AJEHS
African Journal of Environmental
Health Sciences
Volume 4, November, 2017
pp 51-60

Assessing the Potential of Cell lines as Tools for the Cytotoxicity Testing of Acid Mine Drainage Effluent Impacting a Natural Water Resource

Oluwafikemi T. Iji,
Jan G. Myburgh
and Lyndy J. McGaw

Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa

Corresponding Author:
Oluwafikemi T. Iji, as above

Keywords:
Cytotoxicity, Coal mine,
Acid mine drainage,
Polluted stream

Mots-clés:
Cytotoxicité, Mine de charbon,
Drainage minier acide,
Cours d'eau pollué

Abstract

In vitro cytotoxicity was assessed using mammalian (Vero) and fish (RTgill-W1) cell lines in a water compartment impacted by acid mine effluent from a coalmine. Physico-chemical characterisation of the stream showed that Al, Mn, Fe and sulphates were much higher than the maximum recommended values established by South African legislation regarding effluent in receiving streams. Water collection points selected was immediately following a wetland (U) and another downstream (T) of the mixing zone where alkali is used to increase water pH to reduce acidity and precipitate metals. Another unaffected site served as the reference site (Ref). Cell viability assays were employed to detect effects of water samples on cells following exposure for 24h, 48h and 72h, namely the neutral red (NR), tetrazolium-based (MTT) colorimetric assays and the lactate dehydrogenase assay (LDH) which assess lysosomal, mitochondrial functions and cellular necrosis respectively. Toxicity was detected in U and T water samples against both cell lines using the NR and MTT techniques, being maximal at 72h with IC_{50} values of 23.9% and 20.5% (MTT), and 32.2% and 49.4% (NR) for Vero cell lines (U vs. T). IC_{50} values for the RTgill-W1 cell lines were 8.4% and 7.19% (MTT), and 10.5% and 35.3% (NR) for U and T respectively, but no cytotoxicity was recorded for Ref. The loss of cell viability resulting in damage to cellular functions gives rise to concerns regarding the potential risks for aquatic animals and downstream end users.

L'évaluation du potentiel des lignées cellulaires comme des outils pour les tests de cytotoxicité des effluents de drainage minier acide sur une ressource en eau naturelle

Résumé

La cytotoxicité in vitro a été évaluée à l'aide de lignées cellulaires de mammifères (Vero) et de poissons (RTgill-W1) dans un compartiment d'eau touché par l'effluent d'une mine de charbon acide. La caractérisation physico-chimique du courant a montré que l'Al, le Mn, le Fe et les sulfates étaient beaucoup plus élevés que les valeurs maximales recommandées établies par la législation sud-africaine concernant les effluents dans les cours d'eau récepteurs. Les points de collecte d'eau

© African Journal of Environmental Health Sciences

sélectionnés suivaient immédiatement une zone humide (U) et une autre zone aval (T) de la zone de mélange où l'alcali est utilisé pour augmenter le pH de l'eau afin de réduire l'acidité et précipiter les métaux. Un autre site non affecté a servi de site de référence (Ref). Des tests de viabilité cellulaire ont été utilisés pour détecter les effets des échantillons d'eau sur les cellules après 24h, 48h et 72h, à savoir les dosages colorimétriques au rouge neutre (NR), au tétrazolium (MTT) et au lactate déshydrogénase (LDH) qui évaluent les lysosomes, fonctions mitochondriales et la nécrose cellulaire respectivement. La toxicité a été détectée dans les échantillons d'eau U et T contre les deux lignées en utilisant les techniques NR et MTT, étant maximale à 72h avec des valeurs IC50 de 23,9% et 20,5% (MTT), et 32,2% et 49,4% (NR) pour les lignées Vero (U contre T). Les valeurs de CI50 pour les lignées cellulaires RTgill-W1 étaient de 8,4% et 7,19% (MTT), et de 10,5% et 35,3% (NR) pour U et T respectivement, mais aucune cytotoxicité n'a été enregistrée pour Réf. La perte de viabilité cellulaire entraînant des dommages aux fonctions cellulaires soulève des préoccupations concernant les risques potentiels pour les animaux aquatiques.

Introduction

Mining activity has been identified as one contributing to severe water pollution because of the generation of acidic waters (acid mine drainage or AMD) resulting from mining activities and, most importantly, the presence of heavy metals in such waters along with acidic pH negatively affect aquatic ecosystems (Chen *et al*., 2004). Usually in the aquatic environment, toxicity of pollutants does not occur due to individual compounds but from mixtures (e.g. AMD complex), whose combined effects result in mixture toxicity (Faust *et al*., 2001).

