transepithelial Electrical Resistance (TER) of gill epithelium subjected to seawater. FW; Cells from freshwater acclimated fish, SW; Cells from seawater acclimated fish and FW + C; Cells from freshwater acclimated fish cultured in medium supplemented with cortisol. Bars represent the mean  $\pm$  SD (experiment repeated three times,  $n = 6$ ). Two-way analysis of variance done using GraphPad Prism 8.0. Asterisks indicate significant difference at  $p < 0.05$  in TER of cells 24 h after being subjected to seawater.



**Figure 2.** Viability of cells subjected to seawater. FW; Cells from freshwater acclimated fish, SW; Cells from seawater acclimated fish and FW + C; Cells from freshwater acclimated fish cultured in medium supplemented with cortisol. Points represent the mean  $\pm$  SD (experiment repeated three times,  $n = 6$ ). Dotted line indicates viability above 90%. FW + C cells had consistent viability above 90% with little variation among replicates.

$Cd^{2+}$  at all three exposure concentrations. More than 50% of Cd was present in solution as cadmium chloride ( $CdCl^+$ ). At two of the exposure concentrations for Cu (0.00076 and 0.00142 mM), more than 70% of Cu added as  $CuSO_4 \cdot 5H_2O$ , was present as copper II carbonate ( $CuCO_3$ ), only 6.4% of Cu was present in solution as  $Cu^{2+}$ . At the highest exposure concentration of Cu (0.550 mM), only 2.4% of Cu was present as  $Cu^{2+}$ . At all the three exposure concentrations for Ni (0.0336, 0.329 and 2.000 mM), an average of 59% of Ni added as  $NiCl_2 \cdot 6H_2O$ , was present in solution as  $Ni^{2+}$ . At two of the exposure concentrations for Pb (0.00062 and 0.007 mM), 5.7% of Pb added as  $[Pb(NO_3)_2]$  was present in solution as  $Pb^{2+}$ , most of the added Pb was present in solution as lead chloride ( $PbCl^+$ ) and lead II carbonate ( $PbCO_3$ ). At the highest exposure concentration of Pb (29 mM), only 0.008% of Pb was present as  $Pb^{2+}$ , 97% of the Pb was present in solution as  $Pb_4(OH)_4^{4+}$  (Table 2).

##### 3.2.2. Sensitivity of the optimised gill cells to trace element toxicity

At the no observable effect concentration (NOEC) of As (0.017 mM), the rainbow trout optimised gill cells had maximum ( $\geq 100\%$ ) cell viability. At 0.119 mM As, the optimised gill cells had 60% cell viability. At the highest exposure concentration of As (3.8 mM), only 5% cell viability was recorded in the optimised gill cells. At the NOEC of Cd (0.00057 mM), the optimised cells had maximum cell viability. At 0.011 mM Cd, the optimised gill cells had also maximum ( $\geq 100\%$ ) cell viability. At the highest exposure concentration of Cd (2.6 mM), the optimised gill cells had only 16% cell viability. The optimised gill cells had 99% cell viability after exposure to 0.00076 mM and 0.00142 mM of Cu, respectively. At the highest exposure concentration of Cu (0.550 mM), the optimised gill cells had 88% cell viability. At the three exposure concentrations of Ni (0.0336 mM, 0.329 mM and 2.000 mM), the optimised gill cells had maximum cell viability ( $\geq 100\%$ ). The optimised gill cells also had maximum cell viability after exposure to 0.00062 mM and 0.007 mM of Pb. At the highest exposure concentration of Pb (29 mM), the optimised gill cells had only 37% cell viability (Table 3).

