Assessing the cyto-genotoxic impacts of un-neutralised and pH-neutralised acid mine drainage on the human breast cancer cell line, MCF-7

BY

Shirmoné Botha

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Under the supervision of

Prof. A. M. Botha-Oberholster

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Under the supervision of

Mrs B. Genthe

Dr P. Oberholster

December 2015

Declaration

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ABSTRACT

The use of toxicity tests to evaluate the quality of streams affected by mixtures such as acid mine drainage (AMD), adds value to assessments whereby site-specific toxicological data may identify toxicants that pose a threat to humans. To successfully evaluate the risk of combined mixtures, an improved understanding of the individual components, their uptake, metabolism, excretion and mode of action is required. This study aimed to identify the extent of AMD toxicity in a dose dependant manner on the MCF-7 cell line. The first study site associated with gold mining was chosen as the Tweelopies Stream situated in the Gauteng province of South Africa. The AMD effluent (un-neutralised) contaminating the Tweelopies Stream had undergone pH-neutralisation using a reactor-bed limestone technology incorporating the use of both calcium carbonate ($CaCO_3$) powder and limestone beds. The second study site, the Kromdraai River, is situated in the eMalahleni region of South Africa where a predominance of coal mining exists. The pH-neutralisation of the AMD (un-neutralised) contaminated Kromdraai River was performed using a caustic soda (NaOH) precipitation technique. This study demonstrated the rapid and effective application of the comet assay as a screening tool for AMD-associated DNA breakages in the human cell line, MCF-7. Moreover, the study analysed parameters of cellular survival, DNA fragmentation and variations in morphologies indicative of cellular death. Collectively, the cyto-genetic aberrations observed in the MCF-7 cells as a result of exposure to gold and coal mining associated AMD, confirms the urgency of incorporating high-throughput screening in ecological toxicity assessment to evaluate cellular damage at genetic levels in low dose exposures where detection might be missed.

SAMEVATTING

Die gebruik van toksisiteitstoetse om die gehalte van strome te evalueer wat geraak word deur mengsels soos suur mynwater (SM), gee waarde aan spesifieke toksikologiese data van gifstowwe wat 'n bedreiging vir die mens kan identifiseer. Om die risiko van gekombineerde mengsels en hul individuele komponente beter te begrip en suksesvol evalueer, is hul opname, metabolisme, uitskeiding en modus van aksie nodig. Hierdie studie het gepoog om die omvang van SM-toksisiteit in 'n dosis afhanklike wyse op die MCF-7-sellyn te identifiseer. Die eerste studie-area wat gekies is, hou verband met goudmyn-ontginning, en is die Tweelopiesspruit, geleë in die Gauteng-provinsie van Suid-Afrika. Die SM-uitvloeisel (on-geneutraliseerde) wat die Tweelopiesspruit besoedel, het pH-neutralisasie ondergaan met behulp van die integrasie van 'n reaktor-bed kalksorpsietegnologie wat gebruik maak van beide kalsiumkarbonaat ($CaCO_3$) poeier en kalksteenbeddens. Die tweede studie-area, is die Kromdraairivier geleë in die eMalahleni-streek van Suid-Afrika, waar steenkoolontginning die oorheersende aktiwiteit is. Die pH-neutralisasie van die SM (on-geneutraliseerde) in die geval van die Kromdraairivier word met behulp van 'n bytsoda (NaOH) neerslag tegniek, uitgevoer. Hierdie studie het die komeet-toets getoon as 'n vinnige en doeltreffende toepassing vir SM-geassosieerde DNA-breekskade in die menslike sel lyn, MCF-7. Verder het die studie parameters van sellulêre oorlewing, DNA-fragmentasie en variasies in sel morfologieë wat ‘n aanduiding van sellulêre dood is, ontleed. Gesamentlik dui die resultate daarop dat die sitogenetiese afwykings wat in die MCF-7-selle waargeneem is, as 'n gevolg van blootstelling aan goud- en steenkool-geassosieerde SM is. Die studie het verder die dringendheid van die integrasie van hoë-deurset tegnologieë in ekologiese toksisiteitstoetse in selle wat genetiese skade mag ondergaan, na 'n lae dosis blootstelling waar opsporing dalk gemis word, ondersteun.

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I dedicate this thesis to my grandfather Christiaan Voight. You have inspired me to never stop seeking.

Shirmoné Botha

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CHAPTER ONE

1.1 BACKGROUND AND RATIONALE

The generation of acid mine drainage (AMD) and dissolution of metals is a pertinent factor threatening the South African environment (Younger, 2001).

The decant and contaminated catchments in the western basin of South Africa became an issue of concern with an observed decant of acid mine drainage from an abandoned gold mine in 2002 (McCarthy, 2011). The decant occurred at an estimated rate of 20 mega litres of water per day into the Tweelopies Spruit. To restore the ecosystem in the Tweelopies Spruit, the implementation of a limestone neutralisation strategy was initiated (DWAF, 2009). Similarly, a discharge of coal mining water into the Olifants River catchment was reported (Maree et al., 2004). Thus deterioration in water quality was observed with sulphate loads at 70 tons per day (Hodgson & Krantz, 1998).

Approaches to evaluate the ecological impacts of AMD traditionally include aspects of formation, runoff and composition (Hobbs, 2007). These studies have made important strides in qualitatively assessing the impacts of AMD on the surrounding ecosystem (Jooste and Thirion, 1999). However, few studies investigate the accumulative effect of AMD at a tissue or cellular level of organisation. A study conducted on rodent species in a mine shaft revealed the bioaccumulation of heavy metals in tissues (Andrews et al., 1984).). In a study performed by Lewis and Clark (1996), the combinative effects of low pH and high concentrations of metals, such as arsenic and aluminium was shown to have severe toxicological effects on aquatic ecosystems.

The toxic exposure of humans to metals often occur through the consumption of contaminated water, plant roots, deposition of metal contaminants onto plant surfaces, and animals which have fed on metal burdened plants (McBride, 2003). Although toxicity of metals to humans has been reported, several metals form crucial roles in maintaining the biological homeostasis of cells. Transition metals such as copper, zinc, iron and manganese, which are commonly found in AMD, similarly control metabolic and signalling pathways (Valko et al., 2005). However, within a complex mixture such as AMD, metals have the ability to escape control mechanisms of transport, homeostasis, compartmentalization and binding to their designated cellular components (Valko et al., 2005). As a result, metals may displace one another by eliciting blocking signals to natural binding sites. Therefore, metal induced toxicity has the ability to prompt the malfunction of cells which eventually lead to complete toxicity. Metals have unique co-ordination chemistries and redox properties which enable metal toxicity through oxidative stress (Leonard et al., 2004).

The best evidence supporting the hypothesis of the oxidative nature of metal-induced genotoxic damage is provided by the wide spectrum of nucleic products typical for the oxygen attack on DNA in cultured cells and animals exposed to carcinogenic metals (Beyersmann and Hartwig, 2008). The carcinogenic effect of metals may be induced by targeting a number of cellular regulatory proteins or signalling proteins participating in cell growth, apoptosis, cell cycle regulation, DNA repair, and differentiation. These factors control the expression of protective genes that repair damaged DNA, power the immune system, arrest the proliferation of damaged cells, and induce apoptosis (Wang and Shi, 2001). The “decision” to commit to cell death or cell survival will in part depend on the concentration and duration of oxidant exposure and on the cell type involved.

This study represents an investigation of the effects of un-neutralised and pH-neutralised AMD on nucleic DNA. The study considers the dose-response relationships as an integral

factor in investigating cellular toxicity to AMD. However, data gaps do exist, and a number of possibly critical cellular effects remain to be investigated.

Study objectives:

To date, the impact of un-neutralised and pH-neutralised AMD on human cells have not been elucidated. The current study aims to fill this knowledge gap by identifying signal transduction pathways responsible for cyto-genotoxicity in human cells exposed to un-neutralised and pH-neutralised AMD. To accomplish this, the study intends to focus on biomarkers of toxicity such as cellular proliferation rates, morphologies of toxicity and DNA breakages. The MCF 7 cell line was chosen for the study as it was shown to have the excellent ability to withstand harsh conditions which allow the use of higher levels of exposure (Wu et al., 2006). Furthermore the study aims to compare the extent of DNA damage in the gold and coal mining related AMD and pH-neutralised AMD. Thus the investigation presents as a first study exploring the use of molecular techniques in the assessment of environmentally toxic substances such as AMD on a human cell line.

This dissertation is divided into four chapters:

Chapter 2 structured as a literature review, explores the characteristics of acid mine drainage (AMD) and the neutralisation techniques employed. In addition, chapter 2 highlights the main components of cellular and genetic toxicity. Chapter 3 investigates the impact of gold mining related AMD and neutralised AMD from the Western Basin of South Africa, on the MCF-7 cell line. Chapter 4 investigates the impact of coal mining related AMD and neutralised AMD from the Mpumalanga province of South Africa, on the MCF-7 cell line. Lastly, chapter 5 presents a summary of the major findings of the study.

The following outputs were delivered by the study:

Conferences

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Assessing the cyto-genotoxic impact of gold mining-related acid mine drainage on the human breast adenocarcinoma cell line (MCF-7), March, 2013. South Africa. Council for Scientific and Industrial Research (report number 223840).

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CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

An environmental pollutant has the potential to cause harm to living organisms due to its inherent toxic properties. These substances are usually present at levels far beyond a set tolerance limit, thus impairing the welfare of the environment (Duruibe et al., 2007). The mining of minerals such as gold, nickel and iron ore are responsible for long term physical disruption of the environment, giving rise to toxic runoffs such as acid mine drainage (AMD). The production of AMD is a function of the surrounding mineralogy and the rate of pyrite oxidation in the presence of oxygen and water (Nordstrom and Southam, 1997). Un-neutralised AMD is a highly conductive mixture predominantly comprised of metals, sulphates, iron, aluminium, and a low pH (Pastor et al., 2001). The processing of metal and mineral ore can significantly increase the availability and mobility of metals and minerals in the environment which may have toxic implications on the surrounding ecology (Akcil and Koldas, 2006).

In South Africa, mines and governmental organisations commonly employ neutralisation technologies to reduce the severity of AMD contamination in receiving water bodies. The use of active technologies such as limestone and sodium hydroxide have proven to successfully precipitate metals out of the system as metal hydroxides (Maree et al., 1996a, 1996b; Günther, 2003). Active neutralisation technologies often incorporate aeration, metal removal and precipitation, chemical precipitation, membrane processes, ion exchange and the removal of biological sulphate. As a result, AMD is neutralised to an alkaline mixture exhibiting a high pH and low salinity (Akcil and Koldas, 2006). Due to inadequate processing capacities at neutralisation plants, pH-neutralised AMD is often co-released alongside un-neutralised AMD into receiving streams. Consequently, concentrations of common elements (found in AMD)

such as Copper (Cu), Zinc (Zn), Aluminium (Al), Ferrous Iron (Fe), Manganese (Mn) become readily available to surrounding aquatic and terrestrial organisms (Jennings et al., 2008; Savinov et al., 2003).

Bio-monitoring assessments are replete with scientific studies analysing the environmental impacts of AMD on aquatic organisms (Griffith et al, 2004; Hansen et al, 1999). Until recently, the best indicators of AMD toxicity have been the presence of specific algae communities, a high density of chironomids (midge flies), a high concentration of iron hydroxides, pH data and the physiological degeneration of fish species (Jennings et al., 2008). This type of assessment is essential in presenting integrated effects on fauna, single compound toxins, habitat and physical environmental degradation (Gerhardt et al., 2004). However, this type of research does not necessitate the inquiry of toxicity at a cellular level of organisation.

Toxic chemical mixtures in the environment are commonly distributed by complex mechanisms of transport in cells. As a result, environmental pollutants composed of a combination of chemical components often elicit unknown cellular and genetic interactions (ATSDR, 2000a, 2000b; U.S. EPA, 2000a). A study performed by Da Silveira et al. (2009), proved the effectiveness of cellular genotoxicity as an indicator of DNA damage in *Geophagus brasiliensis* exposed to both un-neutralised and pH-neutralised AMD. To date, the impact of un-neutralised and pH-neutralised AMD on human somatic cells have not been elucidated. The current study aims to fill this knowledge gap by identifying signal transduction pathways responsible for cyto-genotoxicity in a human somatic cell line exposed to un-neutralised and pH-neutralised AMD. To accomplish this, the study intends to focus on biomarkers of toxicity such as cellular proliferation rates, morphologies of toxicity and DNA breakages.

Programmed cell death is essential in maintaining cellular homeostasis, development and differentiation in somatic cells. This mechanism of regulation termed apoptosis is commonly responsible for the energy-dependent elimination of cells exhibiting irregular homeostasis and damaged DNA (Elmore, 2007). Thus, when repair mechanisms in human cells are overcome by toxicity, pathways of programmed or direct cellular death ensue (Chen et al., 2001). The apoptotic pathway is characterised by nuclear and cytoplasmic condensation and nuclear fragmentation of damaged DNA into membrane bound vesicles. The cellular membrane is maintained as the cell progresses toward complete death (Fink and Cookson, 2005; Elmore, 2007). In conditions of extreme toxicity, the oncotic pathway is activated. Oncotic cell death is represented by cellular and organelle swelling, membrane blebbing and increased membrane disintegration (Trump et al., 1997). Ultimately, oncosis leads to a reduction in ATP stores and complete failure of membrane ionic pumps (Fink and Cookson, 2005; Elmore, 2007). Necrosis is the morphological sum of changes that have occurred once a cell has undergone either a direct disintegration due to a toxic onslaught (oncosis) or the progression of programmed cellular death (apoptosis). Thus necrosis is characterised by the complete breakdown of cellular matter (Fink and Cookson, 2005; Elmore, 2007). In an *in vivo* environment, these vesicles known as apoptotic bodies are engulfed by phagosomes (Trump et al., 1997). However, in an *in vitro* environment both apoptosis and oncosis progress toward secondary necrosis whereby the cell advances into collapse and has reached equilibrium with its surrounding environment (Fink and Cookson, 2005; Elmore, 2007).

In addition to homeostatic and morphological disruption, the study anticipates the initiation of DNA breakdown through an oxidative mediated cell death. Both apoptosis and necrosis leads to a pathway specific breakdown of DNA (Napirei et al., 2004; Tsukada et al., 2001; Higuchi, 2003). Apoptosis activates endo-peptidase nucleases which cleave specific regions of DNA into fragments known as internucleosomal DNA fragmentation (Trump et al., 1997).

Alternately, necrosis an abrupt form of cell death causes less of an extensive DNA breakdown but rather a total cellular disintegration (Higuchi, 2003). Numerous studies have reported the toxic interaction of metals on nuclear proteins and DNA molecules by eliciting the oxidative breakdown of these cellular components (Chen et al., 2001; Damek-Poprawa and Sawicka-Kapusta, 2004; Leonard et al., 2004). Metals have unique co-ordination chemistries and redox properties which enable metal toxicity through oxidative stress (Chen et al., 2001; Stohs and Bagchi, 1995).

Evaluating biomarkers of cell death and DNA damage in human cells exposed to acid mine drainage by means of high through-put assays, offers a novel South African perspective in the evaluation of the impacts of environmental contaminants on a cellular system.

2.2 THE NATURE OF ACID MINE DRAINAGE

The mining of precious minerals such as gold and iron ore are associated with the development of contaminants such as acid mine drainage (AMD) (Akcil and Koldas, 2006). AMD commonly occurs with the exposure of sulphide aggregated rocks to oxygen and water. Although this process occurs under natural conditions, mining gold and iron ore rock greatly exacerbates the quantity of exposed sulphide (Akcil and Koldas, 2006; Jennings, 2008). Complex effluents such as AMD are characterised by a low pH, high concentrations of minerals such as aluminium, iron and manganese, high conductivity and low concentrations of toxic heavy metals and dissolved solids. These constituents have detrimental implications on the environment and society when it seeps into receiving surface and ground water sources (Akcil and Koldas, 2006; Duruibe, 2007). As a result, chemical conditions in which the bioavailability of metals and minerals in streams exist at exponential levels far beyond what is stipulated by law (DWA, 1996; Coetzee et al., 2005).

The intensity of AMD contamination depends on both the type and amount of sulphide mineral oxidized (Akcil and Koldas, 2006). Subsequently, the acidity of AMD is determined through several reactions:

In the initial reaction of AMD generation, sulphide mineral pyrite is oxidised into dissolved iron, sulphate and hydrogen (Akcil and Koldas, 2006):

$$FeS_2 + 7/2\ O_2 + H_2O \rightarrow Fe^{2+} + 2SO^{2-}_4 + 2H^+ \quad [1]$$

A decrease in pH and increase in total dissolved solids occur when Fe^{2+}, $2SO^{2-}_4$ and $2H^+$ are in a dissolved state [3]. The production of AMD is sustained with the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) according to:

$$Fe^{2+} + ¼\ O_2 + H^+ \rightarrow Fe^{3+} + ½\ H_2O \quad [2]$$

As the pH decreases between 2 and 3.5, ferric iron (Fe^{3+}) precipitates as iron hydroxide ($Fe(OH)_3$). Thus the mixture contains little ferric iron which simultaneously decreases the pH according to:

$$Fe^{3+} + 3\ H_2O \rightarrow Fe(OH)_{3\ solid} + 3H^+ \quad [3]$$

The remaining ferric iron in Equation [2] which does not precipitate through the solution (Equation [3]) is further used to oxidize surrounding pyrite according to:

$$FeS_2 + 14\ Fe^{3+} + 8H_2O \rightarrow 15\ Fe^{2+} + 2SO^{2-}_4 + 16H^+ \quad [4]$$

In its most simplified form, the oxidative precipitation of ferric iron (Fe^{3+}) to iron hydroxide ($Fe(OH)_3$) in a low pH environment occurs as a combination of equations [1] and [3]:

$$FeS_2 + 15/4O_2 + 7/2\ H_2O \rightarrow Fe(OH)_3 + 2SO^{2-}_4 + 4H^+ \quad [5]$$

The most stable form of reaction during the re-oxidation of available pyrite by ferric iron (Fe^{3+}) occurs as a combination of equations [1] and [3]:

$$FeS_2 + 15/8\ O_2 + 13/2Fe^{3+} + 17/4\ H_2O \rightarrow 15/2Fe^{2+} + 2SO^{2-}_4 + 17/2H^+ \quad [6]$$

The complex mixture that is AMD is constituted by the resultant components of the above equations [5] and [6] along with surrounding rock mineralogy and metals mined (Akcil and

Koldas, 2006). Gradually this mixture will continue to seep into receiving water bodies overwhelming all forms of life (DWA, 2010).

2.3 THE PRIMARY FACTORS THAT DRIVE AMD GENERATION

The formation of AMD varies from mine to mine and predicting the potential severity of its production is often challenging. However, rate of AMD production is often driven by primary factors such as pH, temperature, oxygen saturation in the gaseous and aquatic phase, degree of mineral and metal saturation in water, activity of ferric iron, surface area of uncovered metal sulphide, reaction energy required to excite generation and bacterial activity (U.S. EPA., 1994; Akcil and Koldas, 2006) These chemical, biological and physical factors are integral parameters in determining the strength of underground and decanting AMD (U.S. EPA., 1994).

2.4 THE NEUTRALISATION OF AMD

The associated risk of AMD contamination and the practicality of remediation options will vary from site-to-site. However the collective goal of neutralising AMD contaminated waters are to increase pH, decrease the concentration of available metals and remove solids, for the release of water at a quality that supports aquatic life (U.S. EPA., 2000b, 2008). Neutralization options for AMD ideally depend on discharge flow, type and concentration of iron species, acidity and dissolved oxygen (U.S. EPA., 2000b, 2008). This review will focus on active neutralisation technologies utilising metal hydroxide and carbonate precipitation. Although active neutralization technologies are less cost effective than passive neutralization, they have been used for a longer time. Active neutralisation incorporates the use of chemicals to neutralize acidity and precipitate metals out of the solution (Coulton et al., 2003). Hydroxide agents such as lime ($Ca(OH)_2$) or caustic soda (NaOH) are usually utilised as precipitants (U.S. EPA., 2000b, 2008). However, the use of carbonate precipitants such as

limestone ($CaCO_3$) in combination with lime has proven successful (Maree et al., 1996a). The process of metallic precipitation involves the removal of metals from AMD contaminated water by converting soluble heavy metals to insoluble salts. Additionally, metals precipitate as hydroxides, carbonates, phosphates and sulphides (U.S. EPA., 2000b, 2008). Both hydroxide and carbonate precipitation is disadvantaged when iron within AMD is in the ferrous form. Ferrous iron only converts to ferrous hydroxide at a high alkalinity of pH 8.5. In the presence of a high oxygen content, the conversion of ferrous iron to ferric iron precipitates such as ferric hydroxide (or known as yellow boy) at pH>3.5 (Aubé, 2004). However, AMD in its natural state constitutes poor oxygen content. Thus, an efficient method of converting ferrous iron to ferric iron involves the aeration of AMD and out gassing of CO_2. The ferric iron and remaining metals are neutralised by the increase of pH and addition of either a hydroxide or carbonate chemical precipitant (U.S. EPA., 2000b; Aubé, 2004; U.S. EPA., 2008).