Coal mining is a huge industry in South Africa (Mangena and Brent, 2006). There are reports of water contamination from AMD resulting in fish and crocodile die-offs (Paton, 2008), and potential threats to the aquatic environment (de Villiers and Mkwelo, 2009).

Treatment process for AMD abound with the most common being neutralization, which corrects pH and precipitates metal, giving the appearance that the effluents are non-toxic (Madeira et al., 2005). One way to evaluate the efficacy of such systems is by physical and chemical monitoring of the quality of the effluent, alongside the use of biological indicators because they respond to environmental changes on a short-term, as well as on a long-term basis (Ji *et al*., 2004).

In vitro techniques as alternative systems in toxicity assessment are not new concepts in research, mammalian cell lines are used to monitor water quality (Schirmer *et al*., 2001). Cell cultures, derived from fish, have been effectively used as a biological substitute to the use of whole animals (Chasiotis *et al*., 2012; Kolosov and Kelly, 2013). The functional and structural changes caused by pollutants occur at cellular levels within the organism and consequently, pollutants will exert their effects by interacting/ interfering with basic cellular functions (Larsson *et al*., 1985).

In vitro cytotoxicity assays, was used to detect cytotoxicity of AMD water to both Vero and RTgill-W1 cell lines subjected to both passive (wetland) and active treatment (neutralization) methods. Lysosomal function was determined using the Neutral Red (NR) assay, while mitochondrial function was assessed with the use of the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromides) reduction assay, and likewise necrosis through the LDH assay. A parallel physical and chemical characterisation of the stream was done in conjunction with the cytotoxicity assay to assist in interpreting observed cytotoxicity.

Materials and Methods

Location and description of the study area

The study site chosen was a receiving stream established downstream of a colliery, Kromdraai (25°46' 05.5" S; 29° 07' 15.5" E) in the Highveld region, close to eMalahleni (previously known as Witbank) in Mpumalanga Province. The water sample collected immediately flowing from the wetland, was termed the untreated (U), whilst the one collected downstream, beyond the point of an in-stream, neutralizing agent (caustic soda)which is connected to a dosing tank and monitored telemetrically, was termed treated (T).

A reference site outside the influence or impact of a mine, located at 25°47'6"S; 28° 28' 52"E, served as the control site and water samples collected here were referred to as reference (Ref).

Water chemistry

Water samples were analyzed by the Analytical Services Chemistry Department, CSIR, Pretoria, for their physicochemical parameters. All parameters were determined according to standard methods (APHA, 1995).

Collection and preparation of samples

Water samples collected as subsurface grab samples, over a period of two years between July and September. Water samples for bioassays were filtered through a 0.22-µm in-line filter within 2h of collection and were stored at 4°C in the dark (maximum storage time: 3 weeks) at the Faculty of Veterinary Science, University of Pretoria.

Powdered media was reconstituted with whole water samples before being exposed to cell cultures. Powdered Minimum Essential Medium (MEM) (Gibco® Life Technologies ™, UK) was used for Vero cells and Leibovitz medium (L-15M Sigma-Aldrich, St. Louis, MO, USA) was employed for RTgill-W1 cell lines. Essential nutrients were provided by adding 5% fetal calf serum (FCS, Highveld Biological, South Africa). Water samples were pH-adjusted using a pH meter (Thermo Fisher Scientific Inc.).

Cell cultures from the Vero cell line and the RTgill-W1 cell lines were exposed to a range of concentrations (3.75%, 7.5%, 15%, 30%, 45%, 60%, 75%, 90% and 100%) using control medium as diluent. Doxorubicin hydrochloride (Pfizer Laboratories Pty Ltd, SA) served as the positive control.

Cell viability assay

Cells of a sub-confluent culture were plated in a 96-well microtitre plate at a density of 100 µl of 1 x 10^5 cells /ml / well for Vero cell and 100 µl of 2 x 10^5 cells /ml / well for the RTgill-W1 cell lines were incubated for 24h at 37ºC in a 5% CO_2 humidified environment and 20ºC in an air incubator respectively before exposure to water samples. The duration of exposure for the cells were 24h, 48h, and 72h after which cell viability was determined.

The MTT assay was performed as described by Mosmann (1983) while the neutral red assay was performed as described by Repetto *et al.* (2008). Lactate dehydrogenase activity was determined using lactate dehydrogenase (LDH) kit purchased from Roche®. Experiments were conducted in quadruplicate and were repeated at least four times.

Transmission electron microscopy

Cells incubated in media control, Ref, U and T were fixed at 4°C according to Iger *et al.* (1995) for electron microscopy. The samples were examined in a Philips transmission electron microscope at an accelerated voltage of 80 kV.

