

# Generation of reactive oxygen species in relevant cell lines as a bio-indicator of oxidative effects caused by acid mine water

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## ABSTRACT

Reactive oxygen species (ROS) production and resultant oxidative stress (OS) has been implicated as a pathway of toxicity in animal species exposed to pollutants. The gills of aquatic animals and the liver and kidneys of mammalian species are specific cellular sites of toxicity. Oxidative effects of acid mine drainage effluent (following passive and active treatment) impacting a natural stream were assessed using selected cell lines. Levels of pollutants such as heavy metals in acid mine drainage (AMD) effluent can be quantified following treatment, but it is unknown whether this is associated with equivalent reduction in toxicity. ROS production by AMD untreated (U) and after treatment (T) was quantified in a fish gill cell line (RTgill-W1) and in two mammalian cell lines (C3A human liver and Vero monkey kidney). ROS production was determined using the oxidant sensitive fluorogenic probe, 2', 7'-dichlorofluorescein diacetate (DCFH-DA) following exposure to U and T, AMD water. Treatment of AMD water caused reduction in levels of Al, Zn, Fe, Si and Mn while levels of Cr, Cu, Ar and Hg remained unchanged. A dose-dependent increase in ROS production was observed for U and T. ROS formation decreased from 14% to 4.5%, 16.4% to 7.2% and 25.3% to 17.7% in the RTgill-W1, C3A, and Vero cell lines exposed to 100% AMD water, U and T. The presence of Mn and/or other ions in treated water and subsequent ROS formation indicates that water could still be toxic to cells and requires further processing. The DCFH-DA assay in several cell lines can be used to rapidly bio-monitor quality of AMD water related to formation of ROS and subsequent cellular effects. However, cut-off levels for cellular toxicity must be established to ensure safety of this water for aquatic animals and for animal and human consumption.

**Keywords:** acid mine drainage, bio-monitoring, DCFH-DA, reactive oxygen species

## INTRODUCTION

Coal mining is a major industry in South Africa, as coal is the principal energy source for the country (Mangena and Brent, 2006). A number of coal mines are located in the Mpumalanga Province, where over 10 000 km<sup>2</sup> of hydraulically interlinked mines host 8 of the country's 10 operational coal-fired power stations (Heath et al., 2010). Most rivers have been negatively impacted by extensive industrial and mining activities in the Mpumalanga Province, threatening aquatic ecosystems (De Villiers and Mkwelo, 2009). Coal mining is the most important source of acid mine drainage (AMD) contamination affecting streams and rivers in the upper catchment of the Olifants River (Adler et al., 2007; Driescher, 2008). AMD effluent contains substances that contaminate aquatic ecosystems with effects ranging from chemical (bioavailable metal concentration), biological (acute and chronic toxicity) and ecological (loss of habitat and elimination of sensitive species) to others (Gray, 1997). Additionally, human exposure occurs by ingestion of contaminated water through drinking, preparation of food or irrigation of crops (Awofolu et al., 2005), and causes multiple organ toxicity in the brain, liver and kidneys (André et al., 1991; Bouquegneau and Joiris, 1992; Dietz et al., 1998).

AMD is a complex mixture, and of specific concern is the presence of heavy metals that cause oxidative stress (OS), which is characterized by a disruption in the net balance between the production of reactive oxygen species (ROS) or free radicals, and antioxidant defences by redox cycling, depletion of glutathione (GSH) levels, or catalysis of the Fenton reaction (Halliwell and Gutteridge, 1989; Valko et al., 2005; Lushchak, 2008; Sevcikova et al., 2011). Metals such as Fe, Cu, Cr and V are redox active metals that generate ROS by redox cycling through enhancing production of oxyradicals within cells (Kelly et al., 1998). Transition metals like Fe<sup>2+</sup> can react with H<sub>2</sub>O<sub>2</sub>, with the net reaction leading to formation of a hydroxyl anion and radical (Sevcikova et al., 2011). Other metals such as Hg, Ni, Pb and Cd impair antioxidant defence systems, for example, antioxidant enzymes and GSH (depletion via direct binding).

Assessing water quality from natural sources or water returned to the environment as wastewater is crucial to establish that no pollutants are present which could be harmful to various organisms that utilize the water. AMD effluent is usually treated for compliance before being discharged into water courses. A number of treatment technologies exist and these are used to improve the quality of AMD, such as by neutralization (hydrated lime or caustic soda is common), which is targeted at increasing water pH to enhance solubility of metals and consequently metal precipitation as metal hydroxides (Thompson, 1980), thus reducing metal burden and correcting acidity (Madeira et al., 2005). This outcome sometimes gives the appearance that the water is non-toxic.