Neutralisation of metals often only considers pH and the development of metal hydroxides as integral factors for successful precipitation. However, the removal of metals and minerals can result from co-precipitation of metals and minerals at high pH solubilities and site specific water quality (U.S. EPA., 2000b, 2008). The solubility of iron hydroxide at pH3.5 is often considered when AMD water is neutralised. Precipitates such as aluminium hydroxide and mercury hydroxide have solubilities below pH8 thus removal along with iron hydroxide would occur (U.S. EPA., 2000b, 2008). Removal of cobalt hydroxide, copper hydroxide, nickel hydroxide, lead hydroxide and zinc hydroxide would occur minimally alongside iron hydroxide at pH10. It is proposed that treating AMD for iron may not be the feasible option as metals exhibiting solubilities at a high pH would not be removed. Instead neutralising AMD for metals such as manganese presenting minimal solubility at a high pH range would

increase the quality of neutralisation (U.S. EPA., 2000b; Means and Tiff, 2004; U.S. EPA., 2008).

2.4.1 Integrated limestone pH-neutralisation

The integration of limestone and lime in a fluidised bed reactor neutralisation process was developed by the CSIR (Maree et al., 1992, 1994, 1996b). The dissolution of calcite, the primary component of limestone; is used to neutralise acidity, increase the pH, alkalinity and Ca^{2+} ions (Figure 2.1). During the first stage of the technology, the AMD is neutralised with limestone whilst CO_2 is released by aeration (Maree et al., 1998).

This reaction occurs according to:

$$CaCO_3\,(s) + 2H^+ \rightarrow Ca^{2+} + H_2CO_3^* \qquad [1]$$

$$CaCO_3\,(s) + H_2CO_3^* \rightarrow Ca^{2+} + 2HCO_3^- \qquad [2]$$

$$CaCO_3\,(s) + H_2O \rightarrow Ca^{2+} + HCO_3^- + OH^- \qquad [3]$$

The liberation of free hydroxides in Equation 3 initiates metal hydroxide precipitation. The second stage of neutralisation involved the addition of lime to the AMD mixture, further encouraging precipitation of metals and sulphates according to:

$$Metal^{2+} + Ca\,(OH)_2 \rightarrow Metal\,(OH)_2 + Ca^{2+} \qquad [4]$$

$$2Metal^{3+} + 3\,Ca\,(OH)_2 \rightarrow 2\,Metal\,(OH)_3 + 3\,Ca^{3+} \qquad [5]$$

The use of lime liberates calcium iron which increases the total pH of the neutralised mixture. During the final stage of the process, the CO_2 released during stage one is re-routed to the neutralised mixture in stage two. This facilitates the increase of pH to 8.3, subsequently encouraging the precipitation of $CaCO_3$ (Maree et al., 1996b, 1998).

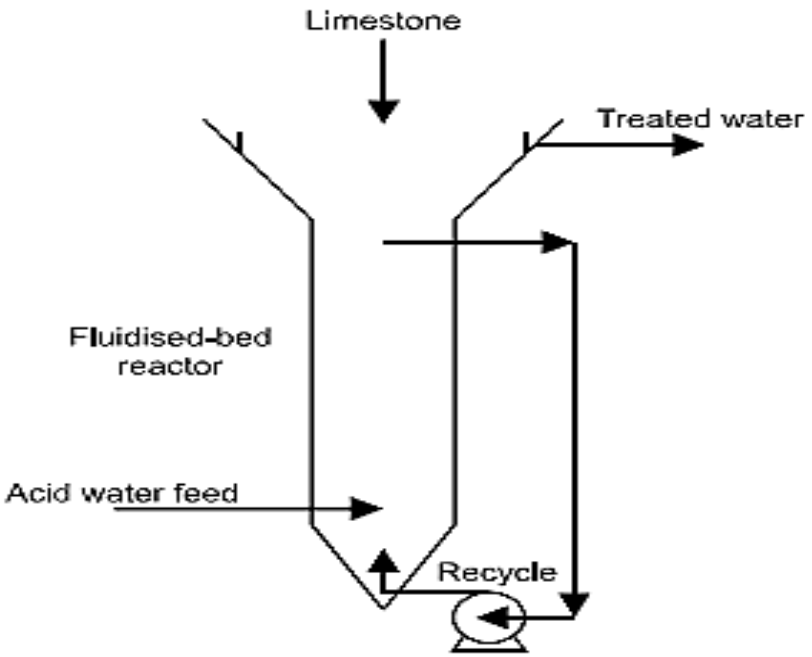

Figure 2.1 In the above configuration, AMD flows through the reactor in which limestone is situated. Neutralisation of AMD occurs along the reactor until sludge, ferric hydroxide and gypsum forms. This waste is released from the system alongside the neutralised AMD. Separation of waste and neutralised AMD is achieved through a clarifier.

2.4.2 Sodium hydroxide neutralisation

Sodium hydroxide (NaOH) neutralisation is typically implemented in low flow, highly acidic water. The solubility rate of NaOH is rapid and pH is quickly increased to an alkaline range. The neutralisation technology involves the direct titration of AMD by dripping a specified aqueous dose of NaOH into affected water (U.S. EPA., 2000b, 2008). Thus the precipitation of metals into metal hydroxides is accomplished according to:

$$Metal^{2+} + 2\ NaOH \rightarrow Metal\ (OH)_2 + 2\ Na^{+} \quad [1]$$

$$Metal^{3+} + 3\ NaOH \rightarrow Metal\ (OH)_3 + 3\ Na^{+} \quad [2]$$

The precipitated metal hydroxides maintain the constant pH of the neutralised mixture until desired pH precipitation levels of metals are reached. When pH levels are achieved, metal ions are eliminated out of the system alongside dense NaOH slurry (U.S. EPA., 2000b, 2008).

2.5 METAL-INDUCED CELLULAR TOXICITY

The effects of metals can be related to the activation of transcription factors which either directly or indirectly target regulatory proteins or signalling proteins responsible for cellular proliferation, cell death, homeostasis, DNA repair mechanisms and differentiation. Further investigation of the activation of transcription factors culminating in a signalling pathway gives insight into the underlying mechanisms of metal toxicity.

2.5.1 The interactions of metals with membranes

Metals and organic compounds differ in that metals can be present as different species in complex mixtures. Thus the parent metal element has the affinity to associate with a variety of membrane ligands whilst never being transformed or metabolised beyond a point of irreversibility. However, organic compounds may no longer resemble the parent compound when absorbed and metabolised. The mechanisms of absorption, distribution and excretion of metals all involve the passage and interaction of the metal with/ across the cell membrane (Manahan, 2002; Finney and O'Halloran, 2003). Therefore it is of immense importance to consider and identify the cues which prompt the interactions of metals contained within AMD with cell membranes. The passage of metals across membranes occurs either by passive processes or by the active contribution of membrane components toward transport. The permeability of most biological membranes tends to favour the passage of water either by diffusion, hydrostatic or osmotic potentials across a cell membrane. Thus inorganic compounds would seem to have a preferential transport across membranes due to a small ionic radius. However, active transport regulates the concentration gradient of inorganic ions.

Since the transport of metals across membranes rely on active transport, a selectively competitive environment ensues between essential minerals and metals. A well known example is the carrier mediated transport of lead via calcium uptake mechanisms (Manahan, 2002; Luk et al., 2003).

An alternative to active membrane transport mechanisms involves the absorption of soluble metal ions by cellular extrusions which engulf and internalize the metal ions into the cytoplasmic space via endocytosis (Finney and O'Halloran, 2003).

2.5.2 Interaction of metals with inter/ intracellular spaces

In order for metals to elicit toxicity, metals interact at specific sites of inter/intra cellular spaces in cells to drive toxic actions and disruptions of homeostatic cellular processes.

At an organism level of organisation, the uptake of metals by aquatic and terrestrial organism occur largely through the oral route. Moreover, aquatic organisms are exposed to metals through the dermal route. The dermal route of exposure is considered to be a minimal contributor of exposure, since the epithelium itself serves as effective barrier in most organisms (Glover and Hogstrand, 2002; Patil et al., 2013).

Therefore the interactions of metals have shown to elicit a greater range of impact on a cellular level of organisation. Several studies have shown the interactions of metals with fish gills, which form an exterior barrier. These studies have reported the inhibition and selective competition of Cu on ion transporters such as Na^{+}/ K^{+} -ATP-ase and Ca^{2+} -ATP-ase. Cadmium (Cd) and Zn has been further implicated in the inhibition of Ca^{2+} influx at apical gill channels (Petering et al., 2000). In addition to selective competition, metals might themselves exert competition on each other. An example might be the binding of Cd to an exposed Zn finger located on a protein. This new found affinity has the potential to initiate

transformational alterations which could inhibit the ability of the protein to transcribe DNA molecules (Glover and Hogstrand, 2002).

2.5.3 Metals and the activation of signalling pathways

Cellular death is either described as apoptosis or necrosis. *In vivo* apoptosis involves the active elimination of cells without eliciting an inflammatory response. Alternately, necrosis is described as a passive process in which cells undergo an abrupt death where upon cells release cellular content into the surrounding environment. The morphological characteristics of apoptosis are defined by nuclear and cytoplasmic condensation followed by nuclear fragmentation. The fragmented nuclear and cellular matter is transported to the intact cell membrane in vesicles which are taken up by circulating phagosomes (Fuentes-Prior and Salvesen, 2004).

Apoptosis plays an integral role in maintaining homeostasis in a cellular system by carefully selecting cells for death and complementing processes of mitosis and cytokinesis. Thus a stable population of cells are maintained. The fine balance required for apoptotic regulation is elicited by serine peptidases known as caspases. Generally caspases are divided into two groups, namely, initiator and effector caspases. Initiator caspases (Caspases 2, 8, 9 and 10) contain long prodomains and primarily activate the caspase cascades (Bortner et al., 1995).

The activation of initiator caspases occur via direct dimerization. The dimerization of initiator caspases are finely mediated by the binding of the long prodomains to adapter molecules either recruiting a caspase recruitment domain or effector domain motif. Once initiator caspases are activated, death inducing signals are circulated by the cascade activation of downstream effector caspases. Effector caspases contain small pro-domains and are mainly responsible for the proteolytic cleavage of cellular substrates. Upon activation of effector caspases, complete remodelling occurs via active proteolysis at aspartic acid (Asp) residues.

Activated effector caspases allow the nucleosomal degradation of DNA which produces fragments of 180 base pairs (bp), a hallmark of apoptosis (Bortner et al., 1995; Elmore, 2007). An alternative programmed counterpart to apoptotic cell death is described as oncosis. During this pathway, the cell undergoes nuclear and organellar swelling, membrane blebbing and an increase in membrane permeability which eventually leads to early membrane rupture. The rupture of the cellular membrane during oncosis is what characteristically differentiates it from the apoptotic pathway. Membrane disintegration is caused by the exhaustion of active energy stores which results in the malfunction of membrane ionic pumps (Krysko et al., 2008).

A key issue in defining necrotic cell death is distinguishing between biochemical necrosis and end-point necrosis. Currently, necrosis is used to describe an aggressive form of cell death other than apoptosis in which a cell completely disintegrates in a programmed manner. Thus in an *in vitro* system the progression of all cells regardless of the active programmed cell death it follows, will reach a state of necrosis when exposed to high levels of toxicity (Rozman and Klaassen, 2001).

2.5.4 Metals and the activation of transcription factors

Transcription factors form part of the epi-genetic control of cells by controlling the gene expression of protective genes which determine the integrity of damaged DNA, influence immunity, cellular arrest of damaged cells and induce apoptosis (Majno, 1995). Severely stressed cellular environments have been shown to induce the activation of redox sensitive transcription factors NF-kB, AP-1 and p53. The AP-1 and NF-kB family is often selectively activated when the concentration of oxidative radicals increase within cells. As a consequence, either proliferation or apoptosis is activated (Baud and Karin, 2001).

The NF-kB further regulates genes which are implicated in the transformation, cellular proliferation and angiogenesis. Furthermore, the role of NF-kB further extends to the maintenance of differentiation of cells, suggesting a possible cancer inducing property.

Therefore the activation of transcription factors is driven by signal transduction pathways that are activated by metals and cellular oxidants such as peroxides (Meplan, 2000).

The transcriptional activator p53 has been well described as an inducer of different functional components such as cell cycle regulatory proteins (p21) and pro-apoptotic factors (CD95, Bax). In the presence of hydrogen peroxide, nuclear transcription factors such as NF-κB, AP-1, and p53 was shown to be upregulated, ultimately leading to the increased activation of death proteins or inhibitors of survival proteins. These factors control the expression of protective genes involved in the repair mechanisms of damaged DNA, command the immune system and arrest potentially harmful mutations by inducing apoptosis (Surova and Zhivotovsky, 2013).

2.6 CELLULAR DAMAGE BY REACTIVE OXYGEN SPECIES

The beneficial roles of reactive oxygen species (ROS) have been reported as being necessary for the homeostatic cellular redox status, cellular function and intracellular signalling. However, at higher concentrations, ROS mediate the damage of cellular components such as lipids, proteins and DNA (Perez-Matute et al., 2009; Valko et al., 2006). The exacerbated damage of these components has been implicated in diseases such as Alzheimer's, rheumatoid arthritis and various carcinomas (Perez-Matute et al., 2009).

2.6.1 Chemistry of reactive oxygen species

Oxidative stress results from the increased production of reactive oxygen species (ROS) beyond the antioxidant balancing capacity of the cell (Fulda et al., 2010). ROS reactive entities comprise of free radicals (O_2 and OH) and non-radical oxidizing derivatives formed

from oxygen (H_2O_2). The formation of these partially reactive species occurs through interactions with oxygen, making it short-lived and highly-reactive. Free radical reactivity is a consequence of unpaired electrons which renders them unstable (Paravicini and Touyz, 2008).

$O_2 \rightarrow O_2^{\bullet -} \rightarrow H_2O_2 \rightarrow OH^{\bullet} \rightarrow H2O$ [1]

The O_2 radical is unique in that it can lead to the formation of many other reactive species, including OH, H_2O_2, identifying it as a stronger oxidative species than O_2 itself (Yu, 1994). The oxidative ability of O_2 on biological systems is short lived due to a superoxide dismutase (SOD) reduction to H_2O_2 (Valko et al., 2006). In addition, the negative charge of O_2 renders it unable to cross cell membranes (Yu, 1994).

The importance of H_2O_2 relates to its participation in the production of OH and subsequent free radical induced cytotoxicity (Yu, 1994). In comparison to free radicals, H_2O_2 is more stable, less reactive and presents a longer half-life. This stable role allows H_2O_2 to engage in intra and extra cellular diffusion (Paravicini and Touyz, 2008).

The OH radical reacts close to its site of formation therefore due to its high reactivity potential it is able to enact oxidative damage on lipids, proteins or nucleic acids. Commonly, the *in vivo* availability of OH is increased through Fe or Cu-catalyzed breakdown of H_2O_2.

The release of unbound Fe^{2+} from Fe-containing molecules is mediated by the presence of excess O_2 under conditions of cellular stress. Therefore, although O_2 itself does not cause direct oxidation of cellular components, it propels free Fe^{2+} to react with H_2O_2 and ultimately generate OH in the Fenton reaction (Equation 1). The iron-catalyzed Haber-Weiss reaction (Equation 4) is the central mechanism by which the OH radical confers its destructive properties on the cellular compartment (Kehrer, 2000).

3 $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$ [2] (Fenton reaction)

4 $Fe^{3+} + O2^{\bullet -} \rightarrow Fe^{2+} + O_2$ [3]

5 Net reaction:

6 $O2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^-$ [4] (Haber-Weiss reaction)

2.6.2 Lipid Peroxidation

The membrane lipid bilayer plays a critical role in the structure and function of the cell. Lipid peroxidation (LPO) is the consequence of free radical formation, usually within close proximity to the membrane. Thus a disruption in the homeostatic role of membranes leads to a cascade including initiation, propagation and termination (Goetz and Luch, 2008). The chain reaction characterising the oxidation of lipids involves a removal of hydrogen atoms from double bonds between polyunsaturated fatty acids (PUFAs), yielding a carbon-centred lipid radical species that interacts with O_2 ions. This lipid peroxyl becomes a mediator of yet another hydrogen ion abstraction (Kehrer, 2000). The by products of LPO such as reactive carbonyl species (RCS) are able to react with both protein (Negre-Salvayre et al., 2008) and DNA (Nair et al., 2007). Further consequences of LPO include ion channel disruption and lipid bilayer permeability, leading to a breakdown in ion homeostasis (Kehrer, 2000).

2.6.3 Protein carbonylation/protein oxidation

The oxidative capability of radicals on proteins might be either indirectly mediated by RCS formed during LPO and carbohydrate oxidation or directly by ROS, leading to the formation of oxidised amino acids.

Protein carbonylation is the reaction of proteins with LPO-derived RCS resulting in adducts known as advanced lipoxidation end products (ALEs). These adducts have been shown to cause protein damage and cellular disruption in diseases such as diabetes, in which high levels of protein carbonyls are detected (Negre-Salvayre et al., 2008). In the presence of increased levels of carbohydrates such as glucose or fructose, glycation leads to elevated RCS levels and ultimately accelerated protein carbonylation, causing irreversible oxidative damage to proteins (O'Brien et al., 2005).

The ROS radical OH has been implicated in the direct oxidation of amino acids such as cysteine, lysine, arginine and histidine (O'Brien et al., 2005; Valko et al., 2006 Goetz and Luch, 2008).

Disruption of amino acid interactions leads to the formation of inter- and intra-protein cross-linkages such as the accumulation of lysine side chains on carbonyl groups of an oxidised protein, OH mediated interaction of two carbon-centred radicals resulting from the removal of hydrogen ions from the polypeptide backbone, the formation of disulphide crosslinkages (-S-S-) from cysteine sulphydroxyl oxidation and finally the formation of tyrosine crosslinkages (-tyr-tyr-) from the oxidation of tyrosine (Valko et al., 2006).

2.6.4 DNA oxidation

The highly damaging potential of the OH radical relates to its direct interaction with the deoxyribose backbone causing purine and pyrimidine base adducts (Valko et al., 2006). This DNA damage results in single or double stranded breaks, base modifications and cross-linkages (DNA-DNA or DNA-protein) (Toyokuni, 1998). In the absence of sufficient DNA and adduct repair, cell death ultimately ensues (Kehrer, 2000). The most extensively studied oxidative modification on DNA is the 8-Hydroxy 2'-deoxyguanosine (8-HO-dG) mutation, formed during guanine oxidation (Valko et al., 2006; Goetz and Luch, 2008). This oxidative indicator presents an essential biomarker for the detection of both DNA-oxidative injury and carcinogenesis.

Additionally, mutagenic adducts can result from the interaction of RCS with DNA thus forming exocyclic-adducts with DNA bases that may induce base-pair substitution mutations (Nair et al., 2007). The reaction of LPO by products with DNA have the ability to form promutagenic adducts such as etheno- and propano-adducts (Valko et al., 2006; Goetz and

Luch, 2008). ROS- and RCS-derived protein and DNA base adducts have the potential of indicating oxidative stress and can be used as predictive targets for oxidative damage prevention.

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CHAPTER THREE

ASSESSING THE CYTO-GENOTOXIC IMPACT OF GOLD MINING-RELATED ACID MINE DRAINAGE ON THE HUMAN BREAST ADENOCARCINOMA CELL LINE (MCF-7)

3.1 INTRODUCTION

Eco-genotoxic assays quantifying the impacts of metal contaminated water have taken the forefront on toxicological assessments. The degree of environmental pollution caused by decanting acid mine drainage (AMD), has been reported at many sites across the world (Nieto et al., 2007; Bird et al., 2005). The quality of AMD contaminated natural water sources have conventionally been restored through the application of a process known as pH-neutralisation (Johnson and Hallberg, 2005). An improved pH-neutralisation technology developed by the Council for Scientific and Industrial Research (CSIR) utilizes a limestone ($CaCO_3$) fluidised bed reactor alongside lime powder (Maree et al, 1996c). The efficiency of the neutralisation process is assessed according to the ability of the integrated limestone treatment technology to neutralise metals out of the system as metal hydroxides, reduce sulphate content, pH (free acid), iron and aluminium ions (Maree et al, 1996c). Commonly, treatment plants have ineffective rates of neutralisation whilst negotiating the rate and volume of decanting AMD. Thus pH-neutralised AMD is often released together with un-neutralised AMD into receiving natural water sources (ETIMC, 2010). Subsequently, streams and rivers undergo a chemical degradation amid the release of highly acidic water containing large concentrations of metal ions and sulphates (ETIMC, 2010).

Traditionally, environmental monitoring programmes analyse the presence or absence of contaminants in water, sediments and/or biota (Coetzee et al., 2004, 2005). These studies

largely examine toxicological endpoints of single compound effects (Cassee et al., 1998). However, they seldom assess the combinative synergistic and antagonistic influence of toxic environmental mixtures on the surrounding ecosystem (Cassee et al., 1998).