## 4. Discussion

Transepithelial resistance is a measure of the formation of tight junctions in gill epithelia and one of the properties that contributes to ionic balance across the gills. Results from this study showed a significant reduction in the TER of trout gill cells subjected to artificial seawater with a salinity beyond 20‰. The gill epithelium of native seawater fish has been reported to be naturally 'leaky' (low resistance) compared to



**Table 2.** Speciation of trace elements in artificial seawater (30%).

Trace element	Added concentration (mM)		Dissolved species		
			%	(mM)	
Arsenic (As <sub>2</sub> O <sub>3</sub> )	0.017	H <sub>3</sub> AsO <sub>3</sub>	87.622	0.015	
		H <sub>2</sub> AsO <sub>3</sub> <sup>-</sup>	12.378	0.0021	
	0.119	H <sub>3</sub> AsO <sub>3</sub>	87.622	0.1043	
		H <sub>2</sub> AsO <sub>3</sub> <sup>-</sup>	12.378	0.0147	
	3.800	H <sub>3</sub> AsO <sub>3</sub>	87.622	3.3296	
		H <sub>2</sub> AsO <sub>3</sub> <sup>-</sup>	12.378	0.4704	
Cadmium (3CdSO <sub>4</sub> ·8H <sub>2</sub> O)	0.00057	Cd <sup>2+</sup>	3.992	0.00002	
		CdCl <sup>+</sup>	50.329	0.00029	
		CdCl <sub>2(aq)</sub>	44.613	0.00025	
	0.011	Cd <sup>2+</sup>	3.992	0.00044	
		CdCl <sup>+</sup>	50.330	0.00536	
		CdCl <sub>2(aq)</sub>	44.612	0.00491	
	2.600	Cd <sup>2+</sup>	4.032	0.1048	
		CdCl <sup>+</sup>	50.471	1.3122	
		CdCl <sub>2(aq)</sub>	44.423	1.155	
	Copper (CuSO <sub>4</sub> ·5H <sub>2</sub> O)	0.00076	Cu <sup>2+</sup>	6.432	0.00005
			CuOH <sup>+</sup>	10.153	0.00008
			Cu(OH) <sub>2(aq)</sub>	1.224	0.000009
CuCl <sup>+</sup>			1.594	0.00001	
CuSO <sub>4(aq)</sub>			1.102	0.000008	
CuCO <sub>3(aq)</sub>			73.545	0.00056	
0.00142		Cu(CO <sub>3</sub> ) <sub>2</sub> <sup>-2</sup>	5.613	0.00004	
		Cu <sup>2+</sup>	6.428	0.00009	
		CuOH <sup>+</sup>	10.147	0.00014	
		Cu(OH) <sub>2(aq)</sub>	1.224	0.00002	
		CuCl <sup>+</sup>	1.593	0.00002	
		CuSO <sub>4(aq)</sub>	1.102	0.00002	
0.550		CuCO <sub>3(aq)</sub>	73.481	0.0010	
		Cu(CO <sub>3</sub> ) <sub>2</sub> <sup>-2</sup>	5.607	0.00008	
		Cu <sup>2+</sup>	2.365	0.0130	
		CuOH <sup>+</sup>	3.734	0.0205	
		Cu <sub>2</sub> (OH) <sub>2</sub> <sup>+2</sup>	8.753	0.0241	
		Cu <sub>3</sub> (OH) <sub>4</sub> <sup>+2</sup>	57.042	0.1046	
Nickel (NiCl <sub>2</sub> ·6H <sub>2</sub> O)		0.0336	CuCO <sub>3(aq)</sub>	24.883	0.1366
			Cu(CO <sub>3</sub> ) <sub>2</sub> <sup>-2</sup>	1.74	0.0096
			Ni <sup>2+</sup>	59.088	0.0199
	NiCl <sup>+</sup>		2.896	0.0010	
	0.329	NiSO <sub>3(aq)</sub>	30.752	0.0103	
		NiCO <sub>3(aq)</sub>	4.268	0.0014	
		NiHCO <sub>3</sub> <sup>+</sup>	2.608	0.0009	
		Ni <sup>2+</sup>	59.205	0.1948	
	2.000	NiCl <sup>+</sup>	2.902	0.0095	
		NiSO <sub>3(aq)</sub>	30.688	0.1009	
		NiCO <sub>3(aq)</sub>	4.231	0.0139	
		NiHCO <sub>3</sub> <sup>+</sup>	2.586	0.0085	
Lead (Pb(NO <sub>3</sub> ) <sub>2</sub> )	0.00062	Ni <sup>2+</sup>	59.853	1.1971	
		NiCl <sup>+</sup>	2.935	0.0587	
		NiSO <sub>3(aq)</sub>	30.322	0.6065	
		NiCO <sub>3(aq)</sub>	4.032	0.0806	
		NiHCO <sub>3</sub> <sup>+</sup>	2.465	0.0492	
		Pb <sup>2+</sup>	5.712	0.00004	
		PbOH <sup>+</sup>	8.037	0.00005	
		PbCl <sup>+</sup>	25.635	0.00016	
		PbCl <sub>2(aq)</sub>	11.856	0.00007	
		PbCl <sub>3</sub>	4.843	0.00003	
PbCl <sub>4</sub> <sup>-2</sup>	1.495	0.000009			
PbCO <sub>3(aq)</sub>	37.670	0.00023			

