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In vitro bioassays as tools for evaluating toxicity of acidic drainage from a coal mine in Mpumalanga, South Africa

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Thesis submitted in fulfilment of the requirements for the degree

Philosophiae Doctor (PhD)

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Declaration

I declare that this thesis, entitled "***In vitro*** **bioassays as tools for evaluating toxicity of acidic drainage from a coal mine in Mpumalanga, South Africa"** which I hereby submit to the University of Pretoria for the degree Doctor of Philosophy is my own original work, and has never been submitted for any academic award to any other institution of higher learning.

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OLUWAFIKEMI IJI **DATE**

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Dedication

To a cleaner and greener environment

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Abstract

Coal mining and coal utilization in Mpumalanga have increased over the years due to national reliance on coal as a source of power generation. In general, this has caused significant deterioration of water quality wherever streams are impacted by acid mine drainage (AMD). The aim of this research was to assess the use of *in vitro* bioassays as a complement to, or potential future replacement of, waste effluent testing in whole animals from AMD impacted watersheds subjected to passive and active treatment, correlating observed changes with water chemistry analysis. To accomplish this goal, water samples were collected and *in vitro* bioassays carried out to investigate generation of reactive oxygen species by the water samples and cytotoxicity against Vero kidney cells, C3A liver cells and trout RTgill-W1 cells. Primary fish gill cultures were established and used as sensitive *in vitro* models for assessing possible contaminants in water, measuring the induction of cytochrome P450 (CYP) 1A and resultant increase in 7-ethoxyresorufin-o-deethylase activity as a potential biomarker in fish gill cells exposed to polycyclic aromatic hydrocarbons. The genotoxic potential of AMD water on commercially available cell lines was also determined.

The study site was an impacted stream located downstream of a coal mine discharge point whose effluent flowed away from the mine. Water chemistry results suggested high AMD impact evidenced by acidity, elevated sulphates, increased conductivity and presence of heavy metals. Al, Fe, Zn, Mn and Si were the major metals of potential concern in the AMD impacted stream; sulphates and major ions like Ca, K, Na and Mg were present at levels above target water quality range (TWQR) for effluents in receiving stream. The AMD impacted stream caused increased generation of reactive oxygen species (ROS) detectable *in vitro* in selected cell lines (Vero, C3A and RTgill-W1 cell lines), an indication of oxidative stress. In-stream, active treatment with caustic soda was efficient at reducing metal burden, with subsequent reduction in ROS generation in fish gill cell lines. For *in vitro* cytotoxicity tests, passive and active treated AMD water was cytotoxic to cell lines (Vero and RTgill-W1), with the fish RTgill-W1 cells exhibiting greater sensitivity compared to the mammalian Vero cells. Mitochondria played a larger role in observed loss in cellular viability (increased vacuolization, mitochondrial membrane swelling and damage), which was detected using mitochondrial specific stains, and by transmission electron microscopy (TEM). Increased dose- dependent cytotoxicity was observed in the fish gill and mammalian cell lines. Cells exposed to water samples (AMD and reference sites) revealed significant differences ($p <$

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0.05) between the AMD impacted watershed and a relatively pristine site (reference site) where exposure to the same cells maintained approximately 100% viability at all concentrations for up to 72h exposure. The observed differences in effect in this study demonstrate that the effluent from the coal mine negatively impacted surface water quality, resulting in toxicity to cell lines, therefore creating an environment that would not be conducive for the survival of biological aquatic communities and potentially of concern for downstream human end users.

The induction of cytochrome P450 (CYP) 1A and resultant increase in 7- ethoxyresorufin-o-deethylase activity in primary fish gill cultures exposed to polycyclic aromatic hydrocarbons B[a]P, a known AhR agonist contaminant associated with coal mining, showed that there was as increase in EROD activity which was not observed using the RTgill-W1 cell lines. Gill epithelial cells isolated from the gills of Tilapia fish (*Oreochromis mossambicus*) bear close similarities to fish gills *in vivo* and their capacity to respond to the presence of AhR indicates that they may serve as a simple, cost-effect screening tool for assessing PAHs and dioxin-like compounds in fresh water.

For genotoxicity evaluation, the Ames test performed without metabolic activation using bacterium *Salmonella typhimurium* TA98 and TA100 strains revealed no indication of genotoxic activity in any of the water samples. Genotoxicity assessment of all water samples using the comet assay however exposed DNA damage to Vero and RTgill-W1 cell lines. A significant reduction in DNA damage was observed following active treatment. The results suggest that neither treatment technologies employed were efficient at removing all potential genotoxicants so further improvements are required. The comet assay proved sensitive enough to detect genotoxicity in reference water samples despite no known untoward effluent inputs at the site, suggesting potential for this assay to be integrated into an environmental monitoring framework.