The cell's initial response to the onslaught of contaminants such as heavy metal oxidation is geared toward restoring cellular homeostasis and genome integrity (Pützer, 2007). However, when insurmountable cellular damage occurs, noxious stimuli activate death signalling pathways such as apoptosis and necrosis (Kastan and Bartek, 2004). The regulation of apoptosis and necrosis has shown to be mutually exclusive in the propagation of cellular demise (Zeiss, 2003). The commitment of a cell to apoptosis often involves the energy dependant activation of a group of cysteine proteases called caspases. These enzymes initiate a cascade of events that lead to the inter-nucleosomal degradation of DNA (Denecker et al., 2001; Wyllie et al., 1992). Thus, producing the hallmark DNA laddering pattern consisting of DNA cleaved into 50-200 bp fragments. Alternately, necrosis is characterised by the random lysosomal cleavage of chromatin DNA which forms a distinctive "smear" on agarose gels (Dong et al., 1997). Biomarkers of DNA fragmentation has successfully demonstrated the extent of cytotoxic and genotoxic responses to complex environmental mixtures (Watson et al., 2004; McClean et al., 2004; Scherer, 2005).

Traditional end points of toxicity are morphological variations of cell death, commonly displaying effects at high-dosage exposures (Wang and Fowler, 2008). However, metals display long half-lives and therefore monitoring genotoxicity at both high and low doses is vital (Silins and Högberg, 2011).

The comet assay has become an invaluable tool for investigating such parameters in order to depict fundamental aspects of DNA damage (Tice et al., 2000). The comet assay, a variation

of gel electrophoresis, allows the separation of single cells. The method is based on unravelling super coiled DNA which has undergone damage. The relaxed loops of DNA migrate upon electrophoresis, allowing free breaks to unwind and give a characteristic comet shape to the cell (Collins et al, 1997).

Recently, there has been a shift in the strategies of toxicological assessments with the use of high throughput assays capable of identifying complex mechanisms of cyto-genotoxicity in organisms habituating AMD contaminated water. This investigation presents a novel and complimentary *in vitro* study of cyto-genotoxic impacts in a human cell line exposed to un-neutralised and pH-neutralised AMD from the Western Basin of South Africa.

3.2 MATERIALS AND METHODS

3.2.1 Study site

3.2.1.1 *Background and pH-neutralisation technology implemented in the study area*

The study area falls within the Mogale City (formally Randfontein) area of the Witwatersrand mining region known as the Western Basin in the Gauteng Province. Cessation of the underground mine water extraction occurred a few years ago, and led to flooded mine voids and decanting of AMD onto the surface. Decant in the Western Basin started in 2002 and is presently occurring from three shafts (Black Reef Incline, 18 Winze and 17 Winze) with seepage from low-lying areas along the Tweelopies Stream and Wonderfontein Stream (DWA, 2002). Due to seasonal changes, the inflow of water into the Western Basin varies widely from between ~10–15 mega-litres per day (Ml/d) in the dry season but has previously peaked at ~60 Ml/d during exceptionally wet summer rainfall seasons (DWA, 2010a; 2010b). The Tweelopies Stream, a surface stream draining into the Krugersdorp Game Reserve, was selected as the study area. The decant of AMD and the discharge of partially treated mine

water via the Tweelopies Stream has had severe impacts on the aquatic ecology (Ramontja et al., 2011). The un-neutralised and pH-neutralised AMD sampling points situated along the Tweelopies Stream is depicted in Figure 3.1. The two sampling sites are situated a metre and a half apart within the study area. Site A shows the location of the un-neutralised AMD, while pH-neutralised AMD water was sampled from Site B immediately before the combined effluent stream enters the Krugersdorp Nature Reserve.

The pH-neutralisation technology employed in the study area utilises a mixture of calcium carbonate (lime) powder and limestone to achieve iron oxidation, neutralisation and gypsum crystallisation of AMD. Discard leachate is collected in a holding tank and treated with limestone ($CaCO_3$) powder in an aerated sludge reactor. Thereafter, the partially oxidised water is passed through a limestone fluidised-bed reactor to allow the increase of pH and liberation of aluminium (Al^{3+}). The pumping capacity of the treatment plant has been reported to provide sub-optimal pH-neutralisation capacities of approximately ~ 12 Ml / d (Coetzee and Venter, 2005). Thus the un-neutralised AMD (partially oxidised) (Figure 3.1, point A) has been directed into the pH-neutralised AMD stream (Figure 3.1, point B) before releasing it into the Tweelopies Stream.

3.2.1.2 *Sampling procedure*

Water samples were collected in March 2012 during the low rainfall season. Un-neutralised (Figure 3.1, point A) and pH-neutralised (Figure 3.1, point B) AMD samples were collected at points where they flow as separate streams before merging into the Tweelopies Stream in Randburg. The pH of un-neutralised and pH-neutralised AMD was measured at pH 3.0 and pH 7.1; respectively. The water samples were collected in polypropylene (PP) bottles according to commonly accepted sampling procedures (EPA., 2000). Sampling comprised the collection of two samples at each site. The PP bottles were washed with HNO_3 before

sampling. The geochemical parameters: temperature (°C), pH, electrical conductivity EC (mS cm^{-1}), and redox potential Eh (mV) were measured directly at the sampling sites using the HACH-DR-3000 multi-meter (Hach, USA). The samples were filtered through 0.2 µm and 0.45 µm low protein binding cellulose acetate membrane filters to remove any organic material present and kept at 4 °C for chemical analysis and later use.

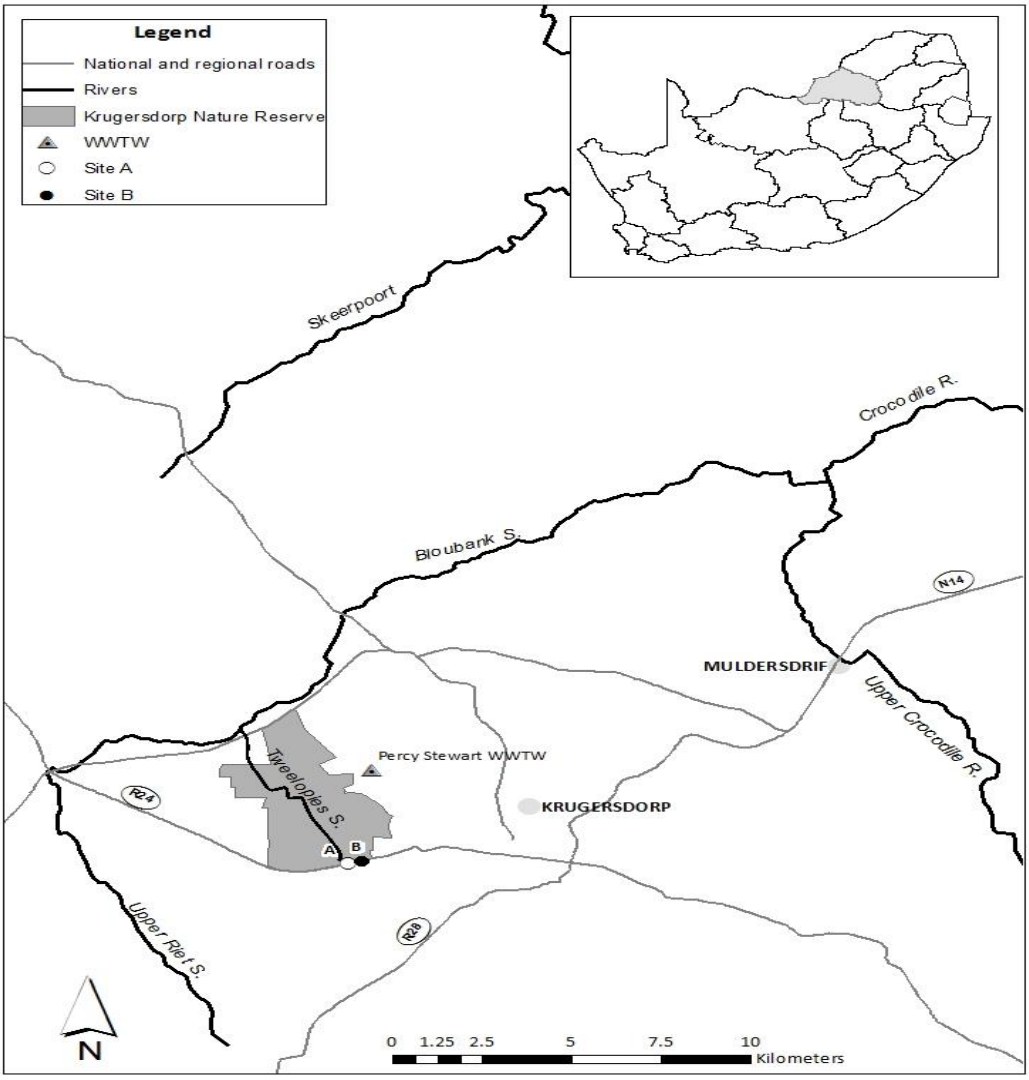


Figure 3.1 Site map of sampling points situated in the Western Basin, Gauteng Province, South Africa. Sampling point A and B indicates the un-neutralised and pH-neutralised AMD sampling points situated along the Tweelopies Stream, respectively.

3.2.2 Cell culture

The MCF-7 cell line was previously described as a prime *in vitro* system in which to study metal interactions (Klutse, 2009). The MCF 7 cell line was chosen for the study as it has been shown to have the excellent ability to withstand harsh conditions which allow the use of higher levels of exposure (Wu et al., 2006).

The human breast adenocarcinoma (MCF-7, HTB-22) (ATCC, 2013) cell line was obtained from Professor Johannes van Wyk at Stellenbosch University. MCF-7, a human breast cancer cell line was derived from metastatic breast adenocarcinoma at the Michigan Cancer Foundation (Soule et al., 1973). The maintenance of MCF-7 growth occurred in Dulbecco's modified Eagle's medium (DMEM) (Lonza, USA) supplemented with 5% fetal calf serum (Lonza, USA) and 1% Penicillin (100 units) and Streptomycin (100 µg) (Lonza, USA), as outlined by ATCC, HTB-22 (ATCC, 2013). The MCF-7 cells were grown in a semi humidified atmosphere of 5% CO_2 at 37°C. After the expansion to a confluence of 4 x 10^6 / ml after approximately 3-4 days, cells were trypsinized with 0.25% Trypsin / 0.45 µM ethylene-diamine-tetra-acetic acid (EDTA) solution in Hanks Balanced Salt Solution (Lonza, USA) for 3-5 minutes (ATCC, 2012). The trypsin-cell mixture was de-activated and centrifuged at 2500 rpm for 10 minutes. The cell pellet was resuspended in DMEM and either reseeded to maintain the MCF-7 culture stock or used for additional end point assays.

3.2.3 Assessing MCF-7 survival rate and growth kinetics

MCF-7 cells were seeded into 24 well plates at a density of 3 x 10^5 per well, and allowed to grow in supplemented DMEM for 4 days. Thereafter, cells were lifted using 0.25% Trypsin / 0.45 µM EDTA solution in Hanks Balanced Salt Solution (Lonza, USA) for 3 minutes (ATCC, 2013). Each well was collected in a 2 ml Eppendorf, deactivated with DMEM and centrifuged down at 2000 rpm for 10 minutes. Thereafter each cell pellet was exposed to

biological controls, experimental controls or test samples, in triplicate. The rate of cellular survival was measured immediately or after 20 minutes, 1, 6, 12, 24, 48, 72 and 96 hours of incubation.

The MCF-7 cells grown in DMEM established a baseline of homeostatic cellular survival and were used as the biological negative control. The experimental positive control included hydrogen peroxide (H_2O_2), indicating oxidative induced cell death.

Varying concentrations of un-neutralised and pH-neutralised AMD were used as the test samples to measure complete (100%), mid-level (1:2 or 50%) and low-level (1:64 or 1.6%) toxicity. Complete toxicity was used as a baseline of cellular starvation at 100% exposure to un-neutralised and pH-neutralised AMD, in a nutrient deficient environment.

All exposures were performed in triplicate. The rate of proliferation of each sample was determined using the Trypan blue exclusion method and an automated TC20 cell counter (Bio-Rad, USA). The rate of cellular survival was plotted as mean percent of three independent counts compared to homeostatic control. Survival data across time points (immediately measured to 48 hours) per exposure were analysed by one-tailed Students *t*-test (percent control). Proliferation data at 96 hours of exposure were analysed by independent Students *t*-test (unequal variances). The level of significance (*P* value) was set at 0.05 for all tests.

3.2.4 Assessing patterns of DNA fragmentation

To determine DNA fragmentation patterns of MCF-7 cells in a completely toxic (100%) environment of un-neutralised and pH-neutralised AMD, cells were exposed for 20 minutes, 1 hour, 48 and 96 hours. A possible deviation of DNA fragmentation patterns were tested with

the exposure of MCF-7 cells to mid-level toxicity (50%) levels of un-neutralised and pH-neutralised AMD, for 20 minutes, 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours and 96 hours.

The isolation of fragmented DNA was performed according to the procedure described by Hermann et al. (1994). This method separates fragmented DNA cleaved by both specific and non-specific endonucleases. Following exposures of MCF-7 cells in 24-well plates at various concentrations and time dependant intervals, cells were harvested, washed with 1 x phosphate buffered saline (PBS) and pelleted by centrifugation at 4500 rpm for 10 minutes. The cell pellets were treated for 10 seconds with 50 µl lysis buffer (1% Nonidet P-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5) and centrifuged for 10 min at 1600 xg and the supernatant was collected. The extraction phase was repeated once more with the same volume of lysis buffer (50 µl lysis buffer). To the supernatant, 1% sodium dodecyl sulphate (SDS) was added and treated overnight with RNase A (final concentration 5 µg/ml) at 56°C. This was followed by digestion with proteinase K (final concentration 2.5 µg/ml) for 4 h at 37°C. After addition of 500 µl of 10 M ammonium acetate, the DNA was precipitated with 250 µl of cold ethanol. Isolated DNA was dissolved in loading buffer (50 mM Tris-HCl, 7.5, 10 mM EDTA containing 0.25% bromophenol blue and 30% glycerol) and electrophoresed on a 8% poly-acrylamide gel (acrylamide : bisacrylamide (29:1)) (Bio-Rad, USA). DNA concentration was measured using the Nanodrop ND-1000 Spectrophotometer, where the ratio of sample absorbance was set at 260 and 280 nm (ThermoScientific, U.S.A.).

3.2.5 Single cell gel electrophoresis assay (Comet assay)

3.2.5.1 *Comet assay slide preparation*

The alkaline comet assay, previously described by Singh et al. (1996), was performed according to the OxiSelect™ Comet Assay Kit (Cell Bio labs, USA). To determine the

distribution of DNA damage in MCF-7 cells, exposures occurred at levels of complete (100%) and low-level (1.6%) toxicity to un-neutralised and pH-neutralised AMD, within 24-well plates for a duration of 96 hours. Thereafter, exposure media was aspirated from each well to successfully cease the reaction. Cells were harvested, pelleted by centrifugation at 4500 rpm for 10 minutes and resuspended in 100 µl of cold PBS at $1x10^5$ cells/ ml.

Slides were coated with 1% low melting agarose before the comet assay commenced. Upon the completion of MCF-7 exposures, cells were harvested, pelleted by centrifugation at 4500 rpm for 10 minutes and resuspended in 1x cold PBS at 1×10^5 cells / ml. Cells at a density of 1×10^5 were mixed with 1% Comet agarose at a 1:10 ratio and 75 µl added to each comet slide well. An additional layer of 2% agarose was placed on top of the cell-agarose layer. The slides were transferred to 4ºC and kept dark for 1 hour to allow for effective drying. This was followed by lysis (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10 and 1% Triton X-100) and immersion in alkaline solution (300 mM NaOH, 1 mM Na_2EDTA) at 4 ºC in the dark for 60 minutes and 30 minutes respectively as described by the manufacturer. Maintaining the slides horizontally, slides were transferred to an electrophoresis chamber and immersed in alkaline electrophoresis solution (300 mM NaOH, pH >13, 1 mM EDTA). The comet slides were electrophoresed at 18 V for 20 minutes at a current of 300 mA. On completion of electrophoresis, the slides were washed in chilled deionised water, followed by a rinse in cold 70% ethanol for 5 minutes and allowed to air dry.

3.2.5.2 *The modified comet assay method: assessment according to cellular morphology.*

Following the alkaline comet assay, air dried slides was stained with 100 µl 4', 6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich, U.S.A.) for 5 minutes. To detect morphological indicators of apoptosis and necrosis all exposures were viewed under translucent and fluorescent light using wide field and confocal DAPI excitation filters (Zeiss Axiomat, Carl Zeiss GmbH, Jena, Germany).

3.2.5.3 *The traditional comet assay: Evaluation of cell death distribution and percentage (%) DNA in tail.*

Each slide was stained with 100 µl of 1:10000 diluted Vista Green DNA dye (Cell Bio labs, USA) for 10 minutes. Slides were counter stained with 100 µl DAPI (Sigma Aldrich, U.S.A.) for 5 minutes, rinsed and analysed under fluorescence microscopy (Zeiss Axiomat, Carl Zeiss GmbH, Jena, Germany) using FITC (Fluorescein isothiocyanate) and DAPI filters. DAPI and Vista green staining allowed the observation of fragmented nucleic DNA and cell membranes, respectively.

To determine the extent of apoptotic and necrotic cell distribution, a total of 100 cells were counted per level of toxicity (complete (100%) and low-level (1.6%)) upon an exposure to un-neutralised and pH-neutralised AMD. Comet cells on each slide were visually scored according to the length of DNA migration in the tail region. Thus each cell was scored as belonging to one of five classes, where 0 represents visually undamaged cells and 4 visually-maximally damaged morphologies (Collins et al., 1997).

Percentage of DNA in tail was determined using the Comet Score® software developed by TriTek (www.tritekcorp.com). The DNA in the tail represents the frequency of DNA strand breaks. A total of 50 comets per undiluted and diluted dose of each treatment group were scored in duplicate, constituting an amount of 100 cells counted in total. Percent DNA in tail for each exposure (complete (100%) and low-level (1.6%) toxicity to un-neutralised and pH-neutralised AMD) is represented as a mean of two replicas of 50 counts each per slide (n=100). Percent DNA in tail data at 96 hours of exposure were analysed by independent Students *t*-test (unequal variances). The level of *P* value was set at 0.05.

3.3 RESULTS

3.3.1 *Chemical analysis*

In Table 3.1 the concentrations of metals and elements measured at observable levels are depicted below.

Table 3.1 Chemical analysis of un-neutralised and pH-neutralised AMD at concentrations of 100% and 1.6%#.

Acid Mine Drainage (AMD)	**pH**	**Mg (µg/l)**	**K (µg/l)**	**Na (µg/l)**	***SO_4 (µg/l)**	**Al (µg/l)**	**Mn (µg/l)**	***Fe (µg/l)**
Un-neutralised 100%	3.2	43100	5300	25360	954300	80	9700	40
pH-neutralised 100%	5.5	99700	8300	43000	1732300	990	62700	259750
Un-neutralised 1.6% (1:64)*	4.0	1500	1600	2800	33000	20	250	0
pH-neutralised 1.6% (1:64)*	6.1	3000	1800	3300	58300	40	1530	4890

* **p value significant between 100% and 1.6%**

\# In the study, un-neutralised and pH-neutralised AMD were diluted using DMEM (homeostatic control). The level of significance was determined as a p value less than 0.05.

A significant difference in SO_4 (µg/l) and Fe (µg/l) levels were determined between the 100% and 1.6% concentrations of un-neutralised and pH-neutralised AMD, respectively. No significant difference was observed in any of the groups.

3.3.2 *Cellular survival rate of the MCF-7 cell line*

The consequence of exposure to hypertonicity, isotonicity or hypotonicity depends largely on the cyto-genotoxic potential of complex mixtures. In the absence of a buffer to minimize the concentration gradient, the proliferation of MCF-7 cells gradually decreased when exposed to all undiluted (hypertonic) treatment groups (Figure 3.2). MCF-7 cells exposed to all treatments at 100% concentrations, displayed a significantly different survival rate compared

to homeostatic control ($p<0.05$) (Table 3.2). Interestingly, the survival rates of MCF-7 cells exposed to 100% concentrated un-neutralised AMD was higher than those of MCF-7 cells exposed to 100% concentrated pH-neutralised AMD (Figure 3.2). This observation is further confirmed with the lack of significant difference in MCF-7 survival rates between pH-neutralised AMD and hydrogen peroxide control at 100% concentrations (Table 3.3). This may suggest a higher oxidative effect of pH-neutralised AMD on MCF-7 cells.

The MCF-7 cells are able to overcome initial impacts of the oxidative stress elicited by un-neutralised and pH-neutralised AMD at a concentration of 50% (Figure 3.2). However, soon the MCF-7 cellular survival rates show a significant difference in proliferation from that of control as cellular survival decreases ($p<0.05$, Table 3.2).