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Received 2 December 2015; accepted in revised form 5 January 2017

In general, water quality is assessed based on its physicochemical characteristics: the pH, temperature, dissolved oxygen (DO), total dissolved solids, specific inorganic elements, etc. (Simpi et al., 2011). This does not furnish information on possible biological or chemical effects on the biota inhabiting the aquatic environment if polluted. When water quality monitoring is applied together with biomarkers, it provides additional detailed assessment of possible pollution effects (Bayne et al., 1988) applicable in environmental quality monitoring (Stegeman et al., 1992). Biomarkers are applied as sensitive indicators of pollution to detect underlying stressful conditions (Stegeman et al., 1992). With regard to pollution, biomarkers of exposure can be assessed based on antioxidant responses and oxidative stress parameters because pollutants enhance intracellular formation of ROS through mechanisms such as redox cycling, or cytochrome P450-dependent metabolism of polycyclic aromatic hydrocarbons and the Fenton reaction in the presence of transition metals (Regoli, 2000; Halliwell and Gutteridge, 2007; Monserrat et al., 2008).

Although ROS formation follows a normal physiological process in cellular metabolism, imbalances in generation and removal of ROS species could result in oxidative stress and biological and cellular damage (Dat et al., 2003; Mittler, 2002). ROS formation initiated by the presence of contaminants, resulting in OS, has been reported in biota exposed to pollutants. This suggests a possible connection between contaminant-stimulated ROS production and resultant OS as an established pathway of toxicity in exposed organisms which is present in aquatic animals to prevent ROS-induced damage (Di Giulio et al., 1995; Kelly et al., 1998; Winston and Di Giulio, 1991). Sustained increase in ROS generation (OH<sup>-</sup> radicals, hydrogen peroxide, and superoxide anions) leads to OS, causing distress in cellular metabolism and regulation, membrane and organelle damage and/or cellular death (Kelly et al., 1998). Environmental pollutants causing OS can modify the balance between pro-oxidants and antioxidant forces (Winston and Di Giulio, 1991).

From the perspective of environmental monitoring and assessment of pollution effects, it is useful to determine potential pro-oxidative effects of polluted waters, which enhances oxyradical production, to give an indication of the total antioxidant capacity or resistance to toxicity triggered by ROS. Studies involving aquatic organisms like fish have reported differences in susceptibility to oxidative damage in organs such as gills, brain, muscle, liver and kidney (Oliveira et al., 2008; Monserrat et al., 2008), some of which was attributed to higher antioxidant basal levels in various tissues. Rather than measuring a limited number of antioxidants or conducting classical oxidative stress studies quantifying antioxidant efficiency individually, an alternative is to evaluate oxidative effects of polluted water on relevant cell lines to serve as a quick and easy screening method when applied together with water quality parameters.

In-vitro techniques determine possible toxicological effects of effluent compounds at the sub-cellular level versus whole or intact animal testing, offering the advantage of rapid, reproducible and more cost-effective research (Castaño et al., 2003; Bols et al., 2005). These in-vitro systems have been extremely useful in the following studies: cytotoxicity (Environment Canada, 1990), drug metabolism and toxicity (LeCluyse, 2001), molecular toxicity (Blaauboer et al., 1998), and predicting biological reactivity of potential toxic compounds (Barratt, 2000).

Cell line use in toxicity testing involving environmental contaminants has been widely reported (Maruoka, 1978;

Kfir et al., 1981; Van Doren et al., 1984; Mochida, 1986; Bols et al., 2005). An advantage to using cell lines is that they allow exposure of cells directly to whole water with little toxicant lost to extraction processes or to further sample processing (Dayeh et al., 2002). Cell types representing specific cellular targets can be used, such as RTgill-W1 cell lines derived from fish gills. Fish cell lines may be used as potential alternatives to whole fish in testing environmental samples for toxicity. The RTgill-W1 and RTL-W1 liver epithelial fish cell lines from rainbow trout have been used successfully (Dayeh et al., 2005). Mammalian cell lines such as HepG2 and Vero kidney cells representing liver and kidneys, respectively, can be used to evaluate mammalian toxicity. Mammalian cell cultures have been used successfully in identifying and understanding possible effects chemicals pose to humans and in studies performed by health laboratories and water quality regulators (Rees, 1980). Richardson et al. (1977) used a mammalian cell culture assay to evaluate water quality of oil refinery effluents.