Statistical analysis

Percent cytotoxicity was calculated using the mean percentage inhibition relative to the unexposed control ± standard deviation (SD) using the formula [100 ((Mean Experimental data/Mean Control data) * 100)]. Control values were set at 0% cytotoxicity and 100% viability. Statistical analyses were carried out using one-way analysis of variance (ANOVA) followed by Hartley's f-test for equal variance using

OpenEpi. Cytotoxicity data was fitted with an appropriate model and IC_{50} values were calculated using linear regression. Significance was set at $p \leq 0.05$.

Results

Water quality parameters of the studied sites and TWQR values are presented in Tables 1 and 2.The electrical conductivity values observed for U and T were much higher (around 320 mS/M)) than those recorded in Ref (5 mS/m), and the pH of U was acidic (pH ≥ 3.65). No cytotoxicity (% loss in cell viability) was found for Ref site water samples against both cell lines (Vero and RTgill-W1) at all concentrations tested (100% to 3.75%) within the different exposure periods; 24h, 48h and 72h employing both the neutral red (NR) and tetrazolium-based (MTT) colorimetric assays (Figure 1).

Metal analyses from U and T showed values that, in some cases, were much higher than the Ref and TWQR for aquatic ecosystems (DWAF, 1996) (Table 2).

Following in-stream neutralization, a decrease in metal concentration was observed for metals such as Al ~(127X), Mn (1.66X), Fe (36X), Si (4.5X) and Zn (72X), in effluent T compared to U.

Dose dependent loss in cellular viability was observed in both cell lines exposed to U and T water samples ($p < 0.05$) at the 48 and 72h exposure periods respectively. Effluent water was acutely toxic to both Vero and RTgill-W1 cell lines as evidenced by decreased cell viability, which varied with duration of exposure and was maximal at 72h (Fig II-III). For Vero cells exposed to U, obtained IC_{50} values were 48h IC_{50}, ≥ (43.7%; 52.9%), and 72h IC_{50}, ≥ (23,9%; 32,2%) for MTT and NR assays respectively. The IC_{50} values obtained for RTgill-W1 cells exposed to U were 48h IC_{50}, ≥ (25.2%; 51.4%), and 72h IC_{50}, ≥ (8.4%;10.5%) for the MTT and the NR assays respectively (Table 3). IC_{50} values for Vero cells exposed to T using MTT and NR assays respectively were 48h Vero IC_{50}, ≥ (28.8%; 64%) and 72h EC_{50}, ≥ (20.5%; 49.4%), while results for RTgill-W1 were 48h EC_{50}, ≥ (19,8%; 43,1%), 72h EC_{50}, ≥ (7.2%; 35.3%).

Duration of exposure likewise influenced cell viability, IC_{50} values were significantly lower ($P<0.05$) after 72h exposure compared to 48h. IC_{50} values revealed a difference in sensitivity of

Table 1: Mean values showing physical and chemical water quality data (mg/l, n = 2 determinations) TWQR

	Reference site	Untreated	Treated	TWQR
pH	6.5	3.65	8	6-9
Alkalinity	6	2.75	6.4	N/A
DO (%)	7.9	7.1	5.05	N/A
COD	<10	10	33	N/A
Conductivity (mS/M)	5.21	320	326	70-250
Ammonia as N	<0.1	6.7	6.45	0.5-2.5
Orthophospate	0.23	0.15	0.15	N/A
Nitrate+ Nitrite	<0.2	2.3	3.6	N/A
TP	<0.2	<0.25	<0.25	<5
Magnesium	<0.5	160	162	N/A
Potassium	<1	9	11	N/A
Sodium	<1	43	120	N/A
Calcium	<0.1	475	561	N/A
Chloride	<5	3.5	3.5	N/A
Sulphate	<5	2215	2336	200

Table 2: Mean values of metal concentration (mg/l, n = 2 determinations) and TWQR

	Reference site	Untreated	Treated	TWQR
Aluminium	<0.03	28	0.22	0.005
Arsenic	<0.001	<0.01	<0.01	0.01
Chromium	<0.01	<0.01	<0.01	0.007-0.012
Copper	<0.01	<0.03	<0.03	0.0003-0.0012
Iron	<0.02	0.36	<0.01	N/A
Mercury	<001	<0.005	<0.005	0.004
Manganese	<0.01	65	39	0.18
Silicon	2.6	9	2	N/A
Zinc	<0.02	3.4	0.044	0.002