To determine low-level toxic impacts, un-neutralised and pH-neutralised AMD were prepared at negligible concentrations of 1.6% in homeostatic control media. MCF-7 cells exposed to un-neutralised and pH-neutralised AMD at 1.6% concentrations display unstable survival rates as MCF-7 cellular viability increases and decreases sharply every 2 days after 24 hours of exposure (Figure 3.2).

When survival rates were compared to homeostatic control, MCF-7 viability when exposed to pH-neutralised AMD at 1.6% concentrations displayed a significant difference. This may suggest a further suppressive role of pH-neutralised AMD on MCF-7 proliferation.

Comparisons of MCF-7 viability per group at 50% and 1.6% concentrations showed no significant difference in MCF-7 proliferation compared to hydrogen peroxide control at 50% and 1.6% concentrations, respectively (Table 3.3). This finding suggests an oxidative effect of

un-neutralised and pH-neutralised AMD on MCF-7 cells even at negligible 1.6% concentrations.

Table 3.2 Statistical description of un-neutralised and pH-neutralised AMD at 100%, 50% and 1.6% concentrations compared to control.

100% concentration	**Un-neutralised AMD TW**	**pH-Neutralised AMD TW**	**Hydrogen Peroxide**	**Homeostatic Control**
Std. Error	12.46	10.6	10.69	0
P value (two tailed)	0.0477	< 0.0001	< 0.0001	
Significant (alpha=0.05)?	Yes	Yes	Yes	
50% concentration	**Un-neutralised AMD TW**	**pH-Neutralised AMD TW**	**Hydrogen Peroxide**	**Homeostatic Control**
Std. Error	12.4	14.5	12.48	0
P value (two tailed)	0.0105	0.0184	0.0011	
Significant (alpha=0.05)?	Yes	Yes	Yes	
1.6% concentration	**Un-neutralised AMD TW**	**pH-Neutralised AMD TW**	**Hydrogen Peroxide**	**Homeostatic Control**
Std. Error	12.15	11.41	10.09	0
P value (two tailed)	0.0594	0.0241	0.1162	
Significant (alpha=0.05)?	No	Yes	No	

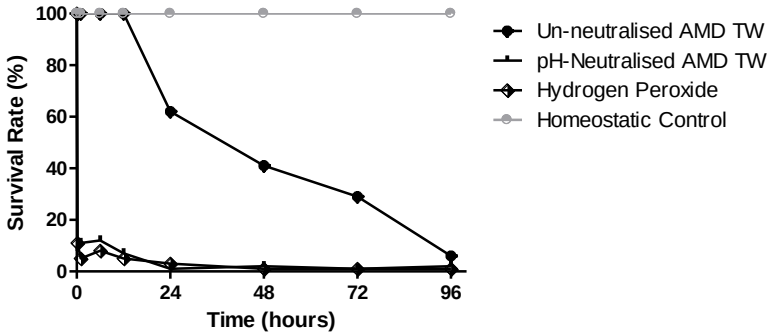
In Table 3.2 statistical analysis of un-neutralised and pH-neutralised AMD at 100%, 50% and 1.6% concentrations are described. The survival rates of all data were tested for significant difference from homeostatic control. A level of significance was confirmed when p was less than 0.05.

Table 3.3 Statistical description of un-neutralised and pH-neutralised AMD at 100%, 50% and 1.6% concentrations compared per group.

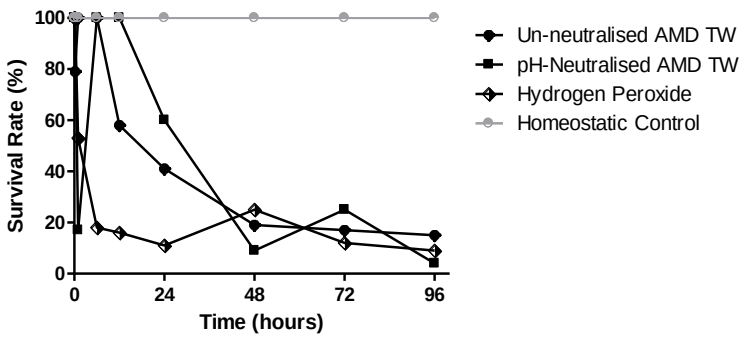
AMD	**Bonferroni's Multiple Comparison Test**	**Significant? P < 0.05?**	**Summary**
100%	Un-neutralised AMD TW vs pH-Neutralised AMD TW	Yes	***
	Un-neutralised AMD TW vs Hydrogen Peroxide	Yes	***
	pH-Neutralised AMD TW vs Hydrogen Peroxide	No	ns
50%	Un-neutralised AMD TW vs pH-Neutralised AMD TW	No	ns
	Un-neutralised AMD TW vs Hydrogen Peroxide	No	ns
	pH-Neutralised AMD TW vs Hydrogen Peroxide	No	ns
1.6%	Un-neutralised AMD TW vs pH-Neutralised AMD TW	No	ns
	Un-neutralised AMD TW vs Hydrogen Peroxide	No	ns
	pH-Neutralised AMD TW vs Hydrogen Peroxide	No	ns

In Table 3.3 the statistical significance of MCF-7 survival rates per group was determined using Bonferroni's Multiple Comparison Test. A level of significance was confirmed was set at 0.05

A. 100% concentrated



B. 50% concentrated



C. 1.6% concentrated

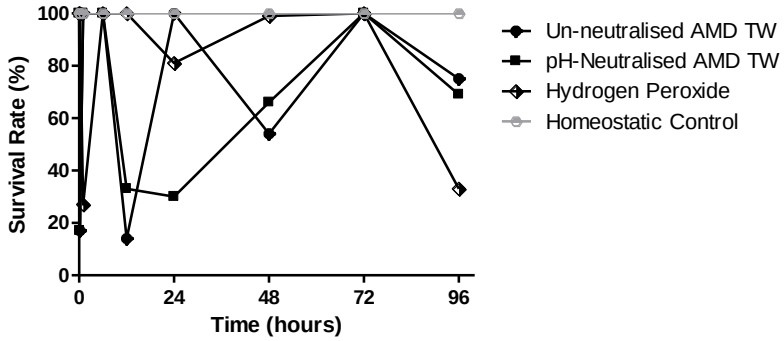


Figure 3.2 The survival rates of MCF-7 cells exposed to un-neutralised and pH-neutralised AMD at 100% (A), 50% (B) and 1.6% (C) concentrations at 20 minutes, 1, 6, 12, 24, 48, 72 and 96 hours. Cellular survival was determined using the trypan blue exclusion method. Average means of each time point per treatment group is plotted.

3.3.3 *MCF-7 DNA fragmentation patterns*

One of the most pivotal issues in cell death is distinguishing between apoptosis and necrosis, as these processes often occur independently, successively as well as concurrently (Hirsch, 1997; Zeiss, 2003). DNA fragmentation is often used as a molecular tool to give insight into the global state of genomic DNA (gDNA). The DNA fragmentation patterns of MCF-7 cells exposed to un-neutralised and pH-neutralised AMD was evaluated in a time and concentration dependant manner (Figures 3.3, 3.4, 3.5 and 3.6).

Exposures of MCF-7 cells to 100% concentrations of un-neutralised and pH-neutralised AMD expectantly did not yield inter-nucleosomal DNA laddering patterns indicative of an apoptotic cellular death. However, gDNA smearing patterns were observed from 20 minutes to 96 hours of exposure to un-neutralised and pH-neutralised AMD at 100% concentrations (Figure 3.3 A and B).

MCF-7 cells exposed to 50% concentrations of un-neutralised AMD prompted an initial gDNA smearing pattern followed by faint inter-nucleosomal degradation of DNA, observed as a fragment ladder at 96 hours of exposure (Figure 3.4 A). MCF-7 cells displayed no visible DNA fragmentation upon exposure to 50% concentrations of pH-neutralised AMD, instead depicting high molecular weight bands (Figure 3.4 B).

The DNA fragmentation potential of remediated water, namely, pH-neutralised AMD was evaluated on MCF-7 cells exposed to a negligible level of 1.6% concentrations (Figure 3.5). As observed, high molecular weight bands appear with slight smearing of gDNA.

Taken together, the results suggest a non-apoptotic mediated cell death and DNA damage mechanism which may be responsible for the observed gDNA smearing patterns observed in

MCF-7 cells exposed to un-neutralised and pH-neutralised at 100%, 50% and 1.6% concentrations (Figure 3.3, 3.4, 3.5 and 3.6).

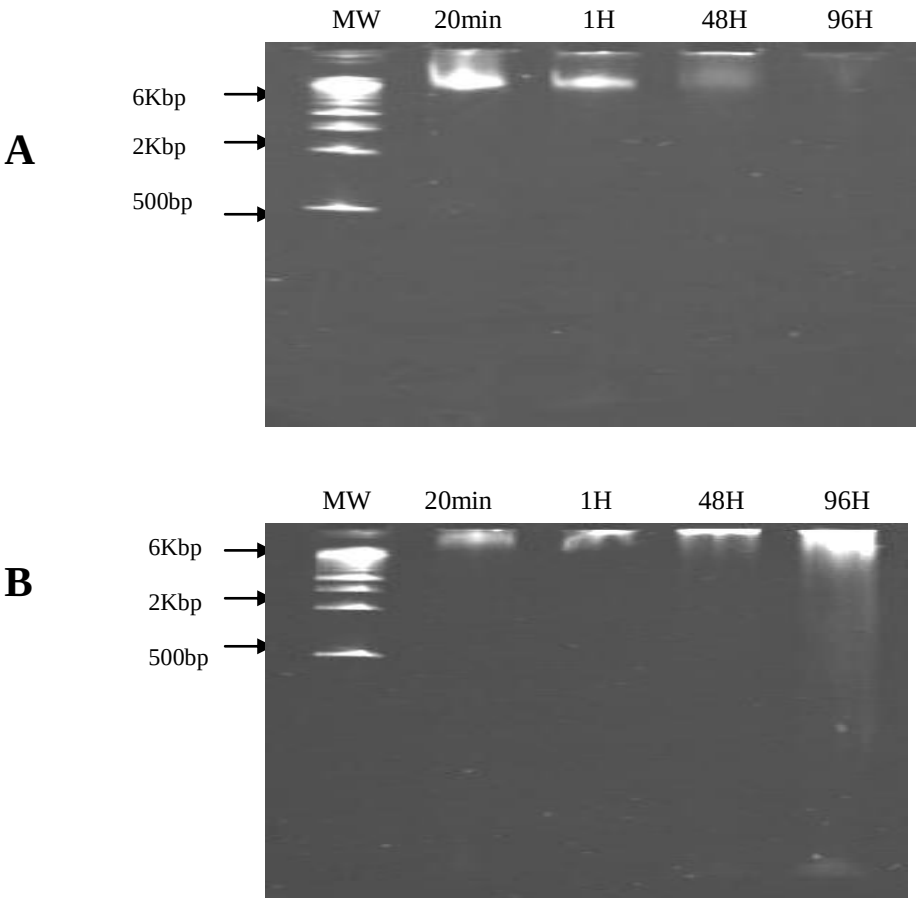


Figure 3.3 DNA fragmentation in MCF-7 cells exposed to 100% concentrations of un-neutralised (A) and pH-neutralised (B) AMD. In A and B, lanes 1, indicate the DNA molecular weight marker ranging in size from 500 bp to 6 Kbp (see arrows).. Similarly, in A and B, lanes 2, 3, 4 and 5 represent DNA fragmentation patterns of MCF-7 cells exposed to un-neutralised and pH-neutralised AMD for 20 minutes, 1, 48 and 96 hours respectively. Ethidium bromide (2%) was utilised to visualize the molecular weight markers and DNA fragmentation patterns.

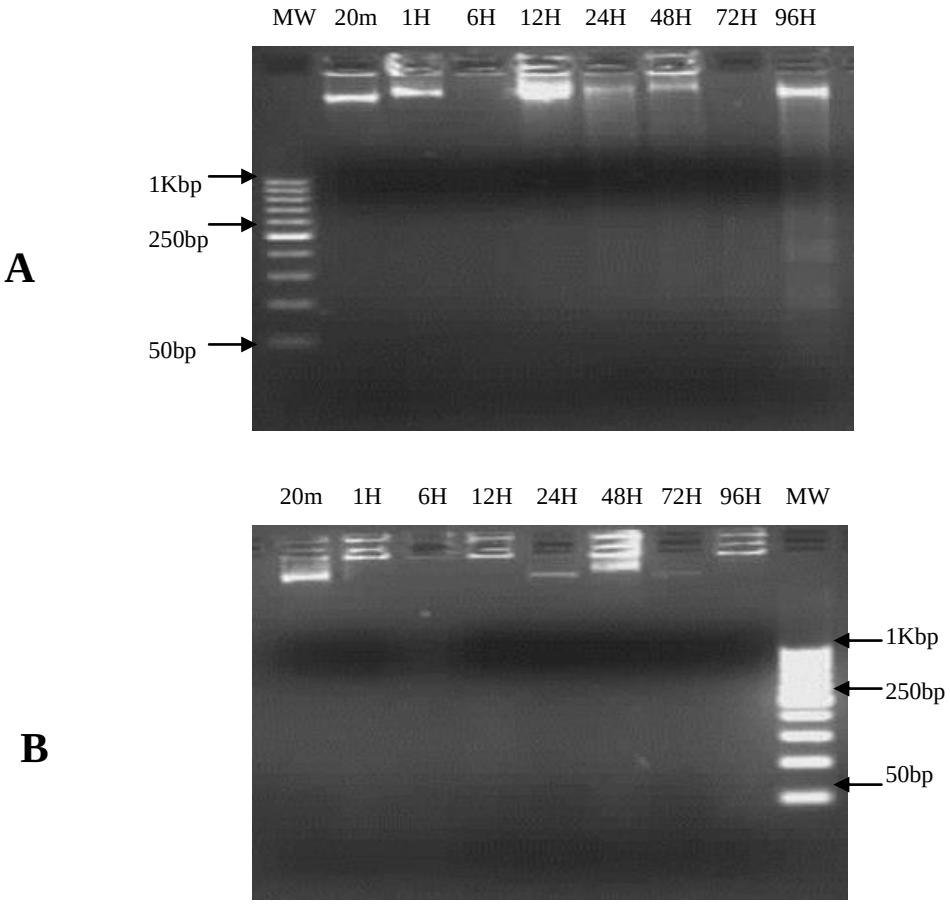


Figure 3.4 DNA fragmentation in MCF-7 cells exposed to 50% concentrations of un-neutralised (A) and pH-neutralised (B) AMD. In A and B, lane 1 (A) and lane 9 (B), represents the DNA molecular weight marker ranging from 50 bp to 1 Kbp (see arrows).. In A, lanes 2-9, depict 20 minutes, 1, 6, 12, 24, 48, 72 and 96 hours of exposure, respectively. In B, lanes 1-8 display exposure times of 20 minutes, 1, 6, 12, 24, 48, 72 and 96 hours, respectively. Ethidium bromide (2%) was utilised to show molecular weight markers and DNA fragmentation.

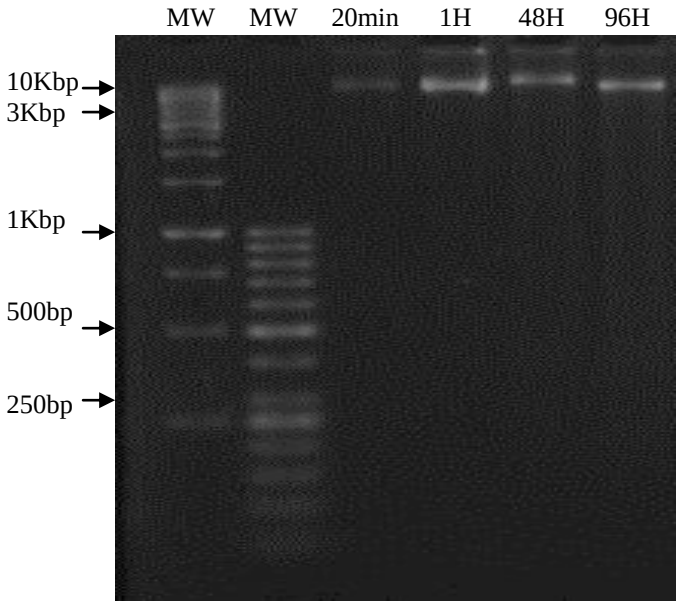


Figure 3.5 DNA fragmentation in MCF-7 cells exposed to 1.6% concentrations of pH-neutralised AMD. Lanes 1 and 2, represents the DNA molecular weight marker ranging from 250bp-10Kbp and 50bp-1Kbp (see arrows), respectively. Lanes 3-6, depict 20 minutes, 1, 48, and 96 hours of exposure, respectively.

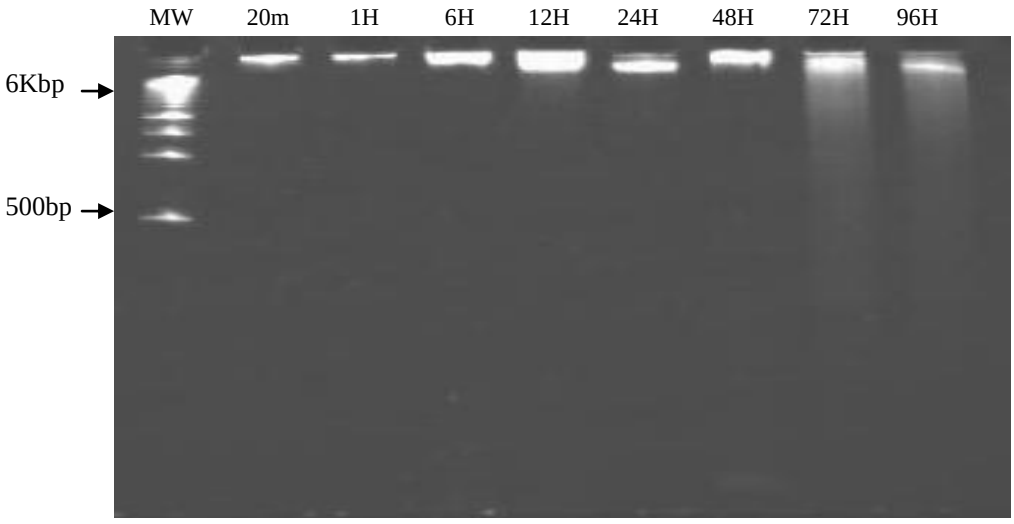


Figure 3.6 DNA fragmentation controls of MCF-7 cells exposed to homeostatic control medium and hydrogen peroxide. Lane 1, represents the DNA molecular weight marker ranging from 500 bp to 6KBp (see arrows). Lanes 2-5, depict homeostatic negative control at 20 minutes, 1, 48, and 96 hours of incubation, respectively. Lanes 6-9, depict hydrogen peroxide positive control at 20 minutes, 1, 48, and 96 hours of incubation, respectively.

3.3.4 *Morphological indicators of cell death in the MCF-7 cell line*

Morphological changes are considered the gold standard for distinguishing between apoptotic, oncotic and necrotic associated cell death. The survival rates of MCF-7 cells exposed to a 1.6% concentration of pH-neutralised AMD was of particular interest to the study. When exposed to pH-neutralised AMD 1.6% concentrations, MCF-7 survival rates showed to be significantly different from homeostatic control but not significantly different from proliferation rates of MCF-7 cell exposed to hydrogen peroxide at 1.6% concentrations. Thus to assess whether, apoptosis may be driving this decline in survival rates, the study analysed MCF-7 cells exposed to 1.6% diluted un-neutralised and pH-neutralise AMD for 96 hours for any morphologies of cell death.

In Figure 3.7, the development of apoptosis and oncosis related cell death leading to necrosis was observed in this study. Apoptotic cells displayed early membrane integrity and cell shrinkage (Figure 3.7 b, white arrow), pyknosis (Figure 3.7 d, white arrow) and karyorrhexis (Figure 3.7 b, black arrow) at late stages of apoptotic death. The onslaught of toxic conditions to a cell elicits the injurious response of oncosis, leading to alterations in cellular shape and membrane disruption (Figure 3.7 a, green arrow), cytoplasmic blebs (Figure 3.7 c, green arrow) and the development of chromatin clumping (Figure 3.7 c, white arrow). The *in vitro* exposures of MCF-7 cells indicative of necrotic death was typically observed with damaged organelles, disintegration of cell membrane, nuclear leakage and complete disintegration of cellular matter (Figure 3.7 c, gray arrow).