Herein, we describe OS attributable to ROS production using an oxidant sensitive fluorogenic probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA), to detect intracellular ROS formation (Rosenkranz et al., 1992) as an early stage marker for xenobiotic-induced OS in cultured fish and mammalian cell lines. DCFH-DA is a cell-permeable, non-fluorescent dye that easily crosses biological membranes because the acetate ether is uncharged; but when cleaved by intracellular esterases it becomes charged and is retained intracellularly (Pereira et al., 2003; Poljsak et al., 2005). On hydrolysis, DCFH-DA changes to non-fluorescent DCFH, but the presence or generation of ROS oxidizes DCFH to highly fluorescent dichlorofluorescein (DCF) (LeBel et al., 1992). The increase in fluorescence can be quantified and used as an index to determine overall cellular OS.

## MATERIALS AND METHODS

### Location and description of the study area

The study was conducted at the receiving stream 'Kromdraai' (25°46' 05.5"S; 29° 07' 15.5"E) located in the Highveld region close to Witbank (Mpumalanga Province, South Africa). AMD effluent from the mine passes through a wetland into a free-flowing receiving stream which is continuously dosed with a neutralizing agent in-stream to increase the water pH and precipitate metals as hydroxides, improving water quality.

In-situ measurements of temperature, dissolved oxygen and pH were conducted at the two sample collection points along the course of the stream. Temperature, dissolved oxygen and pH were measured using a portable multimeter (HACH HQd, USA). The water samples collected immediately after flowing through the wetland (where it undergoes passive treatment) were termed 'untreated AMD water sample' (U), while the water samples collected downstream beyond the dosing tanks (which continuously supplied the neutralization agent) were termed 'post-treated AMD water sample' (T). Samples were collected as subsurface grab samples, and placed on ice immediately for transport back to the laboratory for analysis. Water samples were analysed by the Analytical Services, Chemistry Department, CSIR, Pretoria for nutrients (ammonia, orthophosphate, phosphate, nitrate and nitrite), metals (aluminium, arsenic, chromium, copper, iron, mercury, silicon and zinc) and inorganic constituents (calcium, magnesium, potassium, sodium, chlorine and sulphates). These were all determined according to the methods of APHA (1995). Water samples for bioassays were filtered through a 0.22 µm in-line filter within 2 h of collection

and stored at 4°C at the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria.

### Cell cultivation

The C3A cell line (derivative of HepG2), purchased from the American Type Culture Collection (ATCC CRL-10741), and Vero monkey kidney cells (from the collection of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria) were grown in vented filter-cap flasks at 37°C in Minimal Essential Medium (MEM, Sigma-Aldrich, USA). Vero monkey kidney cell culture medium was supplemented with 0.1% gentamicin (Virbac, PHENIX, SA) and 5% foetal calf serum (FCS, Highveld Biological South Africa), while the C3A cells were grown in antibiotic-free medium supplemented with 10% FCS and 1 mM sodium pyruvate (Sigma-Aldrich, St. MO, USA). Cells of a sub-confluent culture were harvested and centrifuged at 200 x g for 2 min, and re-suspended in growth medium supplemented as above, and 100 µl (5 x 10<sup>4</sup> cells/mL) were plated in each well of columns 2 to 11 of a 96-well microtitre plate. The plates were incubated for 24 h at 37°C in a 95% air 5% CO<sub>2</sub> humidified environment.

The RTgill-W1 cell line (ATCC CRL2523) was cultured in Leibovitz's L15 medium supplemented with 12% foetal calf serum (FCS, Highveld Biological South Africa) in atmospheric air at 20°C. Cells from a sub-confluent culture were re-suspended in cell culture medium supplemented with 10% FCS and 2% penicillin-streptomycin (5 000 U/L penicillin, 5 000 µg/mL streptomycin, Gibco Life Technologies, USA).

### DCFH-DA assay for ROS generation

A 50 µL aliquot of 75 µM DCFH-DA was added to 100 µl of medium per well to give a final concentration of 25 µM DCFH-DA. After the cells were exposed for 45 min in 95% air 5% CO<sub>2</sub> at 37°C for the C3A and Vero monkey kidney cells, and at room temperature in atmospheric air for the RTgill-W1 cells, the excess extracellular DCFH-DA was removed by 2 washes of phosphate-buffered saline (PBS).