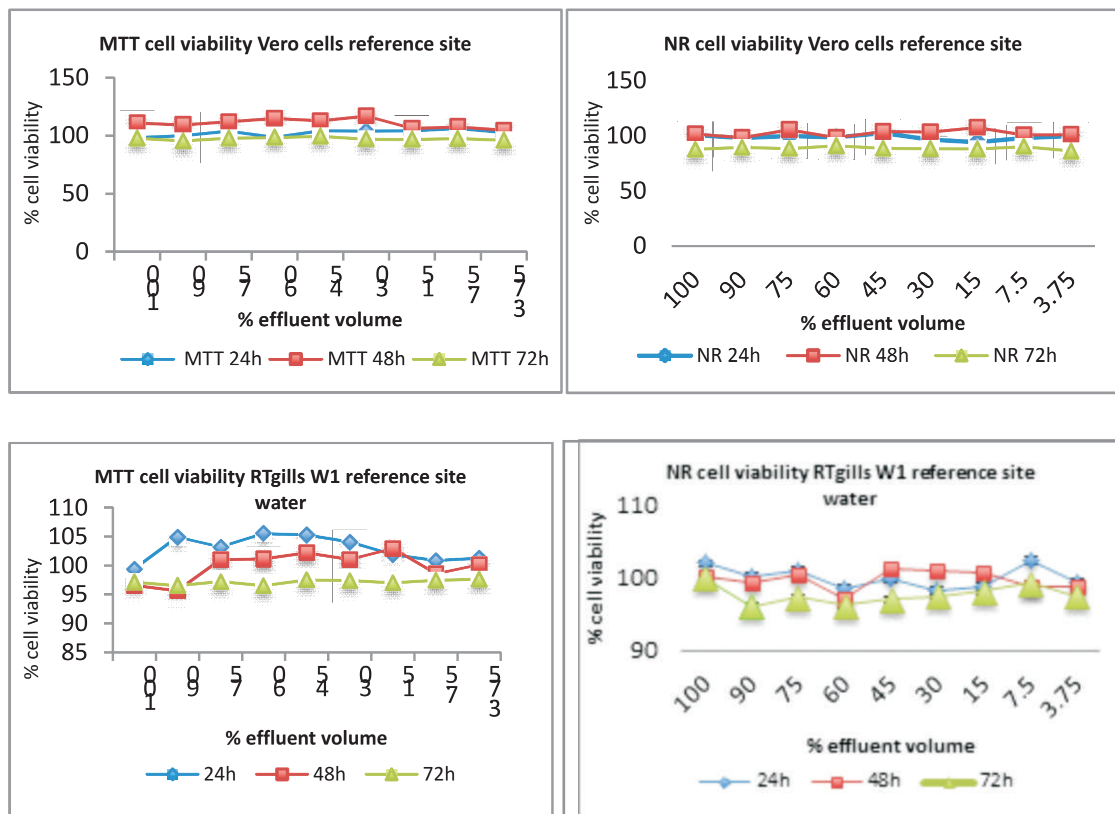

Figure 1: Cell viability effects of different concentrations of water samples from reference site on Vero and RTgill-W1 cell lines. Data is expressed as a percentage of unexposed controls ± SD of four replicates for each exposure concentration (P<0.05) at different concentrations tested exposed for 24, 48 and 72h respectively.

cells when comparing both assay techniques within the same exposure period. The RTgill-W1 cell line exhibited greater sensitivity (reduced cell viability and enhanced cytotoxicity) to effluent U and T detected using the MTT assay technique when compared to the Vero cell lines. MTT IC_{50} values in Vero and RTgill-W1cells were significantly lower than those obtained for the NR