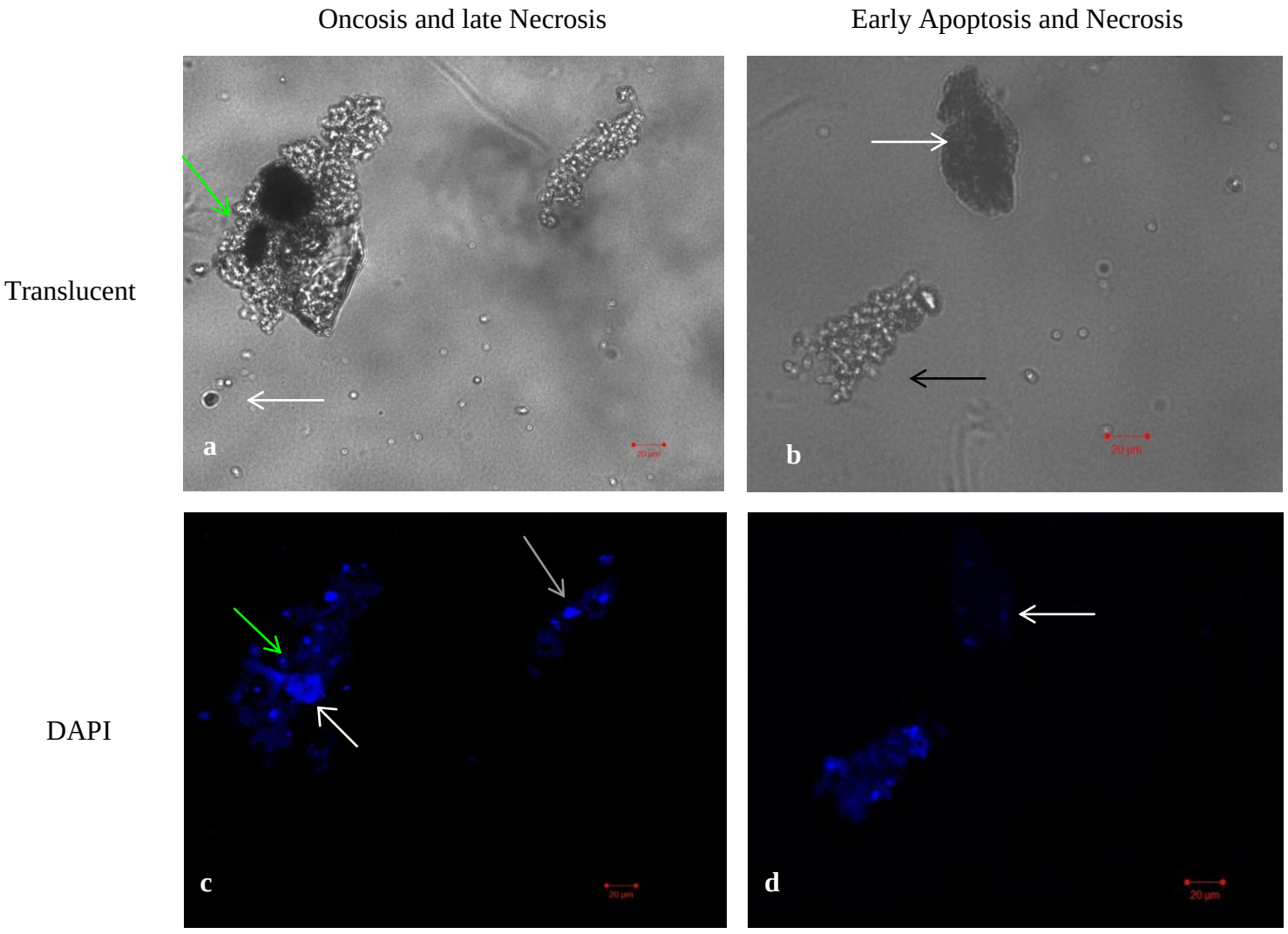


Figure 3.7 Cytotoxic induced apoptosis, oncosis and necrosis in MCF-7 cells exposed to a 1.6% concentration of pH neutralised AMD.

3.3.5 *The distribution of comet cell types undergoing cellular death*

In Figure 3.8 the visual method of classifying comet cell types into five categories (0-4) proposed by Collins et al. (1997) was utilised. Score zero to four denotes undamaged, initial apoptosis, mid-apoptosis, late apoptosis and necrosis, respectively; as determined with TriTek Comet Score®. Late stage apoptotic (score 3) and necrotic (score 4) cells predominantly occurred in MCF-7 cells exposed to 100% concentrations of un-neutralised and pH-neutralised AMD for 96 hours (Figure 3.9 A). However, in MCF-7 cells exposed to 1.6% concentrations of pH-neutralised AMD, a moderate percentage of non-damaged (score 0) comet cells were observed (Figure 3.9 B). When exposed to 1.6% concentrations of pH-neutralised AMD for 96 hours, a large progression toward mid stage (score 2) and late stage (score 3) apoptosis was observed (Figure 3.9 B). Additionally, MCF-7 cells exposed to 1.6% concentrations of un-neutralised AMD, display an increase in necrotic cell development (Figure 3.9 B). Remarkably, the percentage of necrotic cells in MCF-7 cells exposed to 1.6% concentrations of pH-neutralised AMD decreased significantly (Figure 3.9 A and B).

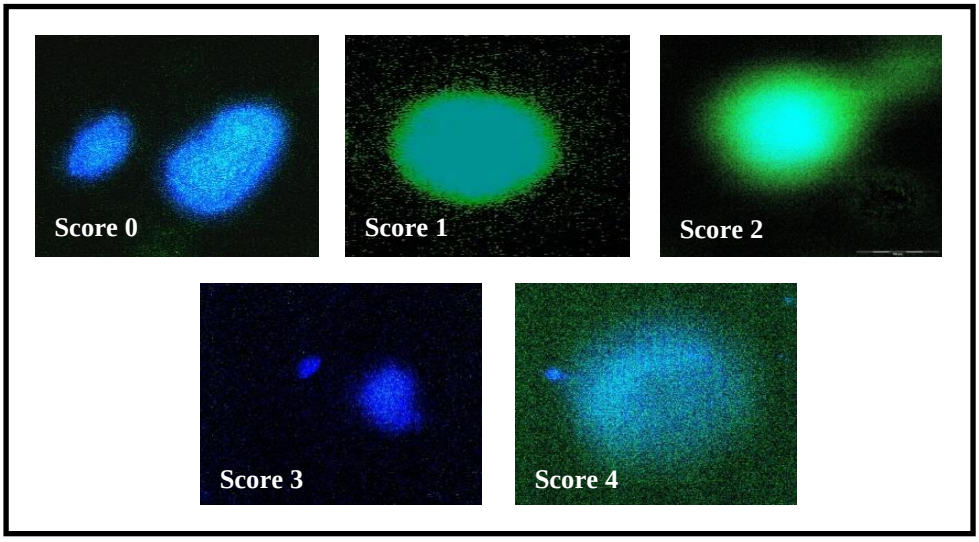
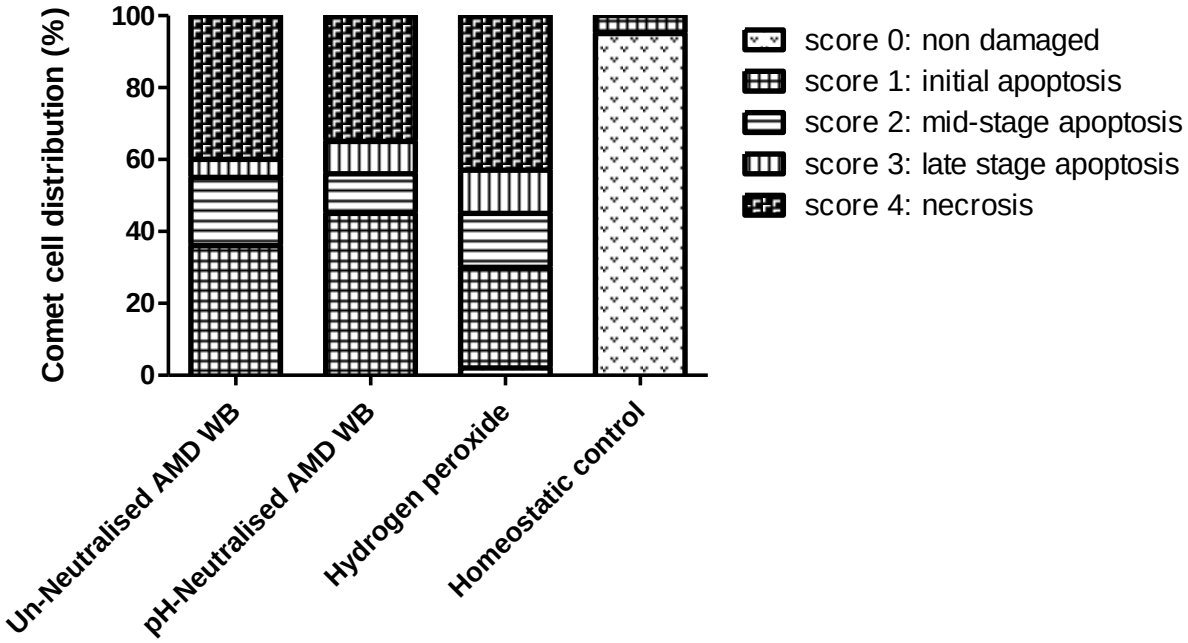


Figure 3.8 Comet cell development of MCF-7 cells exposure to 100% concentrated un-neutralised and pH-neutralised AMD for 96 hours.

A



B

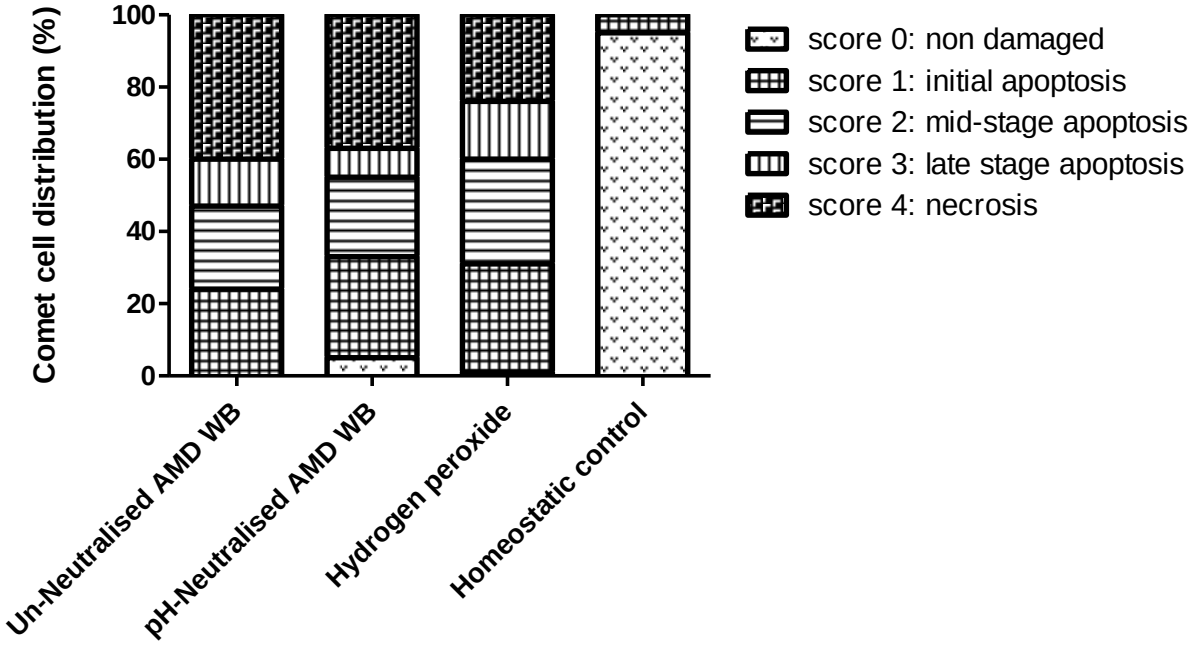


Figure 3.9 Analysis and depiction of various comet cell types upon the exposure of MCF-7 cells to 100% (A) and 1.6% (B) un-neutralised and pH-neutralised AMD for a duration of 96 hours.

3.3.6 *Percentage DNA in Tail observed as single and double stranded breaks in MCF-7 cells*

The genotoxic response of MCF-7 cells to 100% concentrations of pH-neutralised AMD, elicited an expectedly lower percentage of single stranded DNA (ssDNA) and double stranded DNA (dsDNA) breaks when compared to un-neutralised AMD exposure (Figure 3.10). However, an increased percentage of ssDNA and dsDNA breaks were observed with the exposure of MCF-7 cells to 1.6% concentrations of pH-neutralised AMD, suggesting a possible increase in the bio-availability of trace elements at low-level exposures (Figure 3.11).

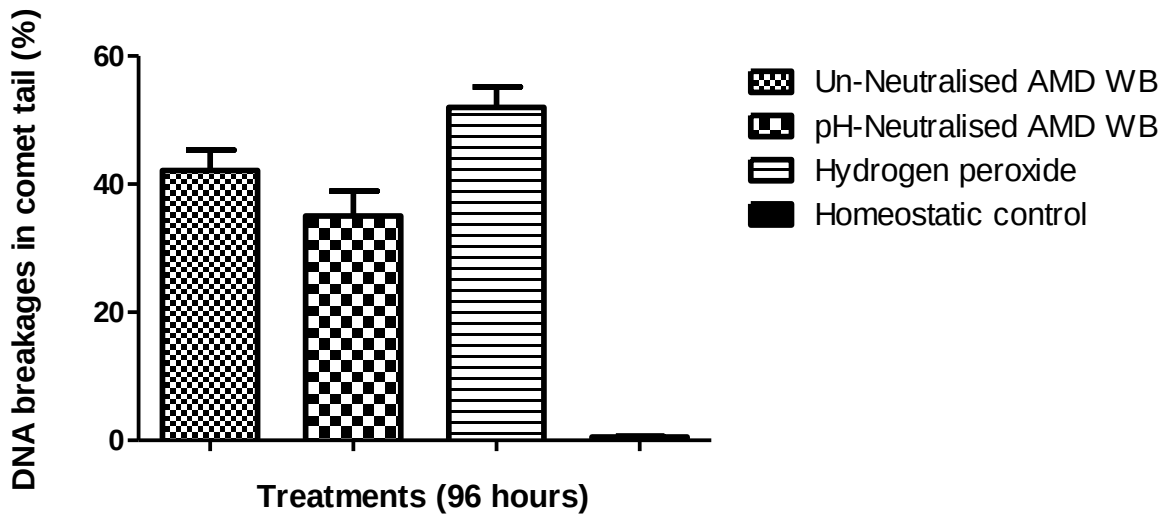


Figure 3.10 Distribution of ssDNA and dsDNA breakages in MCF-7 cells exposed to 100% concentrations of un-neutralised and pH-neutralised AMD for 96 hours. Average means of each treatment group is depicted above.

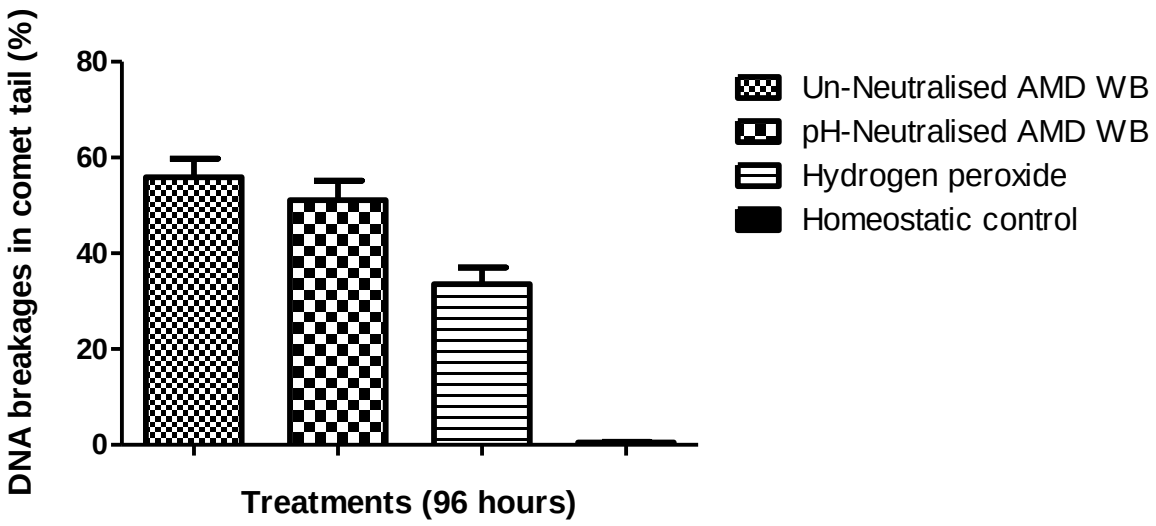


Figure 3.11 Distribution of ssDNA and dsDNA breakages in MCF-7 cells exposed to 1.6% concentrations of un-neutralised and pH-neutralised AMD for 96 hours. The average of 100 measurements per treatment group is depicted.

3.4 DISCUSSION

There are numerous impediments in understanding the toxicological evaluation of metal mixtures such as AMD. Firstly, a single component or compound within the mixture may have multiple affinity sites and the sequential activation of these sites may be regulated by different cellular pathways. Secondly, metals can be converted to metabolites that elicit an activity not resembling the parent compound. Thirdly, two or more compounds exhibiting synergistic or antagonistic effects on one another may alter the metabolism of the effected compound in such a way to produce toxic metabolites or DNA damaging death signals (Carpenter et al., 1998). To our knowledge, there are no published data detailing the impacts of toxicity derived from gold mined AMD, on the MCF-7 cell line. Therefore it was pertinent to investigate the global impacts of MCF-7 cells at both a cellular and genetic level of organisation.

The chemical nature of un-neutralised and pH-neutralised AMD characterises them as oxidative agents comprising of oxygen species (Johnson and Hallberg, 2005). Reactive oxygen species (ROS) species such as hydrogen peroxide (H_2O_2), superoxide (O_2) and hydroxyl radical (OH), have been implicated in oxidative stress in cells which has the potential to cause DNA damage, cancer, cellular aging amongst other degenerative diseases (Hang et al., 2011).As expected, the chemical analysis revealed a low pH and almost negligible levels of Fe ions in both 100% and 1.6% concentrations of un-neutralised AMD. Additionally, the chemical analysis revealed an average pH and a high level of Fe ions in both 100% and 1.6% concentrations of pH-neutralised AMD. Surprisingly, the level of SO_4 mineral was high in both 100% and 1.6% concentrations of un-neutralised and pH-neutralised AMD. This data points to major differences in the mechanism of toxicity of un-neutralised and pH-neutralised AMD on the MCF-7 cells (Table 3.2 and 3.3).

The cellular oxidative stress elicited by contaminants such as H_2S has been largely described as an aggravator of apoptosis in smooth muscle cells by up-regulating extracellular, signal-regulated kinases (Yang et al, 2004). Although commonly observed as a gas, H_2S is able to form sulphur deposits, as observed in AMD (Beauchamp et al, 1984). Indeed, in this study the un-neutralised AMD at 100% concentrations contained a large quantity of sulphur sediment. Therefore consistent with other investigations, the decline in cellular proliferation observed in MCF-7 cells exposed to un-neutralised AMD at 100% concentrations, can be assumed to be mediated by free hydrogen radical and an abundance of SO_4 mineral (Figure 3.2 A).

Likewise, free ionic iron, as a potent initiator of ROS, can enhance oxidative stress and damage to macromolecules, through Fenton reactions (Karbownik et al, 2003). The high level of free iron in pH-neutralised AMD at 100% concentrations (Table 3.1) may explain the negligible growth observed in MCF-7 cells (Figure 3.2 A).

It is well documented that low levels of ROS act physiologically at the signalling level, modulating proliferation (Murel et al, 1990), apoptosis (Kim et al, 2002) and gene expression through the activation of transcription factors such as NF-kappa-B (Schreck et al, 1991). This study observed an increase in cellular proliferation of MCF-7 cells exposed to negligible levels of un-neutralised and pH-neutralised AMD at 1.6% concentrations (Figure 3.2 C). It is known that cells generate a small quantity of hydrogen peroxide for use as a chemical signal that regulates everything from glucose metabolism to cellular growth and proliferation. It is possible that the increase in MCF-7 cellular survival observed in un-neutralised and pH-neutralised AMD at 1.6% concentrations could be a result of ROS cycling through the use of excess glucose and ionic compounds within the homeostatic medium

(Figure 3.2 C) (Dakubo, 2010). Similarly, the lower MCF-7 cellular survival rate in the pH-neutralised AMD at 1.6% concentrations may be attributed to the availability of free iron (Table 3.1).