**Table 2 (continued)**

Trace element	Added concentration (mM)		Dissolved species	
			%	(mM)
Lead	0.007	Pb <sup>2+</sup>	5.716	0.0004
		PbOH <sup>+</sup>	8.042	0.0006
		PbCl <sup>+</sup>	25.652	0.0018
		PbCl <sub>2(aq)</sub>	11.864	0.0008
		PbCl <sub>3</sub>	4.846	0.0003
		PbCl <sub>4</sub> <sup>-2</sup>	1.496	0.0001
	29.000	PbCO <sub>3(aq)</sub>	37.630	0.0026
		Pb <sup>2+</sup>	0.0075	1.8459
		Pb <sub>3</sub> (OH) <sub>4</sub> <sup>+2</sup>	2.443	203.92
		Pb <sub>4</sub> (OH) <sub>4</sub> <sup>+4</sup>	97.301	6091
		PbOH <sup>+</sup>	0.01	2.5481
		PbCl <sup>+</sup>	0.067	16.726
		PbCl <sub>2(aq)</sub>	0.065	16.201
		PbCl <sub>3</sub>	0.053	13.368
		PbCl <sub>4</sub> <sup>-2</sup>	0.035	8.65
		PbCO <sub>3(aq)</sub>	0.015	3.7784

**Table 3.** Sensitivity of the optimised gill cells to trace element toxicity.

Trace element	Concentration (mM)	% Trace element bioavailability	% Cell viability
Arsenic	0.017 <sup>a</sup>	87.60 (Arsenous acid)	>100
	0.119 <sup>b</sup>	87.60 (Arsenous acid)	60
	3.800 <sup>c</sup>	87.60 (Arsenous acid)	5
Cadmium	0.00057 <sup>d</sup>	4.00 (Cd <sup>2+</sup> )	>100
	0.011 <sup>e</sup>	4.00 (Cd <sup>2+</sup> )	>100
	2.600 <sup>f</sup>	4.00 (Cd <sup>2+</sup> )	16
Copper	0.00076 <sup>f</sup>	6.40 (Cu <sup>2+</sup> )	99
	0.00142 <sup>f</sup>	6.40 (Cu <sup>2+</sup> )	99
	0.550 <sup>g</sup>	2.40 (Cu <sup>2+</sup> )	88
Nickel	0.0336 <sup>h</sup>	59.1 (Ni <sup>2+</sup> )	>100
	0.329 <sup>i</sup>	59.2 (Ni <sup>2+</sup> )	>100
	2.000 <sup>g</sup>	59.9 (Ni <sup>2+</sup> )	>100
Lead	0.00062 <sup>h</sup>	5.70 (Pb <sup>2+</sup> )	98
	0.007 <sup>j</sup>	5.70 (Pb <sup>2+</sup> )	>100
	29.000 <sup>c</sup>	0.008 (Pb <sup>2+</sup> )	37

<sup>a</sup> Lian and Wu (2017).  
<sup>b</sup> Bhavani and Karuppasamy (2014).  
<sup>c</sup> Morcillo et al. (2016).  
<sup>d</sup> Diamond et al. (1990).  
<sup>e</sup> Hutchinson et al. (1994).  
<sup>f</sup> Wang et al. (2013).  
<sup>g</sup> Babich et al. (1986).  
<sup>h</sup> PAN Pesticide database.  
<sup>i</sup> Svecevicus (2010).  
<sup>j</sup> Davies et al. (1976).