The results obtained support the use of *in vitro* bioassays for evaluating toxicity of industrial effluent through biological responses in test systems elicited following exposure, improving ability to detect AMD polluted water. This could be beneficial when assessing the degree and extent of impact of AMD in natural water sources, and the possible environmental impact resulting from hazardous elements present in effluent water. In conclusion, these results suggest that *in vitro* techniques involving cell lines and primary cultures from fish may serve

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as simple, rapid and cost-effective tools for assessing risk and potential toxic effects of contaminants in AMD waters.

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Articles prepared from thesis

1. **Iji OT, Serem JC, Bester MJ, Venter EA, Jan G. Myburgh JG, McGaw LJ.** Generation of reactive oxygen species in species relevant cell lines as a bio-indicator of the safety of treated acid mine water. Manuscript submitted to Water SA (Chapter 4)
2. **Iji OT, Myburgh JG, McGaw LJ.** Assessing the potential of cell lines as tools for the cytotoxicity testing of acid mine drainage effluent impacting a natural water resource. Manuscript prepared for submission to Environmental Pollution and Research (Chapter 5)
3. **Iji OT, Myburgh JG, McGaw LJ**. Induction of 7-ethoxyresorufin-*o*-deethylase activity by B[a]P in primary culture of gill epithelial cells from Tilapia (*Oreochromis mossambicus).* Manuscript prepared for submission to Global Veterinaria. (Chapter 6)
4. **Iji OT, Mfotie Njoya E, Madikizela B, Myburgh JG, McGaw LJ.** Evaluation of the genotoxic potential of water impacted by acid mine drainage from a coal mine in the Mpumalanga Province of South Africa using an *in vitro* comet assay and the Ames test. Manuscript prepared for submission to Environmental Toxicology and Pharmacology (Chapter 7)

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Conference presentations

1. **Iji OT, Serem JC, Bester MJ, Venter EA, Myburgh JG, McGaw LJ.** 2015**.** Poster presentation: Generation of reactive oxygen species in species relevant cell lines as a bio-indicator of the safety of treated acid mine water. Europe 25th Annual Meeting of the Society of Environmental Toxicology and Chemistry Conference, Catalonia, Spain. 3-7 May 2015.
2. **Iji OT, Venter EA, Myburgh JG, McGaw LJ.** 2015**.** Poster presentation: Assessing the potential of cell lines as tools for the cytotoxicity testing of acid mine drainage effluent impacting a natural water resource. 7th Society of Environmental Toxicology and Chemistry Africa Conference, Langebaan, Capetown.

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CHAPTER 1: Introduction

1.1 Background information

Power generation in South Africa is largely based on coal-fired power stations with 83% of the total coal mined within the Mpumalanga Province (Mangena and Brent, 2006). Historically, exploitation of coal in the Mpumalanga fields started in the early 1900s in the Witbank (now eMalahleni) area, but gained momentum in the 1970s with the construction of many coal fired power stations. There are over 10 000 km^2 of hydraulically interlinked coal mines in the Mpumalanga Province alone (Vureen, 2009). Mining operations have since affected water quality within the Olifants River catchment area, where runoff and drainage from mines have contaminated streams due to the presence of heavy metals and acidity, raising potential environmental concerns, one of which is acid mine drainage (AMD) (Hodgson and Krantz, 1998).

In order to adequately control water pollution and ensure acceptable water quality, knowledge of the effects of pollutants upon the aquatic environment is required. Physical and chemical analysis of water quality furnishes information on the condition of the stream, but does not indicate the cumulative effects on the biota. Currently, commonly used methods for investigating AMD impacted streams involve the use of macro-invertebrates (Jarvis and Young, 2000; Rosenberg and Resh, 1993) and fish as bioindicators in acute toxicity tests by identifying the concentration of contaminants that cause mortality (OECD, 1992; 1995). These processes involve extended, costly and laborious experiments that take a long time before results are available for interpretation. In South Africa, the Department of Water Affairs and Forestry (DWAF) has been collecting data from the 1970s on the physico-chemical characteristics of the country's water resources, but biological data is lacking, and there is no preference yet for a specific AMD screening tool. An approach to characterize the relative importance of AMD toxicity using *in vitro* techniques such as cellular bioassays to detect acute toxicity from AMD contaminants in conjunction with an environmental water quality surveillance system is needed. This could be innovative in terms of assessing anthropogenic changes in aquatic ecosystems, thereby providing crucial inferences about the health of an AMD-impacted stream and helping policy makers decide which level of change is acceptable.