Initially, MCF-7 cultures were exposed to un-neutralised and pH-neutralised AMD for various time intervals ranging from 20 minutes to 96 hours. The study was able to detect the initiation and execution of dominant cyto-genotoxic patterns, utilizing poly-acrylamide DNA fragmentation gels (Figures 3.3, 3.4 and 3.5). DNA fragmentation laddering has been used by many studies as a biomarker to confirm the presence of either apoptotic or necrotic mediated DNA cleavage (Wyllie, 1980; Takemura et al., 2001). One of the biochemical hallmarks of apoptosis is the double stranded inter-nucleosomal cleavage of DNA. The catalytic cleavage of damaged DNA is activated by the cascade of endo-peptidases known as caspases that cleave specific aspartic acid residues (Broker et al., 2005; Ashkenazi, 2008). Cleavage of DNA occurs at sites between nucleosomes (protein contain structures in chromatin) to fragments of 180-200 base pairs, observed as a laddering pattern on electrophoretic gels (Figure 3.4) (Joza et al., 2001). Fragmented DNA of cells undergoing apoptosis typically contain a high percentage of low molecular weight (LMW) DNA fragments compared to non-apoptotic cells exhibiting a presence of high molecular weight (HMW) DNA fragments (Nagata, 2000; Nagata et al., 2003). However, a study performed by Oberhammer et al. (1993), reported that nucleosomal fragmentation into LMW might not often occur during apoptosis, resulting in the formation of HMW fragments. The absence of LMW gDNA fragments, have been observed in cells that exhibit key morphological features of apoptosis (Brown et al,. 1993; Compton, 1992). A study by Cohen et al. (1994) showed that thymocytes exposed to zinc metal showed apoptotic morphologies, without LMW DNA fragment cleavage but with a yield of HMW gDNA fragments. These findings were

corroborated in the current study, where a presence of HMW gDNA was observed in MCF-7 cells exposed to both un-neutralised and pH-neutralised AMD at 100% and 50% concentrations. Alternately, necrosis is characterised by indiscriminate DNA fragmentation. The chromatin of cells undergoing necrosis, are disintegrated by lysosomal and cytosolic nucleases, which result in a characteristic smear when visualised on gel electrophoresis (Dong et al., 1997) This study showed a progression of extensive to minimal gDNA smearing in MCF-7 cells exposed to un-neutralised and pH-neutralised AMD at 100%, 50% and 1.6% concentrations.

Although DNA fragmentation laddering is a gold standard in qualitatively determining the progression of apoptosis and necrosis, further morphological analysis was required to dispel uncertainties surrounding the presence or absence of apoptosis and necrosis in MCF-7 cells exposed to un-neutralised and pH-neutralised AMD.

The morphological variations which occur as a consequence of either apoptosis or necrosis are key bio-indicators of cellular death (Collins et al., 1997). Apoptosis is the discrete arrangement of morphological changes which result in chromatin compaction, mild convolution of cellular membrane, cell shrinkage leading to the formation of apoptotic bodies known as “blebs” along the cell wall and endo-nuclease cleaved DNA fragmentation (Figure 3.7 b and d) (Formigli et al., 2000; Sperandio et al., 2000). Necrosis is typically signified as aggravated cell death resulting in the degradation of the cell membrane, nuclear swelling, and nuclear leakage and intensified breakdown of all cellular structure and organelles (Figure 3.7 a and c) (Trump et al., 1997). However, recent studies have suggested that necrosis might be propagated by a defined pathway of action. A pre-lethal form of injurious death, known as oncosis or early-onset necrosis, is described as the initiation of necrotic death (Majno and

Joris, 1995). Early-onset necrosis is better characterised by cell rounding and nuclear swelling and membrane rupture (Figure 3.7 a) (Levin et al., 1999). Thus necrosis demonstrates the post mortem result of cellular death (Trump and Berezesky, 1998). Of note, however is the development of *in vitro* apoptosis which leads to membrane permeabilization known as secondary necrosis (Scaffidi et al., 2002; Bianchi and Manfredi, 2004). Several studies have shown that MCF-7 cells may lack central caspases that initiate and maintain apoptotic cell death (Abraham and Shaham, 2004; Nicotera, P. and Melino, 2004).

Thus caspase-independent cell death may provide necrosis as a substitute mechanism of absolute cell death, when classical apoptotic machinery fails (Figure 3.7) (Zheng et al., 2000). The inhibition of caspase-dependent cell death has been shown to further promote controlled early-onset necrosis. Therefore, in an *in vitro* environment of cytotoxicity, apoptosis and early-onset necrosis will both lead to total necrosis (Figure 3.7) (Trump et al., 1997). The similarities observed in this study between apoptotic and necrotic morphologies may be an indicator of caspase-independent cell death (Figure 3.7).

Once the study observed characteristic morphologies of apoptosis and necrosis it was pertinent to confirm the dominant signal transduction mechanisms in MCF-7 cells exposed to un-neutralised and pH-neutralised AMD. The exposure of MCF-7 cells to both 100% and 1.6% concentrations of un-neutralised AMD elicited a surprisingly similar progression of necrotic cells. In the same populations, a moderate progression toward the development of early to mid-stage apoptotic cells was observed (Figure 3.9 A). This observation could point to the intensity of un-neutralised AMD oxidative toxicity compared to pH-neutralised AMD. In addition to toxicity, it is possible that the very nature of un-neutralised AMD oxidation prompts MCF-7 cells to undergo an abrupt cellular death resulting in late stage to final

necrosis. To add to this assumption, the study observed a higher development of early to mid-stage apoptosis in MCF-7 cells exposed to 100% and 1.6% concentrations of pH-neutralised AMD. With specific regard to 1.6% concentrations of pH-neutralised AMD exposures, MCF-7 signal transduction resembled that of the hydrogen peroxide control (Figure 3.9 B). Significantly, a small proportion of undamaged cells were observed in this population. Thus, it can be assumed that 1.6% concentrations of pH-neutralised AMD might encourage a delayed inclination toward cellular death in MCF-7 cells.

To ascertain the extent of DNA damage and gain insight into possible genotoxic mechanisms, the study utilised the comet assay to monitor DNA strand breaks. The maximum level of damage observed in the study was quantified as % DNA in tail. Percent DNA in tail is a representation of single and double stranded DNA intensity within the tail region and gave insight into the kinetics of gDNA unravelling in the presence of harmful substances (Hayashi et al., 2000). Due to comet class scoring data, the study observed an expectedly high percentage of % DNA in tail at both 100% and 1.6% concentrations of un-neutralised AMD. The surprising result of high DNA breakage percentage in MCF-7 cells exposed to 1.6% concentrations of pH-neutralised AMD (Figure 3.11), led us to speculate the mechanism at play. Reactive oxygen species in moderate concentrations may be essential mediators against unwanted cells. Thus, in the presence of antioxidant supplements (such as cell media), free radicals are reduced. This incapacitates the natural cellular defence mechanisms of eliminating pre-cancerous or cancerous cells. In the current study it is clear that cells have not evaded cell death and that homeostatic control medium does not confer an antioxidant property to 1.6% concentrations of pH-neutralised AMD, in order to lower the amount of apoptotic cells. Instead pH-neutralised AMD at 1.6% concentrations may act as both an initiator and mediator of cell death.

3.5 CONCLUSION

Chemical compounds and heavy metals can damage the DNA of living cells. If not repaired, these DNA aberrations can initiate a cascade of biological consequences at the cellular and genetic level of organization (Tseng et al., 1996). In this study, sensitive molecular technologies evaluating short term impacts of metal and free ion oxidation of un-neutralised and pH-neutralised AMD have proven useful to screen the toxic potential within human cell lines. This investigation revealed the abrupt development of necrosis in MCF-7 cells exposed to un-neutralised AMD. Similarly, the study confirmed the slow progression MCF-7 of cells toward final apoptosis when exposed to negligible concentrations of pH-neutralised AMD. The finding that pH-neutralised AMD at negligible concentrations may act as both an initiator and mediator of cell death is cause for much concern. Thus pH-neutralised AMD ($CaCO_3$) have proven ineffective in preventing low levels of DNA damage that could possibly induce DNA mutations. Neutralisation technologies that remove trace amounts of metal and sulphates are recommended as an additional system to the current limestone ($CaCO_3$) neutralisation technique.

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CHAPTER FOUR

EVALUATING THE GENOTOXIC EFFECTS OF ACID MINE DRAINAGE, ARISING FROM THE UPPER OLIFANTS RIVER, ON A HUMAN CARCINOMA CELL LINE (MCF-7)

4.1 INTRODUCTION

A majority of South Africa's coal mining industry is situated in the Mpumalanga region (South Africa Country Analysis Briefs, 2005; Somerset 2003). Although one of the most essential natural resources, the process of mining coal significantly contributes to the generation of acid mine drainage (AMD) (McCarthy, 2011). The formation of AMD is catalysed by the oxidation of pyrite ore in the presence of oxygen and water. What ensues is a complex mixture comprising dissolved iron, sulphate, hydrogen and a continuous oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}). The Fe^{3+} in the mixture continues to precipitate as iron hydroxide ($Fe(OH)_3$) driving the pH of AMD between a range of 2-3 (Coulton et al., 2003; Johnson, 2003). The remaining Fe^{3+} which does not precipitate through the solution, is further utilised to oxidise surrounding pyrite and decrease pH, thereby increasing the load of solubilised metals, total dissolved solids and minerals within AMD (Larsen and Mann, 2005).

The Olifants River, a major river in Mpumalanga, is at present one of the most contaminated water bodies in South Africa (Ballance et al., 2001; Van Vuuren, 2009). The observations of fish and crocodile deaths in the Kruger National Park have raised major concerns about the adequacy of water monitoring strategies in the Olifants River catchment (Van Vuuren, 2009). The runoff of agricultural, mining and industrial waste into the Olifants River has further

complicated the development of a sufficient monitoring system in which a specific pollutant could be directly linked to an effect observed (de Villiers and Mkwelo, 2009). The Kromdraai River, a smaller tributary of the Olifants River has similarly been affected by the direct uncontrolled decant of AMD. In an effort to remediate AMD contaminated water in the Kromdraai River, active pH-neutralisation has been implemented. The pH-neutralisation technology incorporates the use of the chemical precipitant, sodium hydroxide (caustic soda or NaOH). Thus the direct titration of NaOH into the Kromdraai River allows the precipitation of metals and increases pH of the solution between ranges of 7-8 (Günther et al., 2003). The pH-neutralisation of rivers such as the Kromdraai River forms part of the 'Consortium for the Restoration of the Olifants Catchment' initiative and National Chemical Monitoring Programme (NCMP) under the directorate of the Department of Water Affairs (DWA). The key mandate of this initiative is the monitoring and remediation of the Olifants River and its tributaries when affected by elevated concentrations of dissolved solids, sulphates, metals and other properties associated with AMD contamination (Anderson et al., 2000). In addition to the NCMP, the National Toxicity Monitoring Programme (NTMP) aims to assess trends of toxicity in polluted waters such as the Olifants River (DWA, 2009). However, these bio-monitoring strategies do not reveal complex trends of geno-cytotoxicity that might indicate the precise modes and mechanisms of toxicity at a cellular level of organisation in humans. This study proposes to form an integral part of toxicity monitoring strategies in the Kromdraai River.

The study aimed to determine the impact of un-neutralised and pH-neutralised AMD on the MCF-7 cell line. Understanding response to toxic complex mixtures such as un-neutralised and pH-neutralised AMD, gives insight into targeted damage to membrane receptors, enzymes and DNA molecules (Shanker, 2008). The disruption of cellular maintenance

culminates as toxic manifestations whereby death signalling pathways such as apoptosis and necrosis initiates a cascade of biochemical events (Langman and Kapur, 2006). The morphological variations which occur as a consequence of either apoptosis or necrosis are key bio-indicators of cellular death (Collins et al., 1997). Apoptosis is the discrete arrangement of morphological changes which result in chromatin compaction, mild convolution of cellular membrane, cell shrinkage leading to the formation of apoptotic bodies known as "blebs" along the cell wall and endo-nuclease cleaved DNA fragmentation (Formigli et al., 2000; Sperandio et al., 2000). Necrosis is typically signified as aggravated cell death resulting in the degradation of the cell membrane, nuclear swelling, and nuclear leakage and intensified breakdown of all cellular structure and organelles (Trump et al., 1997). As morphological alterations proceed, abrupt DNA fragmentation of cells occurs. Evaluating the extent of DNA damage gives possible indications into genotoxic mechanisms. This exploratory study presented a novel opportunity to investigate specific biomarkers of cellular death, particularly membrane viability, cellular morphology, DNA fragmentation and DNA breakages in the MCF-7 cell exposed to un-neutralised and pH-neutralised AMD from a coal mining area in South Africa.

4.2 MATERIALS AND METHODS

4.2.1 Study area

4.2.1.1 Background and pH-neutralisation technology implemented in the study area

The Kromdraai River, situated upstream of Lake Witbank was selected as the study area affected by the uncontrolled decanting of AMD in an abandoned coal mining region (Figure 4.1 A) (Louw et al., 2001). The active pH-neutralisation of the AMD contaminated

Kromdraai River is accomplished through a sodium hydroxide (NaOH, caustic soda) chemical precipitation process (Figure 4.1 B) (US-EPA, 2000). The pH-neutralisation involves the increase of dissolved oxygen in AMD (approximately pH 3-4) contaminated water and aeration by gravity flow and sprays (Figure 4.1 B). The intensification of dissolved oxygen allows the oxidation of metals into forms of metal hydroxides (US-EPA, 2008). Subsequently, dissolved metals are removed with the addition of sodium hydroxide, creating an insoluble precipitate. This pH-neutralised (approximately pH 7-8) mixture is directed into the receiving Kromdraai River (on site monitoring). Therefore this site was chosen as a site representative of both contaminated (un-neutralised) and pH-neutralised AMD water in a tributary part of the Olifants River system.

4.2.1.2 *Sampling procedure*

AMD-contaminated (un-neutralised AMD) water were sampled from the Kromdraai River situated close to the town of eMalahleni (Figure 4.1 A). The pH-neutralised AMD samples were collected from the Kromdraai treatment plant situated alongside the Kromdraai River (Figure 4.1 B). A volume of 2 litres of each water sample was collected on the 24th July 2012 in nitric acid rinsed plastic bottles and transported to the CSIR laboratories in Stellenbosch for chemical analysis (Table A1, Appendix A). The samples were filtered through 0.2 µm and 0.45 µm low protein-binding cellulose acetate membrane filters to remove any organic matter and stored at 4 °C for later use.

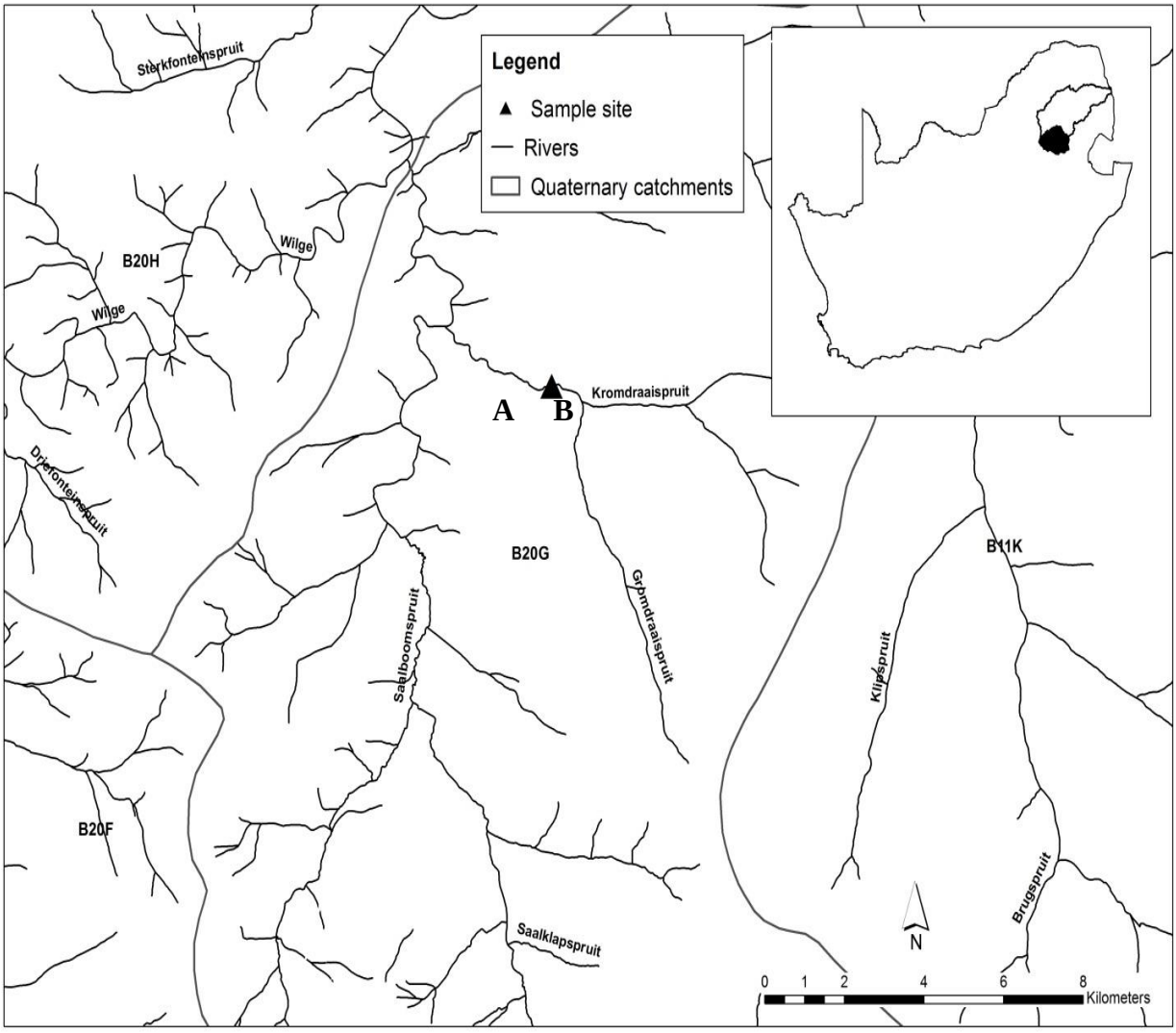


Figure 4.1 A, the Kromdraai River into which contaminated AMD water decants. B, the sodium hydroxide neutralisation plant at Kromdraai. The scale indicates distances in kilometres.

4.2.2 Cell culture

The MCF-7 cell line was previously described as a prime *in vitro* system in which to study metal interactions at high levels of exposure (Klutse, 2009; Wu et al., 2006). The human breast adenocarcinoma (MCF-7, HTB-22) cell line was a kind gift from Professor Johannes van Wyk at Stellenbosch University. The MCF-7 cellular growth was maintained as described in Chapter 3. The cell pellet were resuspended in DMEM and either reseeded to maintain the MCF-7 culture stock or used for additional end point assays.

4.2.3 Assessing MCF-7 survival rate and growth kinetics

MCF-7 cells were seeded into 24-well plates at a density of 3 x 10^5 cells per well in DMEM, and allowed to grow for 4 days. All cellular processing occurred as described in Chapter 3. Cell pellets were exposed to biological controls, experimental controls or test samples, in triplicate. The rate of cellular survival was measured immediately or after 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours and 96 hours of incubation.

The MCF-7 cells grown in DMEM established a baseline of homeostatic cellular survival and were used as the biological control. Hydrogen peroxide served as the experimental positive control, indicating oxidative induced cell death.

Varying concentrations of un-neutralised and pH-neutralised AMD were used as the test samples to measure complete (100%), mid-level (1:2 or 50%) and low-level (1:64 or 1.6%) toxicity. Complete toxicity at 100% exposure to un-neutralised and pH-neutralised AMD, in a nutrient deficient environment, was considered a baseline of complete exposure to AMD.

All exposures were performed in triplicate. The rate of proliferation of each sample was determined using the Trypan blue exclusion method and an automated TC20 cell counter

(Bio-Rad, USA). The rate of cellular survival was plotted as mean percent of three independent counts compared to homeostatic control. Survival data across time points (immediate time zero to 48 hours) per exposure were analysed by one-tailed Students *t*-test (percent control). Proliferation data at 96 hours of exposure were analysed by independent Students *t*-test (unequal variances). The level of significance (*P* value) was set at 0.05 for all tests.

4.2.4 Assessing patterns of DNA fragmentation

To determine DNA fragmentation dynamics of MCF-7 cells in a completely toxic (100%) environment of un-neutralised and pH-neutralised AMD, cells were exposed for 20 minutes, 1 hour, 48 hours and 96 hours. To assess whether DNA fragmentation patterns would differ in lower doses, MCF-7 cells were exposed to mid-level toxicity (50%) of un-neutralised and pH-neutralised AMD, for 20 minutes, 1 hour, 6 hours, 12 hours, 24 hours, 48 hours and 96 hours. The isolation of fragmented DNA was performed according to the procedure described by Hermann et al. (1994) as outlined in Chapter 3.

4.2.5 Single Cell Gel Electrophoresis assay (Comet assay)

4.2.5.1 *Comet assay slide preparation and processing*

The alkaline comet assay, previously described by Singh et al. (1996), was executed as described by the OxiSelect™ Comet Assay Kit (Cell Bio labs, USA). To establish the distribution of DNA damage in MCF-7 cells exposed to un-neutralised and pH-neutralised AMD, exposures occurred at complete (100%) and low-level (1.6%) toxicities. Exposures were carried out in 24-well plates for duration of 96 hours. The procedure used to assay the exposed cells is fully described in Chapter 3.