that of freshwater fish, this characteristic facilitates the excretion of excess monovalent ions for maintenance of osmotic balance within the fish (McCormick, 2001). The reduction in gill epithelium resistance reported in this study can be attributed to an adaptation to seawater to maintain ionic balance within the cells. However, it is of importance to mention that the reduction in TER of cells grown in culture medium supplemented with cortisol was not significant (p > 0.05) when subjected to artificial seawater above 20‰. This may be ascribed to the effect of cortisol on the physiology of the cells enabling them to adapt when subjected to seawater. A study by Legeun et al. (2007) reported that cortisol enhanced transepithelial resistance in trout gill cells cultured on

permeable support and helped to maintain resistance for a longer time compared to control cells (without cortisol).

Cell viability assays are structured to measure activities in cells indicating normal physiological processes and survival (Niles et al., 2009). The activities measured which may include enzyme activities or organelles reduction potential is usually directly proportional to the number of viable cells after exposures to a pollutant or condition (Niles et al., 2009). In this study, viability of cells subjected to seawater was assessed by their ability to convert a tetrazolium salt (MTT) to purple formazan products using mitochondrial succinic dehydrogenases (Hamid et al., 2004). Results showed that cells grown in culture media supplemented with cortisol had a consistent high viability above 90% after being subjected to artificial seawater up to 30‰ for the three repeats of the experiments. The viability of cells obtained from FW or SW acclimated fish grown in culture medium without cortisol was variable and reduced to <80% after being subjected to seawater. These results indicate that addition of cortisol to the culture medium optimised the tolerance of the gill cells to seawater conditions. The role of cortisol in adaptation of euryhaline and anadromous teleost fish to high salinities is well documented in literature (McCormick, 2001; Aruna et al., 2012). Cortisol stimulates  $\text{Na}^+/\text{K}^+$ -ATPase enzyme activity and the differentiation of seawater type chloride cells changing the osmoregulatory capacity of the gill epithelia at high salinities (Madsen, 1990; Bjornsson et al., 2011). Interestingly, more recent studies have shown that cortisol may play a dual role in osmoregulation in teleost fish; working in synergy with growth hormones and insulin-like growth factors at high salinities; and with prolactin at low salinities (McCormick, 2001, 2013).

The optimised gill cells were exposed to trace elements resuspended in artificial seawater with a salinity of 30‰ mimicking typical marine ecosystem conditions. Due to the high salt concentrations and chemical complexity of seawater (Hirose, 2006), it was essential to investigate the speciation of the trace elements in the artificial seawater, to determine the relative percentage of the added trace elements which were bioavailable as cations, triggering toxic effects in the cells. Trace elements are usually precipitated by anions which are present in solution, forming complexes which may not be bioavailable to living systems (Ansari et al., 2004). The results showed that <8% of added Cd, Cu and Pb at all three exposure concentrations of the trace elements respectively were bioavailable to the exposed cells. At the highest exposure concentration of Pb, <0.1% was bioavailable to the cells. Arsenic was present in solution as arsenous acid. Approximately 59% of added Ni was bioavailable to the cells at the three exposure concentrations. The result of the speciation analysis highlights the advantage of the optimised gill cells being able to tolerate exposures to seawater. The cells would give a more accurate representation of the toxic potential of pollutants in marine ecosystems. Cell-based toxicity studies conducted with pollutants resuspended in cell culture medium have a high probability of over estimating toxicity of pollutants in seawater conditions.

The tetrazolium salt (MTT) assay has been used in numerous studies to assess toxicity of trace elements in cell-based experiments (Fotakis and Timbrell, 2006; Aziz et al., 2014; Goswami et al., 2014; Zhou et al., 2017). The assay was used in this study to assess the sensitivity of the optimised gill cells to trace element toxicity in comparison to other fish cells and *in vivo* studies based on results reported in literature. The optimised gill cells were exposed to three different concentrations, sourced from literature, of each of the trace elements: i) NOEC concentrations using whole fish ii) the lowest  $\text{LC}_{50}$  concentrations (representing 50% fish survival) using whole fish and iii) the highest  $\text{EC}_{50}$  concentrations (representing 50% cell viability) for marine fish cells using simple cell viability assays. The highest  $\text{EC}_{50}$  concentrations were selected to establish the relative sensitivity of the optimised gill cells compared to other marine fish cells which would be vital in promoting the use of the optimised gill cells as a viable *in vitro* tool for marine toxicity testing studies. Results showed that the optimised gill cells had a reduced cell viability on exposure to As, Cd and Pb (5%, 16% and 37% respectively)