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In vitro cellular responses (cytotoxicity, oxidative stress, EROD induction) may be correlated with analytical data of water quality, as functional and structural changes occur between pollutants at cellular levels that would interfere with basic cellular functions within the organism (Larsson et al, 1985). Poor water quality will clearly not sustain life and would produce lethal alterations *in vitro* and *in vivo*. This study sought to determine how the concept of cellular responses/toxicity could be applied to evaluate water sources impacted by AMD effluent from a coalmine.

1.2 Properties of Acid Mine Drainage

AMD may have one or more of four complex mixtures of minerals with properties such as high acidity (pH between 2.0 and 4.5), high metal concentrations, elevated sulphate levels and excessive suspended solids and/or siltation, which result in toxicity (Campbell and Stokes 1985). Coal contains pyrites, and wherever pyritic formations become exposed to air and water, acid sulphate is formed, and acid mine water is produced. South Africa's coal contains a considerable amount of pyrites (Thompson, 1980), and hence the oxidation of sulphide minerals, typically iron pyrite or iron di-sulfide (FeS_2) produces AMD (Gray, 1998). AMD impacts the biological, chemical and physical components of the ecosystem and has been identified as the single most significant threat to South Africa's environment, exerting pressure on the fresh water systems and resources (Younger, 2001). The heavy metal pollution of surface waters is worrisome, as an assortment of issues arises, from toxicity to drinking water to food chain disruption, including other biological problems related to pH imbalance and disruption in growth and reproductive patterns of aquatic organisms (Fostner and Whittmann 1981; Lindegaard, 1995). Additionally, coal utilization is associated with polycyclic aromatic hydrocarbons (Guerin, 1978; Ahrens and Morrisey, 2005), which are problematic in the environment due to persistence. There are concerns about the harmful effects of PAHs acting as endocrine disruptors in humans and wildlife and as suspected potential carcinogens (Harrison et al., 1995; Kelce et al., 1995; Kavlock, 1996; Zakaria et al., 2002).

A variety of AMD treatment processes are currently used, one of which involves the use of wetlands as a passive method to treat AMD effluent and reports suggest that they are effective at improving water quality (USDA and EPA, 2002). Active treatment may involve the use of alkali to correct water pH and precipitate metals, which may give the appearance

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that the effluents are non-toxic, before being discharged into receiving streams (Akcil and Koldas, 2006). Occasionally, treatment technologies may become insufficient or uneconomical to maintain, such that a considerable amount of untreated AMD is left, allowing persistence of elevated metal levels and acidity (Diz, 1997). One way to evaluate the efficacy of such systems is by physical and chemical monitoring of the quality of the effluent following treatment, in conjunction with biological effect assessment, because environmental changes on a short-term and on a long-term basis may be detected though bioassays (Burgman and Lindemayer 1998; Mota Marques et al., 2000; Ji et al., 2004; Kelly et al., 2006).

1.3 Problem statement and motivation

Important deductions about the health of a stream can be made by assessing whether change has occurred and to what extent (Chapman et al., 1995). Chemical analyses of water columns only provide an insight into the stream conditions, leaving out the cumulative effects of contaminants such as AMD, thus failing to identify other detrimental disturbances (Karr and Chu, 1999; Chapman, 2007). This deficit may be overcome by combining water chemistry analysis with *in vitro*-based bioassays in order to evaluate contaminant risk and hazards in the environment for possible toxicity. Observed changes could then be correlated with water chemistry parameters that can help target problem areas and monitor recovery.