4.2.5.2 *The traditional Comet assay: evaluation of cell death distribution and percentage (%) DNA in tail.*

Each slide was stained with 100 µl of 1:10000 diluted Vista Green DNA dye (Cell Bio labs, USA) for 10 minutes. Slides were counter stained with 100 µl of 6-diamidino-2-phenylindole (DAPI) dye (Sigma Aldrich, U.S.A.) for 5 minutes, rinsed and, analysed under fluorescence microscopy (Zeiss Axiomat, Carl Zeiss GmbH, Jena, Germany) using FITC (fluorescein isothiocyanate) and DAPI filters. DAPI and Vista green staining allowed the observation of fragmented nucleic DNA and cell membranes (membrane proteins), respectively

To determine the extent of apoptotic and necrotic cell distribution, a total of 100 cells were counted per concentration of un-neutralised and pH-neutralised AMD (complete (100%) and low-level (1.6%)).

Comet cells on each slide were categorised according to a visual scoring method utilising the length of DNA migration in the tail region as a parameter. Thus each cell was scored as belonging to one of five classes, where 0 represents visually undamaged cells and 4 visually-maximally damaged morphologies (Collins et al., 1995).

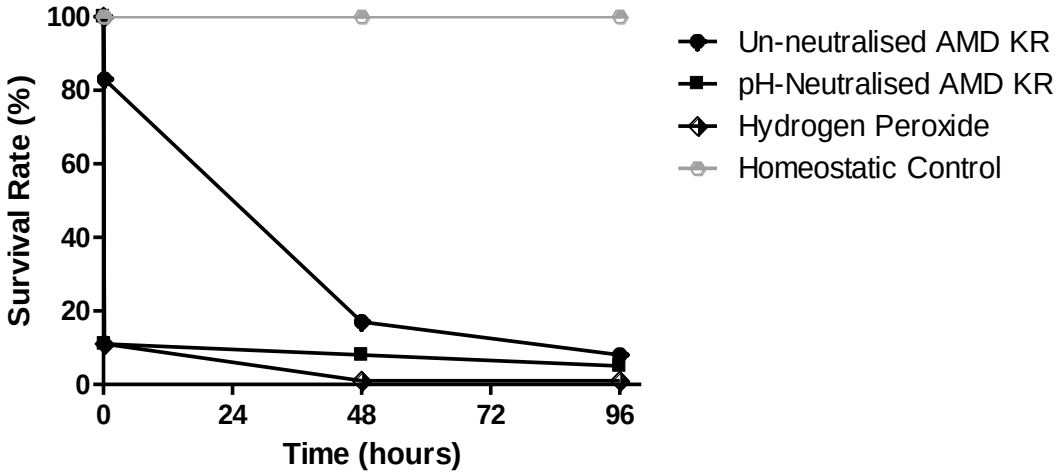
Percentage of DNA in tail was determined using the Comet Score® software developed by TriTek (www.tritekcorp.com). The DNA in the tail represents the frequency of DNA stand breaks. A total of 50 comets per undiluted and diluted concentration of each treatment group were scored in duplicate, constituting an amount of 100 cells counted in total. Percent DNA in tail for each exposure to un-neutralised and pH-neutralised AMD ((complete (100%) and low-level (1.6%) toxicity) is represented as a mean of two replicas of 50 counts each per slide (n=100). Data produced for percent DNA in tail at 96 hours of exposure were analysed by independent Students *t*-test (unequal variances). The level of *P* value was set at 0.05.

4.3 RESULTS

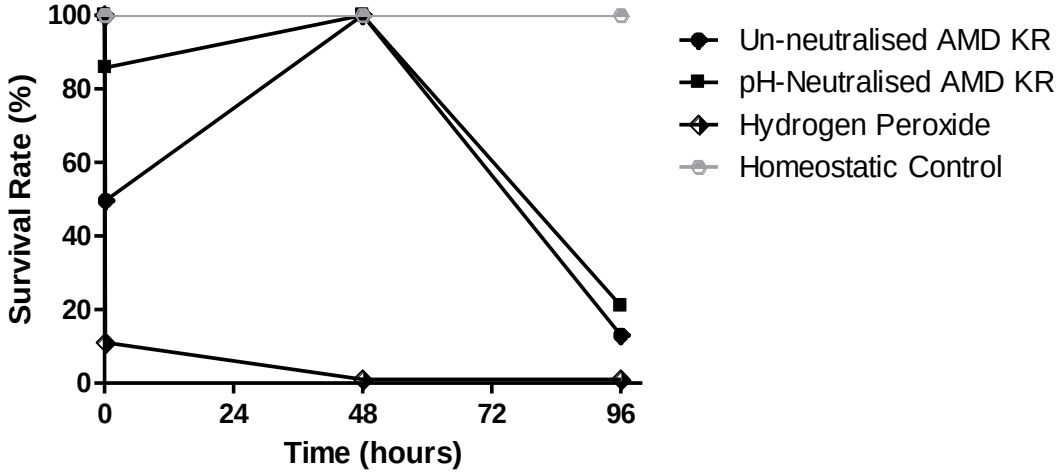
4.3.1 *Cellular viability of the MCF-7 cell line*

The cellular viability of MCF-7 cells is presented as either inhibition or proliferation upon exposure to 100%, 50% and 1.6% concentrations of un-neutralised and pH-neutralised AMD from the Kromdraai River (Figure 4.2). The exposure of MCF-7 cells to un-neutralised and pH-neutralised AMD elicited an overall inhibition of cellular proliferation (Figure 4.2 A) compared to control. Treatment of MCF-7 to 50% concentrations of un-neutralised and pH-neutralised AMD showed an increase in cellular viability up to 48 hours of exposure. MCF-7 cells exposed to 1.6% concentrations of pH-neutralised AMD showed a steady increase in cellular viability. More interestingly, MCF-7 cells underwent an increased recovery after 48 hours of exposure to 1.6% concentrations of un-neutralised AMD.

A. 100% concentrated



B. 50% concentrated



C. 1.6% concentrated

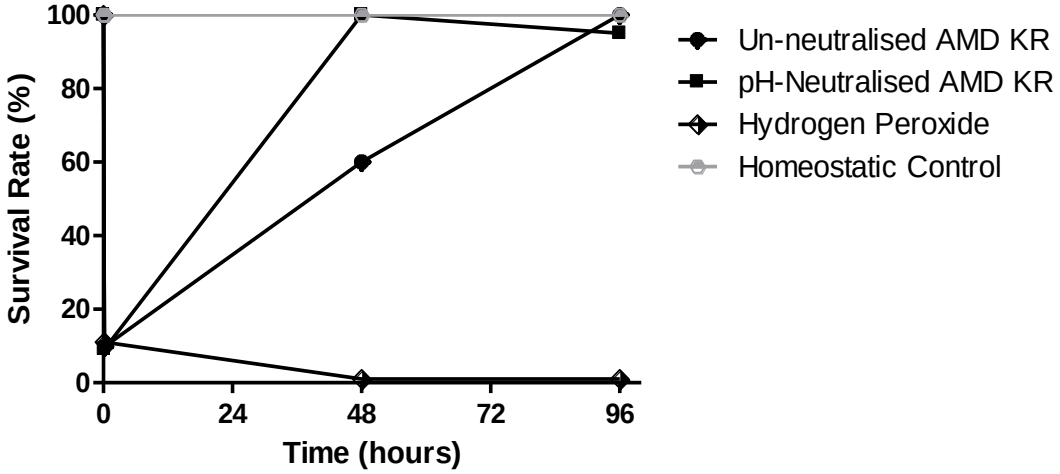


Figure 4.2 Observed cell survival of MCF-7 exposed to 100% (A), 50% (B) and 1.6% (C) concentrations of un-neutralised and pH-neutralised AMD from the Kromdraai neutralisation plant (KR). The average survival measurements per treatment group is depicted.

4.3.2 *DNA fragmentation laddering*

The genomic integrity of MCF-7 cells exposed to 100% concentrations of un-neutralised AMD was observed as high molecular weight genomic DNA (gDNA) bands (Figure 4.3 A). Partial gDNA degradation was observed at 48 and 96 hours of exposure to 100% concentrations of un-neutralised AMD with the appearance of smearing patterns (Figure 4.3 B).

4.3.3 *The fluorescent comet assay: categorising cell death*

In Figure 4.4 A**,** an undamaged cell undergoing replication and chromosomal rearrangement was observed, when exposed to 1.6% concentrations of pH-neutralised AMD. Exposure of MCF-7 cells to undiluted pH-neutralised AMD exposure showed cells undergoing early apoptotic events such as membrane blebbing (white arrow) and membrane shrinkage (gray arrow) (Figure 4.4 B). MCF-7 cells undergoing programmed death when exposed to 1.6% concentrations of pH-neutralised AMD where observed as apoptotic (Figure 4.4 C and D) and necrotic (Figure 4.4 E) cells undergoing various stages of degradation. The visual comet scoring classification into five categories (0-4) was proposed by Collins et al. (1995). Score zero to four denotes undamaged, initial apoptosis, mid-apoptosis, late apoptosis and necrosis, respectively as determined with TriTek Comet Score® software (Figure 4.4).

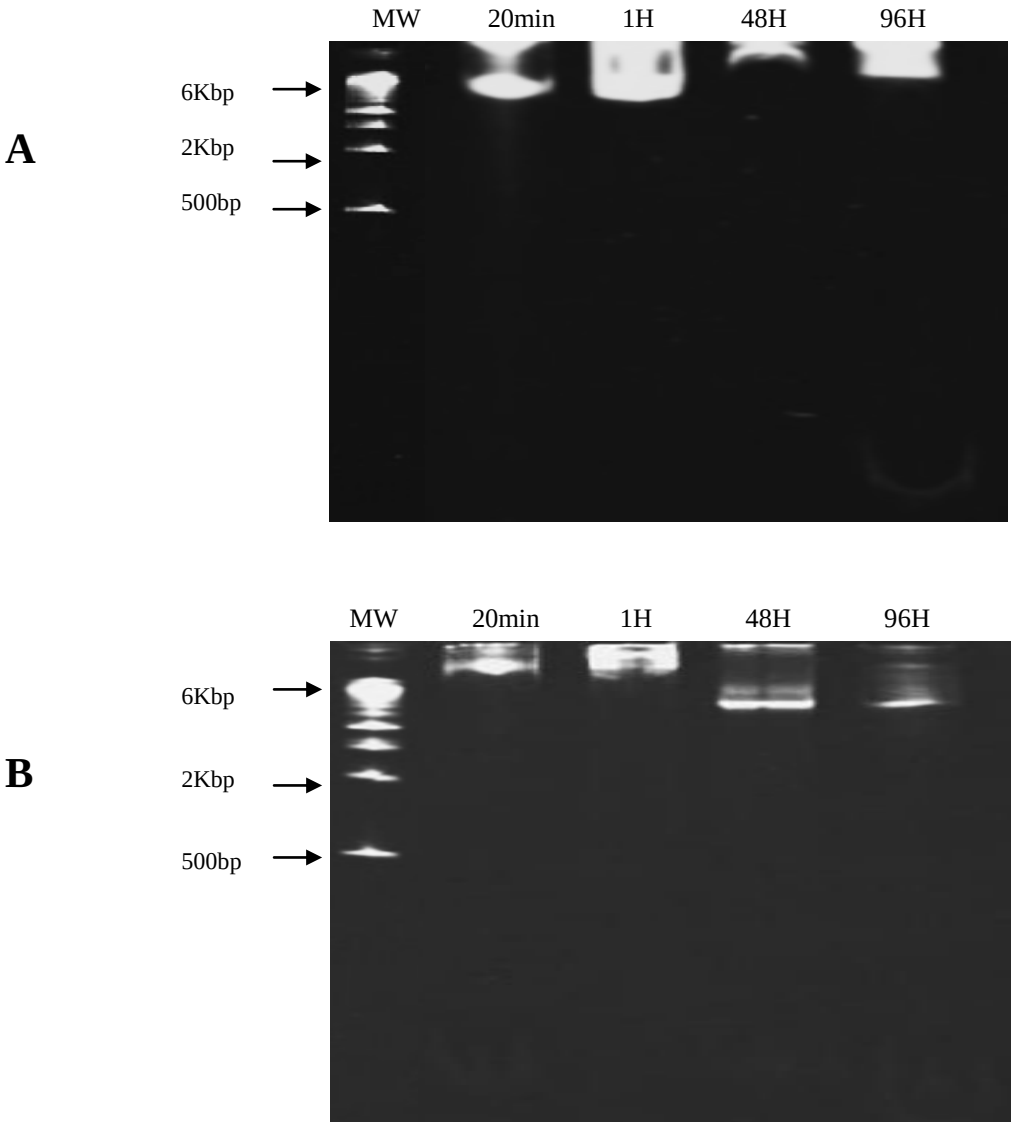


Figure 4.3 DNA fragmentation patterns of MCF-7 cells exposed to 100% concentrations of un-neutralised (A) and pH-neutralised (B) AMD. Lane 1 of A and B show the 500bp to 6KBp DNA molecular size marker (see arrows). In both A and B, lanes 2, 3, 4 and 5 represent exposures times of 20 minutes, 1 hour, 48 hours and 96 hours, respectively.

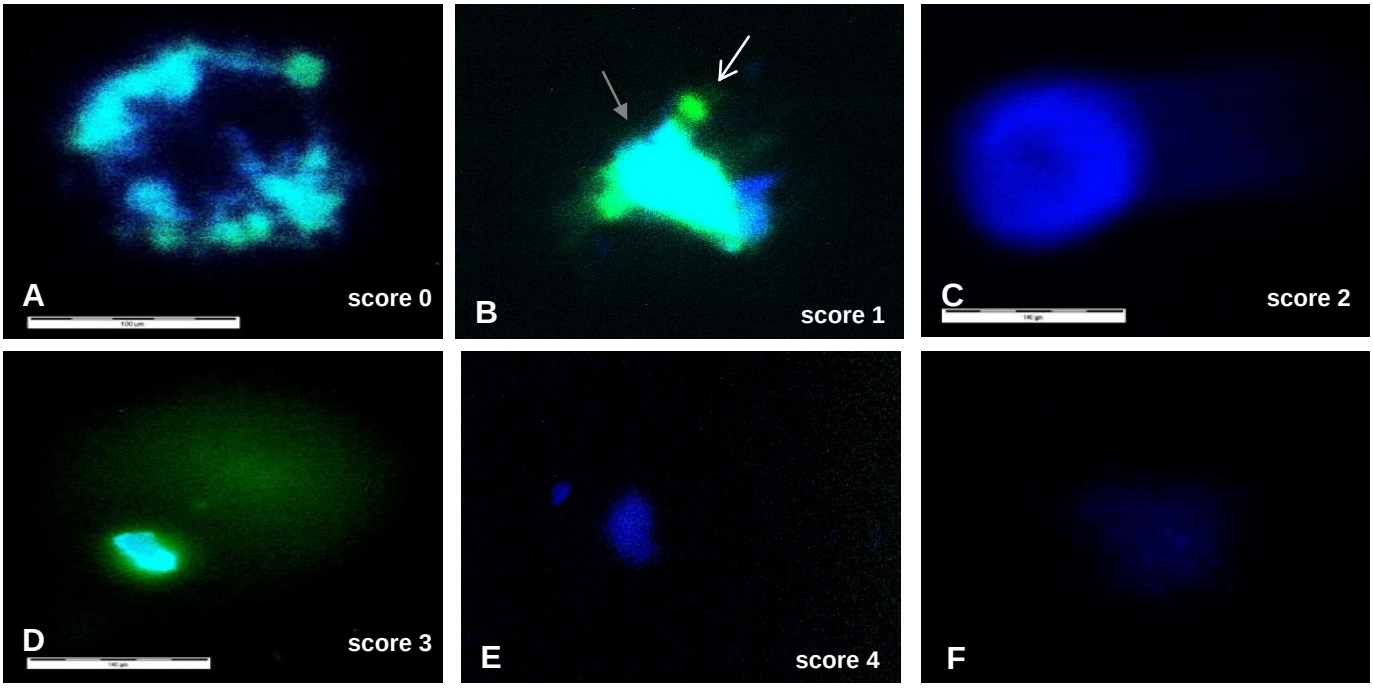


Figure 4.4 Morphological comet cell categories of MCF-7 cells exposed to 1.6% concentrations of pH-neutralised AMD for 96 hours. **A,** depicts an undamaged cell. **B**, **C** and **D** depict comet cells undergoing early, mid and late apoptosis. **E**, represents a cell undergoing necrosis. **F**, shows a cell which have undergone complete degradation. Cells where stained with DAPI and Vista green dye to allow fluorescent visualisation. Images were captured on a 20x magnification on a Zeiss Axiomat Fluorescent microscope. Scale bars depict 100 µm.

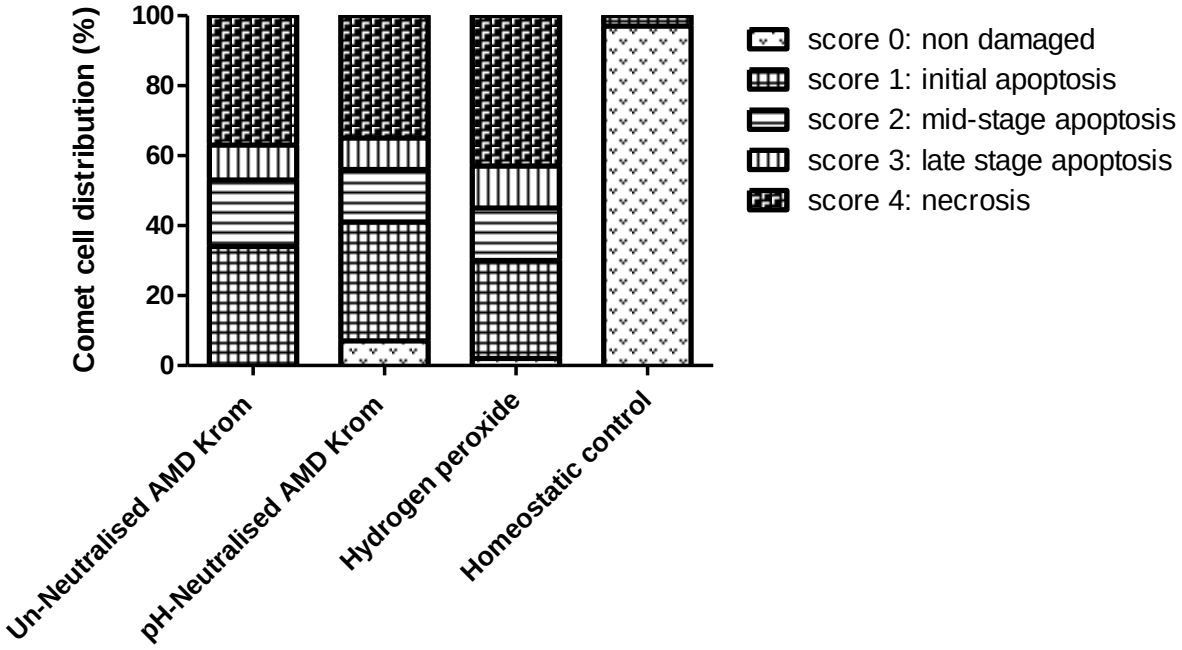
4.3.4 *The fluorescent comet assay: distribution of comet cell types*

In MCF-7 cells exposed to 100% concentrations of pH-neutralised AMD a large proportion, of non-damaged (score 0) and initial apoptotic (score 1) were identified (Figure 4.5 A). When exposed to 1.6% concentrations of pH-neutralised AMD, a progression toward late stage (score 3) apoptosis was observed (Figure 4.5 B). Interestingly, a minute proportion of score 0 undamaged MCF-7 cells were identified when exposed to 100% concentrations of un-neutralised AMD (Figure 4.5 B).

4.3.5 *DNA stranded breakages as an indicator of geno-toxicity*

The genotoxic response of MCF-7 cells to 100% concentrations of pH-neutralised AMD, elicited an expectedly lower percentage of DNA stranded breakages when compared to that of cells exposed to 100% concentrations of un-neutralised AMD. However, an increased percentage of DNA stranded breakages were observed with the exposure of MCF-7 cells to 1.6% concentrations of pH-neutralised AMD, suggesting a possible increase in the bio-availability of trace elements at 1.6% concentrations (Table A1, Appendix A) as reflected by the increase of electrical conductivity values as well as higher concentrations of Na, Ca, Mg and SO_4.