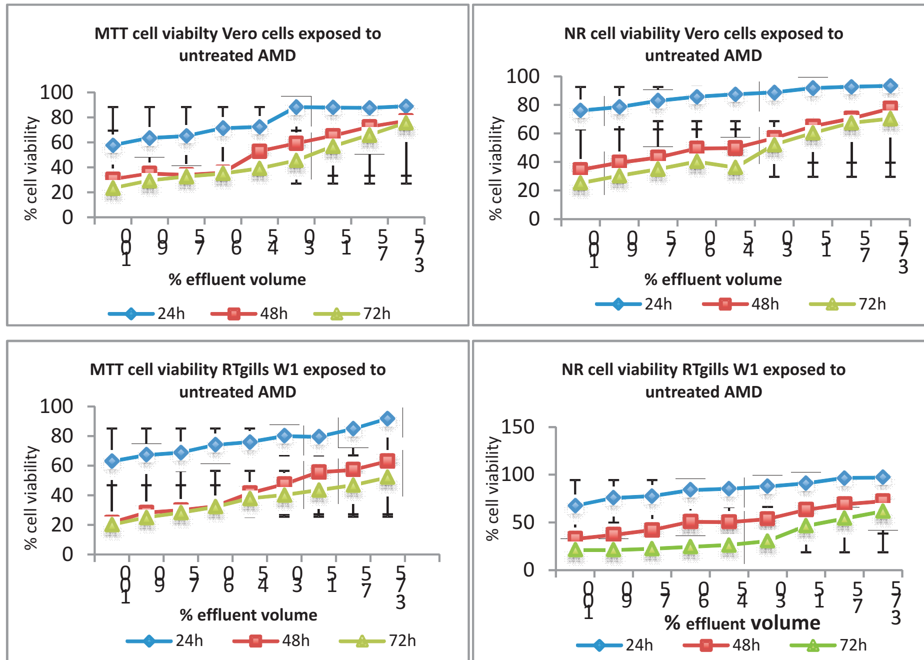

Figure 2: Cell viability effects of different concentrations of water samples from impacted AMD stream U exposed to Vero and RTgill-W1 cell lines. Data is expressed as a percentage of unexposed controls ± SD of four replicates for each exposure concentration. P<0.05 at different concentrations tested exposed for 24, 48 and 72h respectively.

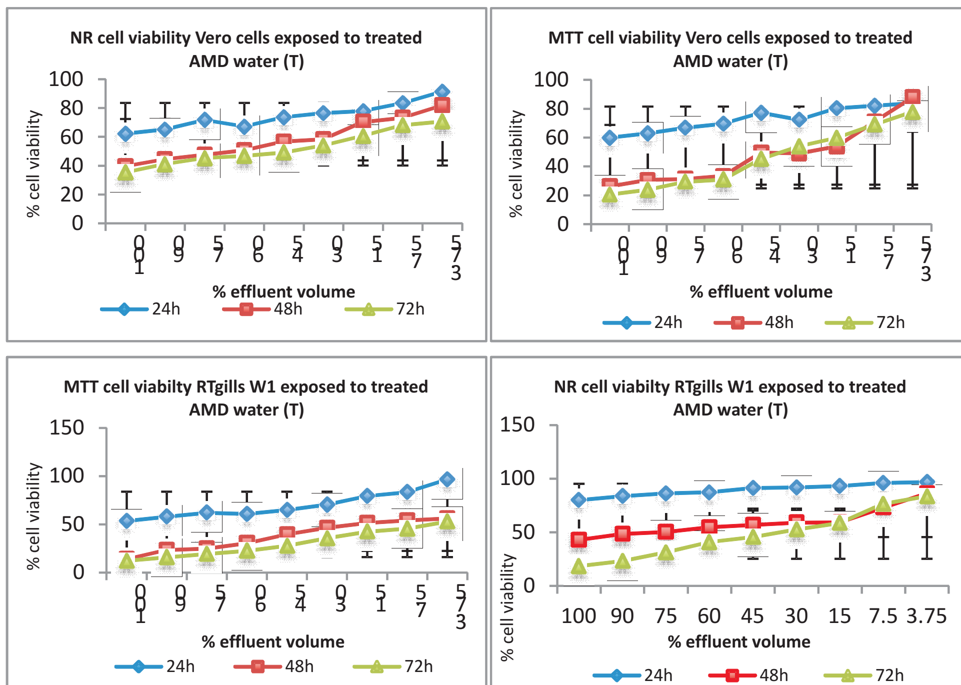

Figure 3: The cell viability effects of different concentrations of water samples from impacted AMD stream T exposed to Vero and RTgill-W1 cell lines. Data is expressed as a percentage of unexposed controls ± SD of four replicates for each exposure concentration. P<0.05 at different concentrations tested at exposure periods of 24, 48 and 72h respectively.

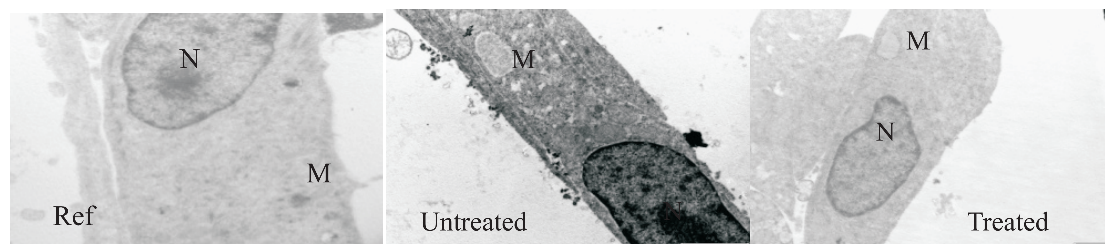

Figure 4: TEM findings of RTgill-W1cells exposed to media Ref site, untreated and treated AMD water samples (N= nucleus, M= mitochondria). Intact mitochondria observed in Ref but swollen and loss of inner and outer membrane seen in cells exposed to U and T.

assays comparing the similar duration of exposure ($P<0.05$).