In order to assess ROS formation, the adherent cells with intracellular DCFH-DA were exposed to varying concentrations of AMD from the untreated and treated AMD samples. Dilutions of 100% (whole effluent), i.e., 75%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.562% and 0.781%, were prepared using ultra-pure Milli-Q water that also served as the negative control. For the positive control, 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) was used at 4 mg/mL dissolved in Milli-Q water. The experiments were performed in triplicate for each concentration and treatment, and the entire experiment was repeated twice to verify results. AAPH is a water-soluble azo compound, which is used extensively as a free radical generator; it is used often in the study of lipid peroxidation and the characterization of antioxidants (Liégeois et al., 2000). Following the breakdown of AAPH, molecular nitrogen and 2 carbon radicals are produced which may then combine to produce stable products or react with molecular oxygen to give peroxy radicals. The fluorescence was measured using a FLUOstar fluorescence plate reader (BMG, LABTECH, Germany) with temperature maintained at 37°C using a 485/520 nm excitation/emission filter. The fluorescence from each well was captured, digitized, and stored on a computer using OPTIMA software. Readings were taken at intervals of 120 s in a selected 30-cycle period up to 1 h.

### Data analysis

Reactive oxygen species formation was expressed as a gradient of the fluorescent units of the oxidized dye DCFH-DA from 0 to 1 h in the different concentration ranges expressed as a percentage of the positive control, AAPH, which was given a value of 100% toxicity. The Milli-Q water, which served as the negative control, was given a value of 0%.

ROS formation was calculated using the formula:

$$\frac{\text{Gradient of sample} - \text{gradient of Milli-Q water}}{(\text{gradient of AAPH} - \text{gradient of Milli-Q water})} \times 100$$

The net change in fluorescence per well was calculated by taking an average of the change in fluorescence over time. The advantage of this method is that it calculates the net changes in fluorescence, such that the calculated data directly reflect the percentage changes of fluorescence over time from the cells in the same well. This method further cancels out the background fluorescence in each well and, therefore, does not require a 'no cell' control. Statistical analyses were performed with SPSS version 8.0 (SPSS Inc., Chicago IL) software, using analysis of variance (ANOVA) followed by Fisher's Protected Least Squares difference post-hoc test for individual comparisons.

### RESULTS

The pH measured at the two sample collection stations varied considerably (Table 1). Significant acidification of the water pH at 3.65 was observed from the collection point emanating from the wetland U into the receiving stream T.

EC values obtained for U and T (320 and 326 mS/m) water samples were higher than the recommended guidelines stipulated for industrial and domestic use (250 mS/m, 70 mS/m). Comparable levels for some nutrients, i.e., total phosphorus, orthophosphate and ammonia were obtained for U and T, whilst a small increase in the level of nitrate and nitrite for T vs. U was observed. Specific inorganic constituents like Na, Mg, K, and Ca were present in high concentrations in U and T although set guideline levels were unavailable in some instances.

The metals Al, Cr, Cu, Fe, Zn, As, Hg, Mn and Si were analysed in their dissolved states. Metal analyses performed on water samples from the discharge site showed values for Fe, Zn and Mn to be much higher than the maximum recommended values for discharged wastewater used for industrial purposes (Government Gazette, 1984; DWAF, 2004). Guideline values for wastewater or effluent produced by or resulting from the use of water for industrial purposes for rivers and dams and suitable for protection of aquatic life are also presented in Table 1 (DWAFEC, 1980). However, downstream end-users access water for drinking and food preparation and so guidelines relating to the safety of water for domestic use and human consumption are also presented in Table 1. No set guideline values were available for Al and Si. The concentrations of As, Hg and Cr remained unchanged in U and T.

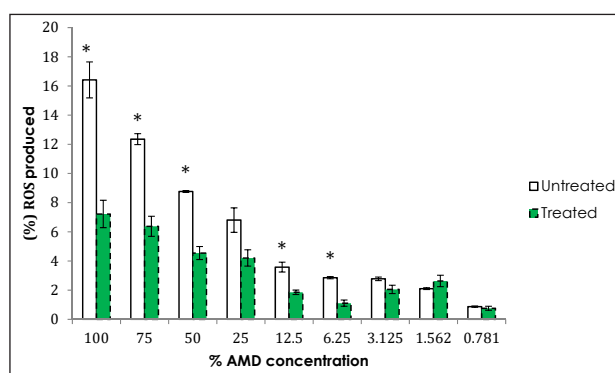
The in-stream neutralization process effectively removed Fe, Al, Zn and Si from the water and the levels of Fe and Zn post-treatment fell within recommended guidelines for industrial effluent and domestic use. In general, U and T water samples presented higher metal levels than recommended guidelines and this was especially noticeable for Mn, Al, and Zn. A slight increase in sulphate levels following neutralization was observed, and the levels of Mn remained high post treatment at 39 mg/L. Following neutralization as a treatment option, total dissolved solids (TDS) levels of Na, Ca, K, and Mg increased downstream when comparing T and U, and an increase in alkalinity was observed.