A



B

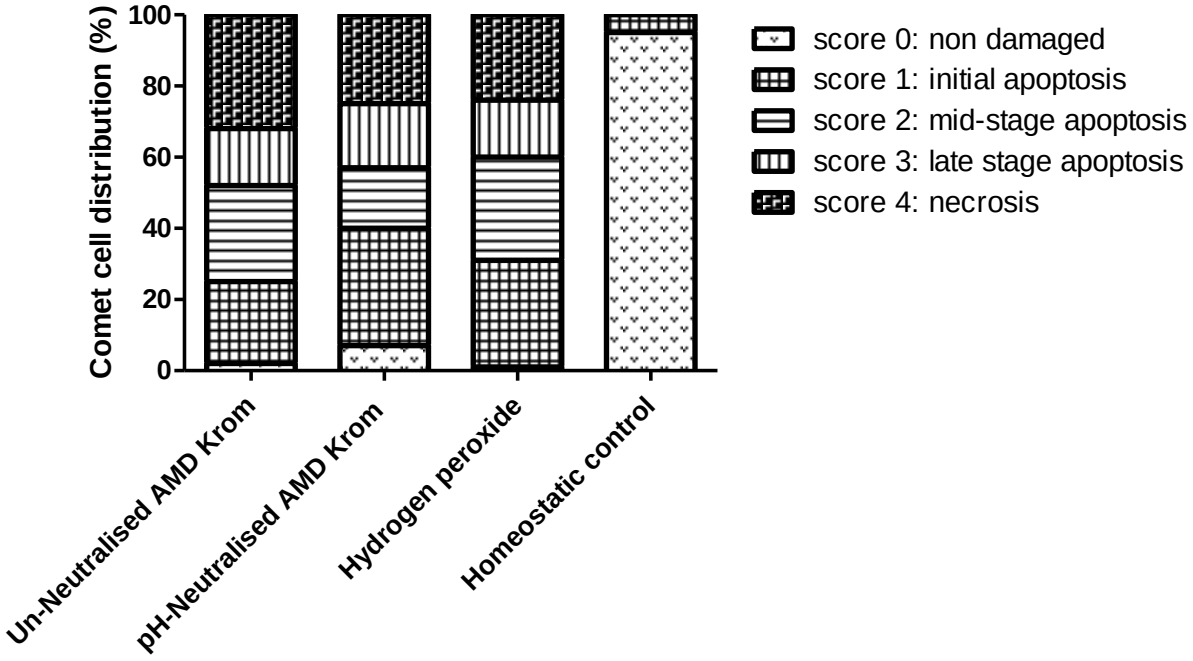


Figure 4.5. Distribution of comet cell catagories in the MCF-7 cell line exposed to 100% (A) and 1.6% (B) concentrations of un-neutralised and pH-neutralised AMD for 96 hours.

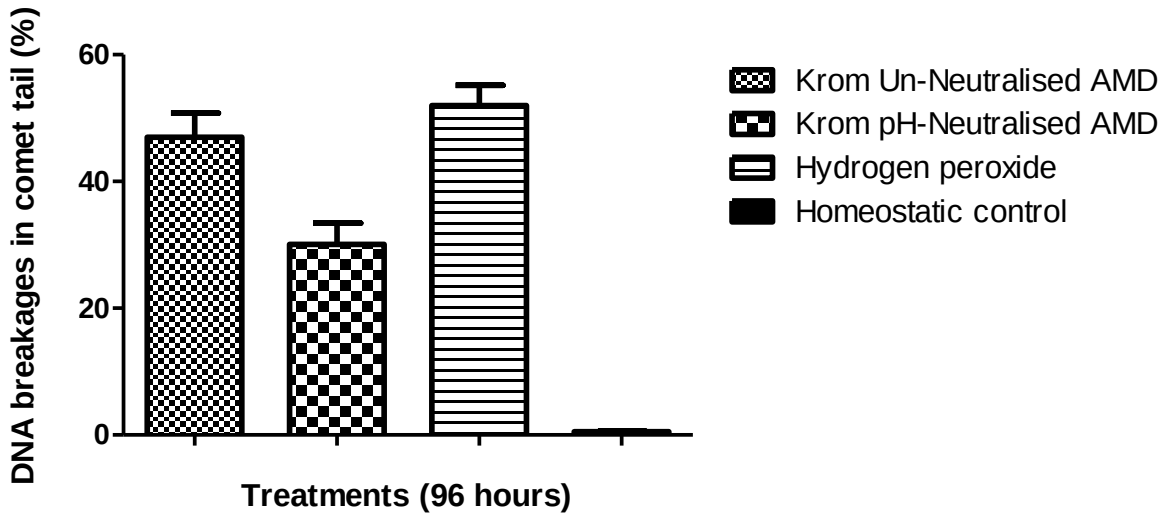


Figure 4.6 Development of DNA breakages in MCF-7 cells exposed to 100% concentrations of un-neutralised and pH-neutralised AMD for 96 hours. Average means of 100 measurements per treatment group is depicted.

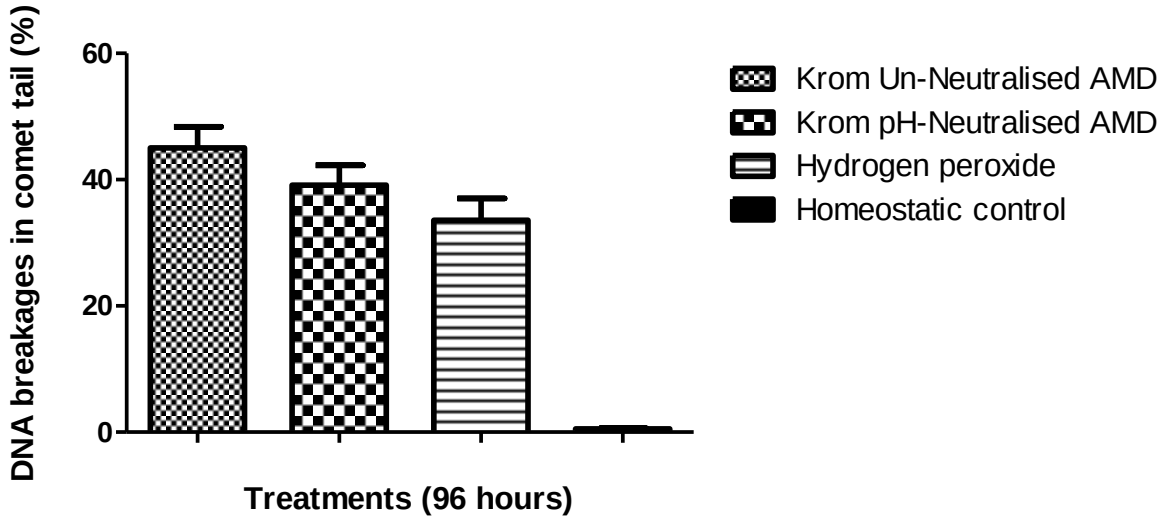


Figure 4.7 Development of DNA breakages in MCF-7 cells exposed to 1.6% concentrations of un-neutralised and pH-neutralised AMD for a duration of 96 hours. The average of 100 measurements per treatment group is depicted.

4.5 DISCUSSION

The accumulation of a cytotoxic effect at its target is facilitated by biological processes that involve absorption, distribution to the location of action, re-absorption and metabolic activation (Shanker, 2008). Any of these integral modes of cellular regulation during metal exposure may contribute to advanced stages of cellular damage and regulatory disturbances (Langman and Kapur, 2006).

Cellular viability of the MCF-7 cell line

Trypan blue exclusion is an effective technique used to monitor cellular mediation in the presence of toxicity (Burow et al., 1998). The survival of cells exposed to a toxicant largely depends on the maintained homeostatic equilibrium between the intra-cellular compartment and its surrounding environment. Once the membrane of a cell is compromised and degraded, quantitative dyes such as Trypan blue can be used to evaluate the degree of cellular stress (Lockshin and Zakeri, 2007). The high-dose exposure of MCF-7 cells to 100% concentrations of un-neutralised and pH-neutralised AMD, established a baseline of high level toxic response (Figure 4.2 A). This study has shown that at a diluted 1.6% concentration of pH-neutralised AMD the MCF-7 survival increases radically, resembling viability similar to control (Figure 4.2 C).

The fragmentation of MCF-7 DNA

In our study, it was essential to determine the mechanism of cellular death elicited by the exposure of MCF-7 cells to undiluted and diluted un-neutralised and pH-neutralised AMD. DNA fragmentation is considered a classic hallmark of the apoptosis and necrosis pathways (Oberhammer et al., 1993). Studies investigating DNA fragmentation have shown that apoptosis activates one or more caspase (cysteine-dependent aspartate-specific proteases)-

activated deoxyribonuclease (CAD) enzymes (Slee et al., 1999). These enzymes promote the breakdown of inter-nucleosomal regions giving rise to the oligo-nucleosome ladder pattern on electrophoretic analysis of multiples of 50-200 base pair (bp) fragments (Vaux, 1993). These fragments are of low molecular weight (LMW) and are consistent biomarkers of an apoptotic pathway (Nagata, 2000) (Figures 4.3 A). A study performed by Oberhammer et al. (1993), reported that DNA fragmentation into LMW fragments does not always occur during apoptosis, resulting in the formation of high molecular weight fragments (HMW). The integrity of the HMW fragments suggests a commitment of cells to either early or late stage apoptosis (Oberhammer et al., 1993). Thus, as observed, MCF-7 cells exposed to 100% concentrations of un-neutralised AMD for 96 hours, exhibits slightly degraded genomic DNA, suggesting a possible activation of a dormant cellular cycle. The apoptotic disruption observed in 100% concentrations of un-neutralised AMD exposures may be a result of cells inappropriately escaping apoptosis and potentially proliferating or becoming dormant. In contrast, MCF-7 cells exposed to 100% concentrations of pH-neutralised AMD for 96 hours indicated highly degraded genomic DNA, suggesting an activated cell death pathway such as either apoptosis or necrosis (Huang et al., 2004) (Figures 4.3 B).

The fluorescent comet assay: Distribution of comet cell types

To gain insight into the mechanism in the activation of signal transduction pathways such as apoptosis and necrosis, it was important to categorise the extent and degree of morphological damage. The morphological classification of cells into comet categories has been perceived as a useful biomarker of cellular death (Collins et al., 1995) (Figure 4.4). The technique incorporates the visual determination of DNA damage based on the migration of tail length (Collins et al., 1995, Tice et al., 2000). In Figure 4.4, comet cells show typical comet cell types as described by Collins et al. (1995). In Figure 4.4, cells display morphological

biomarkers indicative of apoptotic and necrotic cell death. Apoptosis is initiated with the disintegration of nuclear matter and subsequent transport of the fragmented DNA to the membrane in the form of vesicles (Figure 4.4 B, white arrow) (Elmore, 2007). However, in an environment in which cells are exposed to harmful contaminants, cells undergo an aggravated loss of nuclear matter, when regulatory mechanisms are overcome. As a result, nuclear leakage occurs, eventually leading to abrupt necrosis (Figure 4.4 E and F) (Nicholson and Thornberry, 1997).

DNA stranded breakages as an indicator of geno-toxicity

In this study, the toxicity elicited by metal mixtures such as AMD stimulated biochemical events that become apparent at various levels of cellular organisation. The maximum level of damage observed in the study was quantified as % DNA in tail. The quantification of DNA breakages was used as a powerful genotypic biomarker. Percent DNA in tail is a representation of abasic sites. Thus the tail region of a comet cell demonstrates the intensity of single and double stranded DNA breakages (Hayashi et al., 2000). Toxic aberrations elicited on MCF-7 cells exposed to AMD may prompt innate MCF-7 repair mechanisms to overcome genetic instability and restore genetic integrity (Figure 4.4 A). However, in our study it became evident that defective repair mechanisms were at play even at low dose exposures of MCF-7 to 1.6% concentrated un-neutralised and pH-neutralised AMD. In our study, an increase in % DNA in tail was observed in MCF-7 cells exposed to 1.6% concentrated pH-neutralised AMD. This suggests the breakdown of repair mechanisms in the presence of excessive DNA breaks, leaving an abundance of enzymes cleaving away additional bio-molecules (Barnes et al., 1993). An increase in salinity (Table A 1, Appendix A) presents as a possible cause for the elevated DNA breakages observed in MCF-7 exposed to 1.6% concentration of pH-neutralised AMD.

4.6 CONCLUSION

Chemical compounds and heavy metals can damage the DNA of living cells. If not repaired these DNA aberrations can initiate a cascade of biological consequences at the cellular and genetic level of organization (Tseng et al., 1996). In our study, bio-assays evaluating short term impacts of trace metal contamination found in un-neutralised and pH-neutralised AMD have proven useful to screen its toxic potential within human cell lines. This study successfully identified DNA fragmentation and the Comet assay as effective molecular tools to evaluate DNA damage caused by un-neutralised and pH-neutralised AMD. Through the use of these methods, the study was able to identify cellular viability, DNA fragmentation and single-stranded DNA breakages as reliable biomarkers of cytotoxic exposure to un-neutralised and pH-neutralised AMD. The identification of MCF-7 DNA fragmentation in low dosage exposure to a 1.6% concentration of pH-neutralised AMD is cause for major concern and highlights the necessity for evaluating the impacts of AMD. Thus sodium hydroxide pH-neutralisation of AMD have proven ineffective in preventing low levels of DNA breaks that could possibly induce DNA mutations. Technologies that remove trace amounts of metal and sulphates are recommended as an additional system to the current pH-neutralisation techniques. Alternately the study proposes frequent evaluation of DNA damage in human cell lines inherent of known DNA mutations which confer diseases such as cancer.

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APPENDIX

Table A 1. Chemical analysis of un-neutralised and pH-neutralised AMD collected from the Kromdraai River and Kromdraai treatment plant, respectively.

Parameter	**Un-Neutralised AMD (mg/ℓ)**	**pH-Neutralised AMD (mg/ℓ)**
Potassium	6	8
Sodium	28	110
Calcium	450	570
Magnesium	150	170
Aluminium	8.2	0.12
Sulphate as SO_4	2100	2600
Chromium	<0.01	<0.01
Copper	<0.05	<0.05
Iron	0.33	<0.01
Manganese	54	43
Zinc	2.6	0.06
Arsenic	<0.02	<0.02
Mercury	<0.01	<0.01
pH (Lab 20°C)	3.7	7.6
Electrical conductivity (mS/m, 25°C)	325	335*

CHAPTER FIVE

PERSPECTIVES AND RECOMMENDATIONS

5.1 PERSPECTIVES

The *in vitro* exposures of the MCF-7 cell line to un-neutralised and pH-neutralised AMD from gold and coal mining areas revealed extensive DNA damage. A surprising observation was the increased development of MCF-7 DNA breakages at 1.6% diluted exposures of pH-neutralised AMD from both the Tweelopies Stream and Kromdraai River.

The genetic analysis revealed a larger degree of DNA breakages in MCF-7 cells exposed to 1.6% pH-neutralised AMD from the gold mining contaminated Tweelopies Stream (51% DNA damage in Tail region of comet cell) compared to the coal mining contaminated Kromdraai River (39% DNA damage in Tail region of comet cell). This difference in genetic degradation in the two sites may be due to a multitude of factors, namely:

- The neutralisation of the Tweelopies Stream with the limestone reactor bed technology may not be sufficient enough to eliminate trace metals and elements which have DNA oxidising and damaging properties. Thus the sodium hydroxide neutralisation technology utilised for the Kromdraai River may be slightly improved in removing trace metals and/or elements.
- Simultaneously, the study acknowledges the possibility that the metabolism of MCF-7 cells may have been perturbed in exposures to low concentrations of trace metals and ions in the mixtures of pH-neutralised AMD. This trend is possibly observed with the high percentage of % DNA breakages alongside a large proportion of initial

apoptotic cells in MCF-7 cells exposed to 100% and 1.6% concentrations of un-neutralised and pH-neutralised AMD from both sites. This further raises the question of possible mutations which may be conferred to the next cellular generation if repair occurs in this toxic environment.

An unexpected activation of the oncotic pathway was observed in MCF-7 cells exposed to a 1.6% concentration of un-neutralised AMD from the gold mining region (Tweelopies Stream). This finding confirms the difference in the activation of initial factors driving cell death in MCF-7 cells exposed to un-neutralised AMD from the two study sites. This difference may be attributed to the actual content of gold versus coal mining associated AMD.

The levels of DNA breakages in MCF-7 cells exposed to 100% concentrations of un-neutralised AMD from both sites were found to be significantly different (Figure5.1). This could suggest two alternate mechanisms mitigated by un-neutralised AMD from the gold and coal mining regions to inflict DNA damage in MCF-7 cells. Surprisingly, pH-neutralised AMD at from both sites 100% and 1.6% concentrations do not elicit a difference in the level of MCF-7 DNA breakages measured.

Generally, cancer cells show an incredible capacity to adapt to harsh conditions such as in the case of MCF-7 cells exposed to 100% concentrations of un-neutralised and pH-neutralised AMD. In this environment, MCF-7 cells are proliferating in a glucose-free microenvironment. To survive, cells accumulate glycogen to propagate (Wu et al., 2006). It is possible that in a glucose rich environment, as with MCF-7 cells exposed to 1.6% concentrations of un-neutralised and pH-neutralised AMD, MCF-7 cells do not produce as much glycogen to survive. In doing this the “defence’’ mechanism is decreased and trace elements and metals contained within the 1.6% concentrations of un-neutralised and pH-

neutralised AMD are readily taken up by MCF-7 and in turn exerts a harmful oxidative driven DNA damaging mechanism. Regardless of the apoptotic or necrotic mechanisms initiated as a result of MCF-7 exposure to 1.6% concentration of pH-neutralised AMD, this study has shown the undeniably higher cytotoxic and genotoxic impact of remediated AMD from gold and coal mining regions on cellular state and metabolism.

This study presents a first assessment of cyto-genotoxic impacts of un-neutralised and pH-neutralised AMD arising from the gold and coal mining regions of South Africa on the human cell line, MCF-7.

Table 5.1 Describes significant differences in DNA breakages of MCF-7 mitigated by exposures to 100% and 1.6% concentrations of un-neutralised and pH-neutralised AMD from both.

100%	**Bonferroni's Multiple Comparison Test**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
	Un-Neutralised AMD WB vs Krom Un-Neutralised AMD	Yes	*	0.2523 to 23.83
	pH-Neutralised AMD WB vs Krom pH-Neutralised AMD	No	ns	-6.843 to 16.74
1.6%	**Bonferroni's Multiple Comparison Test**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
	Un-Neutralised AMD WB vs Krom Un-Neutralised AMD	No	ns	-2.046 to 23.81
	pH-Neutralised AMD WB vs Krom Un-Neutralised AMD	No	ns	-6.872 to 18.99

5.2 RECOMMENDATIONS

In the current study, the alkaline comet assay was utilised as a bio-monitoring tool in which key mechanisms of MCF-7 response to un-neutralised (Tweelopies and Kromdraai) and pH-neutralised (limestone and sodium hydroxide) AMD were identified. A major advantage of the comet assay is the rapid identification of cells committed to programmed and necrotic cell death through the quantification of DNA breakages. Thus the increase of DNA damage in MCF-7 cells exposed to 1.6% concentrated pH-neutralised AMD from both the Tweelopies and Kromdraai sites is cause for major concern and highlights the necessity for evaluating the efficiency of the sodium hydroxide (Kromdraai River) and limestone (Tweelopies) neutralisation technologies currently utilised.

Further recommendations for this investigation are proposed:

- It is recommended to extend the level of genotoxic monitoring to whole blood samples such as analysing toxicity in lymphocyte populations. These analyses could be performed in aquatic organisms and other vertebrates which inhabit the site of contamination and rely on the surrounding environment for food sources.
- Further inclusion of environmental controls such as non-contaminated water sources could be incorporated to increase our understanding of the impacts of mixtures on cytotoxicity. In addition the study recommends producing controls with metal combinations known to have synergistic or antagonistic relationships.
- Additional parameters such as oxidative cellular stress load and chromosomal aberrations could increase our knowledge of the mutative capacity of un-neutralised and pH-neutralised mixtures.

5.3 LIMITATIONS OF THE STUDY

Experimental studies that can generate important or new data concerning possible biological mechanisms of action have unavoidable limitations when there is a requirement to extrapolate data between high exposure levels used in the laboratory to lower exposure levels to which humans are commonly exposed in the environment. This aspect remains an important challenge to the interpretation and application of experimental data in the assessments of human risk. This study cannot directly extrapolate MCF-7 DNA damage data to possible health risks in humans. However, the findings of this study present a strong illustration of DNA damage at lose dose exposures in the presence of trace metals, particularly in complex mixtures such as acid mine drainage and its remediated counterparts.

Indeed biological methods such as cellular survival determination and the comet assay are attractive due to their high sensitivity and specificity. However, biological methods are not as easily and accurately calibrated as chemistry based methods. Furthermore, it is commonplace to use chemistry based methods to assess environmental damage of water and soil since the empirical date could provide compelling biophysical and biochemical clues to toxicity observed in the surrounding habitat.

The major advantage biological methods; such as the comet assay; has over chemistry based methods is the powerful ability in their detection of oxidative damage to DNA. This by far provides a vital link in uncovering molecular mechanisms through which exposure to harmful contaminant could play a role in disease causation. Thus it was the goal of this study to shed light on the oxidative DNA damaging potentials of un-neutralised and pH-neutralised AMD on a human cell line.