LDH leakage assay technique (data not shown), in contrast to the MTT or NR did not result in apparent cytotoxicity; no significant differences in LDH activity were detected among any of the treatments compared to the control.

Ultra-structurally, mitochondria of cells exposed to U and T (Figure 4) displayed morphological alterations, as they became progressively more swollen and in some cases, rupture of outer mitochondrial membranes was observed. The photomicrograph of RTgill-W1 (not shown) likewise presented with fewer fluorescing mitochondria in cells exposed to U and T compared to controls using Mitotracker® and Rhodamine®.

Discussion

In this study, chemical and cytotoxicity tests were used to obtain information from polluted AMD sites compared with a cleaner site. Physicochemical water analyses showed that a wide range of contaminants usually present in polluted AMD waters were absent in Ref samples (Tables 1 and 2). Test sites had differences in water column (Tables 1 and 2) in terms of pH, metal load, sulphates and presence of salt.

The cell lines showed differences in cytotoxic responses based on potential contaminants present in the water samples. Using the water sample, Ref, from the cleaner site indicated there were no losses in cell viability in both cell lines at all concentrations tested (Figure 1). However, an indication that toxic contaminants may have a localized effect within the AMD sites was reinforced by the results obtained in the bioassays. For the Vero and RTgill-W1 cells, cytotoxicity assays showed substantial deleterious effects of the water column on cell viability, reducing cell viability by at least 50% within a 48h exposure period (MTT, NR).

Cytotoxicity to cell lines resulting from exposure to industrial effluents has been reported in RTgill-W1 cell line, (Dayeh *et al*., 2002). Dayeh *et al.* (2004) reported a dose-dependent decline in cell viability in both RTgill-W1 and RTL-W1 cells exposed to metals common in mining effluent, measured with alamar blue and 5-carboxyfluorescein diacetateacetoxymethyl ester indicator dyes. Heavy metals presence may be a possible explanation for the loss in cell viability. Ercal *et al*. (2001) reported that metals such as Fe, Cu, Cr and As could generate ROS by redox recycling, RTgill-W1 cells exposed to Cu produced a dose-dependent elevation in cytotoxicity and enhanced ROS formation (Bopp *et al*., 2008). A previous experiment confirmed increased ROS generation in cell lines exposed to water samples from our study site (Iji *et al*., 2017).

Ultra-structural changes observed in the mitochondria of the RTgill-W1 cells (Fig IV) revealed a possible link to cell death in cells exposed to U and T. Mitochondrial pore opening as a consequence of mitochondrial swelling is widely documented as promoting apoptosis (Halestrap *et al*., 2002). A reduction in fluorescing mitochondria in U and T further substantiates, an indication of possible effects of contaminants on mitochondria activity. Mitochondrial damage is known to be closely associated with the

generation of intracellular ROS that leads to disruption of respiratory chain and possible DNA damage (Pulido, 2003). Koizumi *et al.*, 1996 reported that inhibition of mitochondrial respiration stimulates forceful oxygen related cell death. Cadmium was reported to disrupt mitochondrial function both *in vivo* and *in vitro* (Pourahmad and Brien, 2000).

Difference in sensitivity could be due to different uptake mechanisms by the two cell lines. Kramer *et al.* (2009) reported that in most cases, mammalian cell assays appear to be less sensitive than fish acute toxicity studies when assessing the cytotoxicity of chemicals. Target site concentration may play a role in the observed differences in cell sensitivity, as is expected that the response of an organism to a toxic compound would be in relation to its concentration (Escher and Hermens, 2002). The fact that RTgill-W1 cells are derived from gills which are a primary target for the uptake of waterborne toxicants and an organ prone to damage by metals (Castaño, 2003) explains its sensitivity and perhaps, it being a better choice for elucidating cytotoxicity to waterborne contaminants compared to mammalian cell lines.

Different profiles in cytotoxicity assay techniques employed showed MTT> NR>LDH in sensitivity, suggesting that cell viability (mitochondrial activity) measured with MTT was more sensitive to contaminants present in the effluents compared to lysosomal activity and LDH leakage.AMD contaminants apparently appeared to target the mitochondria rather than plasma membranes as its specific mechanism of toxicity. Lund *et al.*, 1993, described the mitochondria as a more important site in metal induced toxicity because respiratory impairment appears to precede plasma membrane breakdown. Mitsuyoshi *et al.* (1999) reported that the XTT (2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]- 2H-tetrazolium-5-carboxanilide) assay, which is similar to the MTT assay, showed more sensitivity than the LDH leakage assay. LDH release, which is an indicator of necrosis arising from cell membrane damage, did not seem to play a big role in loss of cellular viability, a possible indication of an energy-dependent cell death (apoptosis) at least preceding necrosis.