compared to 50% cell viability reported for isolated marine fish blood cells (Morcillo et al., 2016) at the same trace element concentrations. The optimised gill cells had a higher cell viability on exposure to Cu and Ni (88% and >100% respectively) compared to 50% cell viability reported for the Bluegill sunfish BF-2 cell lines (Babich et al., 1986) at the same trace element concentrations. The results have shown that the optimised primary gill cells are more sensitive than marine fish primary blood cells but less sensitive than the BF-2 cell line, to trace element toxicity. Primary cells have been reported to differ in their responses to pollutants compared to cell lines and may be less sensitive than cell lines on exposure to certain pollutants including trace elements (Castaño et al., 2003). On exposure to  $\text{LC}_{50}$  concentrations (representing 50% fish survival) using whole fish reported in literature (Davies et al., 1976; Hutchinson et al., 1994; Svecevicus, 2010; Wang et al., 2013; Bhavani and Karuppasamy, 2014), the optimised gill cells had between 60% and 100% cell viability for the respective trace elements (As, Cd, Cu, Ni and Pb). These results were not unexpected as cell cultures have been reported to be less sensitive to pollutant toxicity than whole organisms (Schirmer, 2006). However, it is vital to mention that speciation analysis was also done for reference studies which documented physicochemical parameters of exposure media (L-15 medium, DMEM and freshwater) and results showed that bioavailability of the trace elements in artificial seawater was less than bioavailability in exposure media in reference studies. This finding imply that lesser concentrations of the trace elements caused toxic effects against the optimised gill cells compared to the reference studies and supports the deduction that the optimised primary gill cells are more sensitive than marine fish primary blood cells due to reduced cell viability recorded and may have similar sensitivity to the BF-2 cell line and whole fish despite similar or higher cell viability recorded.

The MTT assay used in this study expresses cell viability as a measure of mitochondrial activity reducing the tetrazolium salt to purple formazan product. Mitochondrial activity >100% compared to control (100%) were recorded at low trace element exposure concentrations (NOEC concentrations) in this study. Results from a study by Fotakis and Timbrell (2006) also showed cell viability >100% at low exposure concentrations compared to control using MTT assay, on exposure of HTC hepatoma cell line to CdCl<sub>2</sub>. The higher mitochondrial activity, compared to control, recorded in this study can be attributed to the number of cells at the time of exposure to trace elements. Although, similar number of cells were seeded on inserts during cell culture, the cells in inserts are ready to use in experiments 9–12 days after cell culture, which would have allowed for cell growth and differentiation. This period of growth may account for relatively uneven number of cells at time of exposures. However, the higher mitochondrial activity reported in cells exposed to low trace element concentrations were not significantly different to activity in control cells.

## 5. Conclusion

This present study has shown that primary rainbow trout gill epithelial cells can be optimised to tolerate seawater when grown in culture medium supplemented with cortisol. The study has also established the better sensitivity of the optimised gill cells to toxic pollutants compared to other primary cells isolated from marine species. This gill cell system can serve as a viable *in vitro* tool for the toxicological studies of pollutants in marine ecosystems. Pollutants, particularly trace elements undergo complexation and speciation in seawater which may greatly affect bioavailability and toxicity (Hirose 2006) as shown by the speciation analysis in this study. Testing pollutants in the form which they occur in the environment (dissolved in seawater) will enable the accurate assessment of bioavailability and toxic effects in seawater organisms. The gill system can also be used for environmental monitoring of marine ecosystems enabling early detection of harmful contamination and timely deployment of remediation strategies.

## Declarations

### Author contribution statement

Kafilat Adebola Bawa-Allah: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Adebayo Otitolaju: Conceived and designed the experiments.

Christer Hogstrand: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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### Data availability statement

Data included in article/supplementary material/referenced in article.

### Declaration of interests statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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