**TABLE 1**  
**Water chemistry analytes of AMD water samples (n = 2 determinations) collected at Kromdraai, Mpumalanga, South Africa compared to guideline values (DWAF, 1996, 2004, 2007) for effluent produced by or resulting from the use of water for industrial purposes, and South African water guidelines for domestic use.**

Metals present as ions dissolved in water					
Analysis	Unit	Untreated AMD	Treated AMD	Guidelines for industrial use	Guidelines for domestic use
Potassium (K <sup>+</sup> )	mg/L	9	11	0.1	N/A*
Sodium (Na <sup>+</sup> )	mg/L	43	120	N/A	100
Calcium (Ca <sup>2+</sup> )	mg/L	475	561	N/A	N/A
Magnesium (Mg <sup>2+</sup> )	mg/L	160	162	N/A	170
Alkalinity (CaCO <sub>3</sub> levels)	mg/L	2.75	6.4	20	20–300
Sulphate (SO <sub>4</sub> <sup>2-</sup> )	mg/L	2 315	2 336	1 400	200
Chloride (Cl <sup>-</sup> )	mg/L	3.5	3.5	N/A	250
Ammonia (NH <sub>4</sub> <sup>+</sup> )	mg/L	6.7	6.45	1	N/A
Nitrate & nitrite (NO <sub>3</sub> <sup>-</sup> )	mg/L	2.3	3.6	1.5	6
Orthophosphate (PO <sub>4</sub> <sup>3-</sup> )	mg/L	0.15	0.15	1	N/A
Total phosphorus (TP)	mg/L	<0.25	< 0.25	N/A	N/A
Electrical conductivity	mS/m 25°C	320	326	250	70
pH	20°C	3.65	8	5.5–7.5	6–9
Chemical oxygen demand (COD)	mg/L	10	33	30	N/A
Dissolved oxygen (DO)	mg/L	7.1	5.05	5.8	N/A
Iron (Fe <sup>2+/3+</sup> )	mg/L	0.36	< 0.01	0.03	0.1
Manganese (Mn <sup>2+/3+</sup> )	mg/L	65	39	0.1	0.05
Zinc (Zn <sup>2+</sup> )	mg/L	3.4	0.044	0.3	1
Arsenic (As <sup>3+</sup> )	mg/L	<0.001	< 0.001	0.1	0.1
Mercury (Hg <sup>2+</sup> )	mg/L	<0.005	< 0.005	0.02	0.005
Silica (Si <sup>4+/4-</sup> )	mg/L	9	2	N/A	N/A
Aluminium (Al <sup>3+</sup> )	mg/L	28	0.22	N/A	N/A
Chromium (Cr <sup>2+/3+</sup> )	mg/L	<0.01	< 0.01	0.05	N/A
Copper (Cu <sup>+/2+</sup> )	mg/L	<0.03	0.03	0.02	N/A

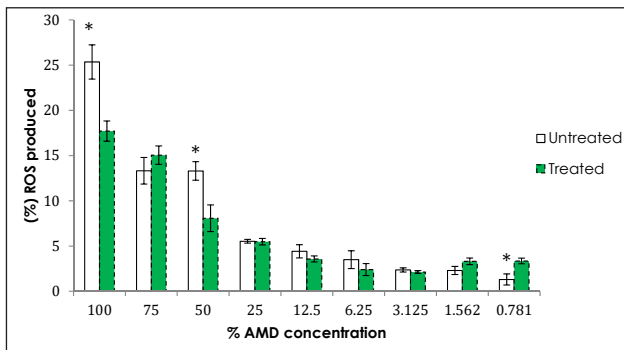
N/A = not available

In this study 3 cell lines were exposed to serial dilutions of U and T water samples (Fig 1–3). An increase in ROS production was observed following exposure to increasing concentrations of AMD water (U and T). The maximum amount of ROS generated using whole effluent for the untreated AMD water was 14%, 16.3% and 26.5%, for the RTgill-W1, C3A and Vero cells, respectively. A direct correlation was found between % AMD concentration and % ROS formation for U, with a R<sup>2</sup> correlation of 0.912, 0.985 and 0.945 for the RTgill-W1, C3A and Vero cell lines, respectively. The rate of decrease in ROS production with decreasing % AMD concentration was 0.1044, 0.144 and 0.212 fluorescence units/h for the RTgill-W1, C3A and Vero cell lines, respectively. Vero cells were most sensitive to the effects of U water as shown by the highest level of ROS production (% of positive control) observed for all cell lines following exposure to whole effluent.