The increase in the presence of sulfates and salts in the following alkali neutralization may have contributed to its sustained reduction in cell viability (MTT assay).Nobergh-King *et al.* (2005), in a toxicity reduction evaluation case study, reported TDS toxicity in the invertebrate *Cerio daphniadubia* even when heavy metals present in discharged effluent met chemical-specific permit limits. Increased TDS is also associated with enhanced ROS generation, which could initiate oxidative damage (Liu *et al.*, 2007).

The current experiment suggests that site-specific information on the toxicity of contaminants present in the aquatic system to cell lines can be obtained to assess the threats posed by point source discharges of this nature in local freshwater resources. Hence, a correlation between cytotoxic alterations in the cells can be made with the analytic data of water quality, as poor water quality will not sustain life. There are benefits to using cell lines; the amount of samples needed for testing is reduced, as well as the time required to complete testing, this can aid decision-making where an effect is observed.

Further work is required to understand the complex relationship between metals and other inorganic ions and organic ligands in complex mixtures. The lack of dose–response data for individual metals made it difficult to rank the potential contributions of each metal to overall toxicity.

Conclusion

Additional findings should be considered to further show the reliability of the RTgill-W1 bioassay as a replacement for the rainbow trout 96-h lethality test and enough data generated to establish better correlations between *in vivo* and *in vitro* toxicity assays.

References

APHA-AWWA-WPCF. 1995. Standard methods for the examination of water and wastewater. American Public Health Association, American Water Works Association and Water Environment Federation, Washington, D.C.

Bopp S.K, Abicht H.K, Knauer K (2008). Copper-induced oxidative stress in rainbow trout gill cells. *Aquat Toxicol.* 86:197–20. Doi.org/10.1016/j.aquatox.2007.10.014.

Castano A, Bols N, Braunbeck T, Dierickx P, Halder M, Isomaa, B, Kawahara, K, Lee L.E.J, Mothersill P, Pärt P, Repetto G, Sintes J.R, Wood C, Segner H (2003). The use of fish cell in ecotoxicology. The report and recommendations of ECVAM Workshop 47 PMID: 15612875.

Chasiotis H, Kolosov D, Kelly SP (2012). Permeability properties of the teleost gill epithelium under ion-poor conditions. *Am. J. Physiol.* 302: R727-R739. Doi.10.1152/ajpregu.00577.2011.

Chen J, Meyerhof DP, Tracy BL (2004). Model results of kidney burdens from uranium intakes. *Health Phys.* 86:3–11. PMID: 14695003.

Dayeh VR, Schirmer K, Bols NC (2002) Applying whole water samples directly to fish cell cultures in order to evaluate the toxicity of industrial effluent. *Water Res* 36: 3727–3738.PMID: 12369520.

Dayeh VR. Chow SL, Schirmer, K, Lynn DH, Bols NC (2004). Evaluating the toxicity of TritonX-100 to protozoan, fish and mammalian cells using fluorescent dyes as indicators of cell viability. *Ecotoxicol. Environ. Saf.* 57: 375-382. Doi: 10.1016/S0147-6513 (03) 00083-6.

DE Villiers S, Mkwelo S.T (2009). Has monitoring failed the Olifants River, Mpumalanga? *Water SA* 35:671-676. Doi:10.4314/wsa.v35i5. 49193.

Ercal N, Gurer-orhan H, Aykin-burns N (2001). Toxic metals and oxidative stress Part I: Mechanisms involved in metal induced oxidative damage. *Current Topics in Medicinal Chemistry 1:*529-539.Doi.10.2174/1568026013394831.

Escher B.I, Hermens J.L.M (2002). Modes of action in ecotoxicology: their role in body burdens, species sensitivity, QSARs, and mixture effects. *Environ. Sci. Technol.* 36:4201–4217. PMID: 12387389.

Faust M, Altenburger R, Backhaus T, Blanck H, Boedeker W, Gramatica P, Hamer V, Scholze M, Vighi M, Grimme L.H (2001). Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. *Aquat. Toxicol*. 56:13. doi: 10.1016 / j.chemosphere.2008.12.026.

Halestrap A.P, Kerr P.M, Javadov S, Woodfield K.Y (1998). Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. *Biochim. Biophys. Acta* 1366: 79-94. Doi./10.1016/S0005-2728 (98) 00122-4

Iji O.T, Serem J.C, Bester M.J, Venter E.A, Myburgh J.G, McGaw L.J (2017). Generation of reactive oxygen species in relevant cell lines as a bio-indicator of oxidative e ects caused by acid mine water. *Water SA* 43: 166-174. Doi.10.4314/wsa.v43i1.18

Ji S.W, Ko J.I, Kim S.J (2004). The status of the passive treatment systems for acid` mine drainage in South Korea. Proceeding of National Meeting of The American Society of Minerals and Rec. and The 25th West Virginia Surf. Mine Drainage. Task Force (Lexington, KY, April 18-24, 2004).