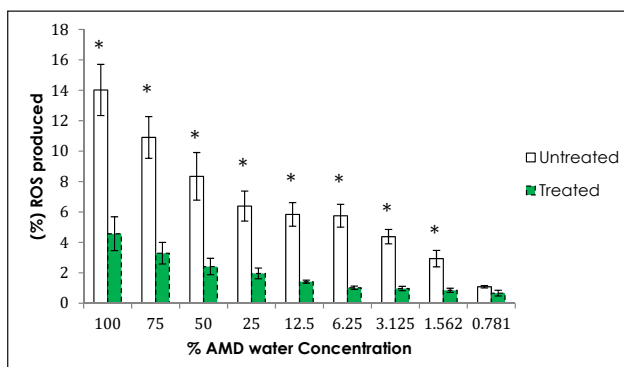
**Figure 1**

Percentage (%) ROS produced in C3A cells. Data is an average of 9 data points ± SEM. \*refers to statistical significance determined using one-way ANOVA ( $p < 0.05$ ).



**Figure 2**

Percentage (%) ROS produced in Vero cells. Data is an average of 9 data points  $\pm$  SEM. \*refers to statistical significance determined using one-way ANOVA ( $p < 0.05$ ).



**Figure 3**

Percentage (%) ROS produced in RTgill-W1 cells. Data is an average of 9 data points  $\pm$  SEM. \*refers to statistical significance determined using one-way ANOVA ( $p < 0.05$ ).

Following treatment, the maximum amount of ROS generated for the treated AMD water was 4.56%, 7.22% and 17.71% for the RTgill-W1, C3A and Vero cells, respectively. A direct correlation was found between % AMD concentration and % ROS formation for T with a  $R^2$  correlation of 0.983, 0.902 and 0.969 for the RTgill-W1, C3A and Vero cell lines, respectively. The rate of decrease in ROS production expressed in terms of fluorescence units/h with decreasing % AMD concentration was 0.0358, 0.0601 and 0.156 for the RTgill-W1, C3A and Vero cell lines, respectively. Fold change in gradient U vs. T was 2.916, 2.39 and 1.36 for each respective cell line, indicating that treatment of AMD water resulted in a decrease in ROS.

Vero cells were most sensitive to treated AMD water at varying concentrations. They were consistently sensitive in terms of % ROS generation using untreated AMD water samples when compared to the C3A and the RTgill-W1 cell lines.

## DISCUSSION

A number of anthropogenic activities resulting in pollution have been associated with pro-oxidant or oxyradical production in aquatic organisms within the aquatic environment (Winston and Di Giulio, 1991; Bairy et al., 1996). As an indicator of oxidative stress, evaluating loss of specific antioxidants and/or generation of oxidation products is employed. A commonly used method is the TOSC (total oxyradical scavenging capacity) that quantifies the overall tissue capacity to scavenge different forms of ROS,

hence providing a general picture of the oxidative stress of that particular tissue (Regoli and Winston, 1999; Regoli et al., 2002). Regoli et al. (2004) reported the successful use of the TOSC method at detecting depleted antioxidant defences in mussels (*Mytilus galloprovincialis*) transplanted to polluted areas, by correlating the influence of pollution to oxidative DNA damage. The advantage of TOSC (and, by extension, an equivalent methodology) is that it establishes an integrated antioxidant response of an organism or tissue against a particular type of ROS, like peroxy, or radicals.

Effluent discharge from mining activity impacts water bodies negatively, usually causing a resultant increase in dissolved metals, such as Al, Fe, Zn, Mn, Cu, or Cd (Grippio and Dunson, 1996). Aquatic pollutants have potential to induce formation of ROS in organisms resulting in OS (Lesser, 2006) that can lead to cellular dysfunction and possibly death. The presence of transition redox-active transition metals can initiate a reaction to produce a highly reactive oxygen species (ROS), the hydroxyl radical (OH $\cdot$ ), via the Fenton reaction (Kelly et al., 1998).