Koizumi T, Shirakura H, Kumagai H, Tatsumoto H, Suzuki KT (1996). Mechanism of cadmium-induced cytotoxicity in rat hepatocytes: cadmium-induced active oxygen-related permeability changes of the plasma membrane. *Toxicology.*114:125–134. Doi 10.1016/S0300-483X (96) 03477-4.

Kolosov D, Kelly S.P 2013. A role for tricellulin in the regulation of gill epithelium`permeability. *Am. J. Physiol.* 304: R1139-R1148. Doi:10.1152/ajpregu. 00086.2013.

Kramer N.I, Hermens J.L.M, Schirmer K (2009). The influence of modes of action and physicochemical properties of chemicals on the correlation between in vitro and acute fish toxicity data. *Toxicol in vitro* 23: 1372- 1379. Doi:10.1016/ j.tiv. 2009. 07.029.

Larsson P. (1985). Contaminated sediments of lakes and oceans act as sources of chlorinated hydrocarbons for release to water and atmosphere. *Nature* 317: 347- 349. Doi: 10.1038/317347a0.

Liu W., Au D., Anderson D.M, Lam P., Wu R. (2007). Effects of nutrients, salinity, pH and light:dark cycle on the production of reactive oxygen species in the alga Chattonella marina. *Journal of Experimental Marine Biology and Ecology* 346:76-86. Doi: 10.1016/j.jembe.2007.03.007.

Lund B.O, Miller D.M, Woods J.S 1993. Studies on Hg(II)-induced H2O2 formation and oxidative stress in vivo and in vitro in rat kidney mitochondria. *Biochem Pharmacol*. 45: 2017–2024.PMID: 8512585.

Madeira V.S, Echert A.B, Schneider C.H, Schneider I.A.H 2005 Sulfate reduction in acid mine drainage through anaerobic treatment with sulphate reducing bacteria. In: Proceedings of the XXI Ores National Treatment Dating Extractive Agents Metallurgy and interference processes,

degradation and environmental damage November, Natal RN.

Mangena S.J, Brent A.C. 2006. Application of a Life Cycle Impact Assessment framework to evaluate and compare environmental performances with economic values of supplied coal products. *J Cleaner Prod* 14:1071-1084. Doi.10.1.1.950.3729.

Mitsuyoshi, H., Nakashima, T., Sumida, Y., Yoh, T., Nakajima, Y., Ishikawa, H., Inaba, K., Sakamoto, Y., Okanoue, T., Kashima, K., 1999. Ursodeoxycholic acid protects hepatocytes against oxidative injury via induction of antioxidants. *Biochem. Biophys. Res. Commun*. 263: 537–542. PMID: 14999689.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65: 55–63.

Nobergh-King T.J, Ausley L.W, Burton D.T Goodfellow W.I, Miller J.L, Waller W.T 2005. Toxicity Reduction and Toxicity Identification Evaluations for effluents, ambient waters and other aqueous Media. Pansacola, Fl, USA : SETAC. Volume 1:152-163.

Paton C. 2008. Dam Dirty. F.M. BDFM Publishers (Pty) Ltd., Cape Town, South Africa, pp: 32–39.

Pourahmad J, O'Brien P.J 2000. A comparison of hepatocyte cytotoxic mechanisms for Cu^{2+} and Cd^{2+}. *Toxicology.* 143:263–273. PMID: 10755712.

Pulido M.D, Parrish A.R 2003. Metal-induced apoptosis: mechanisms *Mutat* 533:227–241. Doi: 10.1016/j.mrfmmm.2003.07.015.

Repetto G, Peso A.D, Zurita J.L 2008. Neutral red uptake assay for the estimation of cell viability / cytotoxicity. Nature protocols 3: 1125-1131. Doi: 10.1038/nprot.2008.75.

Schirmer K., Tom D.J, Bols N.C, Sherry J.P 2001. Ability of fractionated petroleum refinery effluent to elicit cyto and photocytotoxic responses and to induce 7 ethoxyresorufin-O- deethylase activity in fish cell lines. *Sci.Total Environ*. 271: 61-78.doi.org/10.1016/S0048-9697 (00) 00831-7.

*Oluwafikemi T. Iji, Jan G. Myburgh and Lyndy J. McGaw
© *African Journal of Environmental Health Sciences*
Volume 4, November, 2017
ISSN: 2476-8030
pp 51-60