The use of in-vitro contaminant-stimulated ROS production has been demonstrated in a number of species for a number of contaminants (Livingstone, 2001). In this study the RTgill-W1, and 2 mammalian cell lines (C3A and Vero) that represent specific cellular sites of oxidative damage in mammalian species, including humans, were included. An increased ROS generation was observed following exposure to U and T. Net increases in ROS indicates a state of oxidative stress (Livingstone, 2001). Synergistic effects between different metals can cause increased levels of oxidative damage. Al, Fe, Zn and Mn levels were present at concentrations that were above permissible guidelines and as such may have played a role in the observed increased ROS formation in U (Table 1). DiToro et al. (2001) reported that the presence of free metal ion concentration is suggestive of availability and likely toxicity. Of the metals present in the effluent, Fe, Cu, Cr and Ar are reported to generate ROS by redox recycling, while Hg depletes GSH and thiol-containing antioxidant enzymes. Both processes result in an increase in ROS production (see review by Ercal et al., 2001).

Metals such as Al, Cd, U, Zn, Co, Ni, Pb, Mn and Cu have been recognized to be of potential ecotoxicological concern arising as a consequence of mining (Markich and Camiller, 1997). Metals present as free metal ions or those that form weak complexes readily become bioavailable compared to those bound to particulate matter or with strong bonds (Batley, 2004).

A direct correlation, with  $R^2$  close to a value of 1, was seen with % AMD concentration and ROS generation. This implies that the presence of certain pollutants was responsible for the observed increase in ROS production. A number of environmental stressors exert toxic effects via different pathways based on their metabolism and reactivity; metals are reported to exert direct pro-oxidant effects through biochemical oxidation/reduction mechanisms by triggering apoptosis and oxidative stress (Assefa et al., 2005; Valko et al., 2005). Contreras et al. (2005) reported a significant increase in ROS and lipo-peroxide levels in seaweeds around the discharges of copper mines, while Bopp et al. (2008) reported a 25- to 35-fold ROS induction in rainbow trout gill cells exposed to Cu (total Cu 100  $\mu$ M, pH 7). Exposure to a mixture of heavy metals (Cu, Cd, Fe and Ni) caused a decrease in the levels of antioxidant enzymes, reduced GSH and an increase in lipid peroxidation in the gills of the freshwater fish *Channa punctata* (Pandey et al., 2008). Heavy metal cations have a great propensity for the -SH group of GSH (Viarengo, 1993). Garcia-Alfonso (1995) also reported changes in the antioxidant activities in cultured Vero cells after 24 h

exposure to Fe<sup>2+</sup> and Fe<sup>3+</sup>. ROS formation was thought to have followed the process of interconverting through the Haber-Weiss reaction.

The decrease in ROS generation in T could be explained by the decrease in the metal load of the water sample. When low-pH AMD water containing elevated concentrations of dissolved metals was treated in-stream by dosing with the neutralizing agent, the pH increased abruptly, and metals such as Fe, Al, Zn and Si precipitated rapidly into colloidal and particulate forms. The rise in pH of the stream achieved through the in-stream alkali neutralization process resulted in complexation phenomena and consequent metal hydroxide precipitation (Madeira et al., 2005). This may have accounted for the reduced toxicity in effluent water T, as % ROS generated using whole effluent showed a decrease from 26.5% to 17.7% in Vero monkey kidney cells, in C3A cells from 16.3% to 7.2% in C3A and 14% to 4.5% in RTgill-W1 cell lines. The persistence of Mn in T may be because the optimal precipitation of Mn occurs at a higher pH of between 9 and 10 (Means, 2004).

Another observation was the increase in inorganic ions post-treatment in T – this involved major cations like Ca and Mg which are reported to reduce metal bioavailability as they compete with metals for binding sites on the cell surface (Twining, 2000). A decrease in toxicity of effluent from an abandoned uranium mine was described to be concurrent with an increase in pH of effluent (Franklin et al., 2000).

Water chemistry parameters for metals such as Al, Fe, Zn and Si were reduced by 21-, 19-, 146- and 4.5-fold, respectively, in effluent water T (Table 1). Consistent with these findings were those of Moore (1992), where differences in ROS formation were observed in isolated hepatocytes of dab (*Limanda limanda*) from polluted sites versus cleaner sites in the German Bight, North Sea (Schleizinger et al., 2000). Choi and Oris (2000) likewise reported increased ROS production involving a number of water-borne contaminants like duroquinone (component of pulp mill effluent) and benzo[a]pyrene (BaP) diones.

A number of water parameters fell short of the recommended standards. High concentrations of SO<sub>4</sub>, together with higher EC and TDS values, were observed. Increase in conductivity, which is an index for TDS pollutant, indicates the level of pollution (USEPA, 1982). Inorganic ions such as K<sup>2+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Na<sup>+</sup> and Mg<sup>2+</sup> were present in relatively high concentrations in the AMD effluent and concentrations increased downstream post-neutralization. Richness in inorganic ions influences metal bioavailability and toxicity (Antunes, 2007) and water chemistry parameters strongly influence toxicity of metals to aquatic organisms (Paquin et al., 2002). In categorizing the level of AMD impact using water quality parameters that suggest AMD impact, the site could be termed as being highly impacted (Federal Water Pollution Control Administration, 1968) raising a question of compliance to effluent discharge limits, toxicity to water life and suitability for downstream end-users.

Differences in ROS formation were observed in the three cell lines (Table 2). The Vero cell line was the most sensitive (least antioxidant capacity), as it generated the most ROS across all concentrations in T and in higher concentrations exposed to U. There are reports suggesting differences in vulnerability to oxidative damage (Monserrat et al., 2008; Oliveira et al., 2008). The reason for Vero cell sensitivity is unknown, but the kidneys from which they are derived are important target organs usually related to heavy metal toxicity (Gardner et al., 2006; Barbieri et al., 2009). These cell lines may also have lower antioxidant basal levels, making them more prone to ROS formation, and unsuitable for this purpose.

% AMD water	Untreated	Treated
100	Vero	Vero
75	C3A /Vero/RTgill-W1	Vero
50	Vero	Vero
25	Vero/C3A	Vero/C3A
12.5	C3A /Vero/RTgill-W1	Vero
6.25	RTgill-W1	Vero
3.125	RTgill-W1	Vero/C3A
1.562	C3A /Vero/RTgill-W1	Vero/C3A
0.781	C3A /Vero/RTgill-W1	Vero

The C3A and the RTgill-W1 cells derived from human liver and fish gills, respectively, displayed lower ROS formation and, by extension, higher antioxidant capacity on exposure to U and T AMD water. This may be connected with the fact that the cells were derived from organs actively involved with metabolism of xenobiotics. Both cell lines exhibited a decrease in the rate of fluorescence (ROS formation) with decreasing % AMD concentration. We observed a more consistent dose-response relationship using the C3A and the RTgill-W1 cells. A higher fold change in gradient U vs T observed in these cell lines suggests their adaptability and possibly suitability to changes in water conditions. The changes with regard to this experiment stemmed from treatment of U to T, which resulted in lower metal concentration and concurrent decrease in ROS.

In the whole animal, the intact gill is described as one of the most critical sites of toxicity to waterborne contaminants in fish (Wood, 2001), because it is immersed in water and its function involves the uptake of minerals including metals (Bury et al., 2003). Gills have previously been reported to become fatally damaged following exposure to metals (Castaño, 2003). Generally, the use of fish cell lines in aquatic research involving aquatic contaminants is believed to be better suited than mammalian cell lines, because cells from fish cell lines most probably better reflect the properties of the fish from which they were initiated than cells of mammalian origin (Bols et al., 2005).

In parallel to ROS measurements, cytotoxicity of effluents was determined within a 1 h period using whole effluent (results not shown). Since a clear positive correlation has been reported between the number of viable cells and consequent increase in DCF fluorescence (Bopp et al., 2007), this was necessary so that exposed cells do not become damaged from cytotoxic effects, leading to an underestimate of potential ROS formation.

## CONCLUSION

This study showed that the water quality parameters of the receiving stream were adversely impacted by AMD discharge and the presence of metals at environmentally-relevant concentrations may have contributed to observed increased ROS formation in cell lines exposed to U and T. Since biological diversity and physiological state of the biota are direct indices

of water quality, in-vitro techniques involving cellular ROS formation may serve as an alternative relevant model for evaluating potential oxidative effects of polluted waters. This model system using cell lines that represent the target tissues of toxicity can be routinely implemented as a fast, easy and straightforward manner to estimate the possible consequence of exposure to certain aquatic environmental pollutants involving AMD, because antioxidant competence is a mutually-inclusive measurement in biomonitoring research (Monserrat et al., 2008). A limitation is that there is no standard cut-off level for ROS formation that indicates the degree of safety. Another drawback might be that specific biomarkers to link to the antioxidant response were not used in this study.

Bearing in mind that antioxidant competence is measured by ROS determination, this method, applied together with other bioassays related to environment health research, could be a useful alternative to whole animal testing and exposure to polluted streams for detection of onset of OS.

## ACKNOWLEDGEMENTS

OTI is grateful to the Schlumberger Stichting Fund, The Netherlands for a fellowship. The National Research Foundation and the Department of Paraclinical Sciences (University of Pretoria) are acknowledged for research funding [Project number: V027-12].

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