By convention, the impact of contaminants on aquatic environment is assessed through the use of a single species (e.g. use of fish such as fathead minnow, zebra fish, and rainbow trout), or small invertebrates (*Daphnia magna*), bacteria, and algae (Fentem and Balls, 1993). The Organization for Economic Cooperation and Development (OECD, 1992) targets the endpoint of *in vivo* testing which is aimed at identifying the concentration lethal to 50% of the exposed fish, referred to as LC_{50}. These laborious, expensive and extensive bioassay protocols have the limitation of differences in sensitivity of single organisms to environmental contaminants (Cairns, 1986; Chapman, 1981). Other factors like the life stage, nutritional status, species behavior, and food preference, may influence species' sensitivity to toxic agents (Mothersill and Austin, 2003). Lethality, which is usually an endpoint, is a matter of concern and hence there is a need to promote alternative techniques, which have become widely explored in environmental health research. These alternative techniques which comprise *in vitro* or laboratory based methods, satisfy the 3R's (reduce, replace and

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refine) in current research and toxicological studies involving wastewater effluent testing, by providing an alternative to whole animal testing, since large numbers of samples can be screened rapidly at a reasonable cost and fewer laboratory animals are used. Its use in investigating and monitoring of AMD impact in receiving streams helps detect fluctuations in water quality that may have been missed by periodic chemical analyses alone, as well as detecting biological responses within a given period as it is impracticable to obtain a complete profile of all potential toxicants in water columns at all times.

1.4 Hypothesis

The research question may be stated as follows: are *in vitro* cellular responses in cell lines useful to investigate and study AMD toxicity?

1.5 Study aim and objectives

The aim of this study was to develop an *in vitro* based monitoring system for assessing toxicity associated with AMD effluent from coal mines.

The following objectives were undertaken to achieve the aim of this study:

1. Investigate the generation of reactive oxygen species (an indicator of oxidative stress) in species relevant cell lines as a bio-indicator of the safety of treated acid mine water.
2. Determine, *in vitro*, cytotoxic end-points of bioavailable water contaminants in cell lines exposed to AMD, to determine if cytotoxic responses correlate strongly with water chemical parameters in streams.
3. Establish and validate primary fish gill cultures in freshwater fish (*Oreochromis mossambicus*) as an *in vitro* toxicity monitoring system using specific endpoints such as CYP1A induction as biomarker of exposure to environmental contaminants for potential toxicity evaluation of environmental samples.
4. Determine the genotoxic potential of impacted stream by AMD effluent using the comet assay and the Ames test in human and fish cell lines.

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1.6 Thesis structure

Chapter 1

Chapter one describes the background and motivation for the research; it addresses the problem statement and provides a general overview of AMD as well as the goals and objectives of the research.

Chapter 2

This chapter provides a general insight into water resources in South Africa and water quality. It highlights the economic and domestic importance of coal mining and its environmental toll. It also describes monitoring and bio monitoring of South African aquatic systems, the economic, ecological and environmental importance of AMD, and the chemistry and formation of AMD along with currently available treatment options.

Chapter 3

Chapter three describes the main environmental characteristics of the study sites in terms of the location, geography and mining history. It also describes the water chemistry analysis techniques employed and incorporates a discussion of the water chemistry results.

Chapter 4

This chapter describes the use of reactive oxygen species generation (an index of oxidative stress) in species relevant cell lines (mammalian cell lines and a fish gill cell line) as a monitoring tool for treated and untreated AMD water. This study showed the potential use of a simple technique involving a dye, DCFH-DA, for rapid biomonitoring of the quality of treated AMD water relating to the formation of ROS and subsequent cellular effects (Objective 1).

Chapter 5

The *in vitro* cellular toxicity of AMD water samples to mammalian cell lines and a fish gill cell line are assessed in this section. *In vitro* cytotoxicity, which usually precedes *in vivo* lethality, was examined. Cell viability was determined using the tetrazolium-based colorimetric (MTT) assay and the neutral red viability test. Ultrastructural changes following exposure were assessed by TEM and finally, efforts to correlate toxicity with changes in the

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physicochemical parameters of polluted water versus clean water were made (Objective 2).

Chapter 6

Chapter six addresses the need to assess biological responses in primary cultures arising from fish gills because these cultures bear closer similarities than continuous, transformed commercial cell lines to *in vivo* systems for possible detection of contaminants present in water samples. Primary gill cultures from fish *(Oreochromis mossambicus)* were established and validated for their ability to act as an *in vitro* toxicity monitoring system for contaminants such as benzo(a)pyrene, a polycyclic aromatic hydrocarbon, which is commonly associated with coal utilization (Objective 3).

Chapter 7

This chapter evaluates the possibility of using a mammalian (Vero monkey kidney cell line) and a fish gill cell line (RTgill-W1) in assessing the genotoxicity of water samples from an AMD impacted stream, following passive treatment (a wetland) and active treatment by conventional physical and chemical processes (Objective 4).

Chapter 8

This section comprises the General Discussion and Conclusions, summarizing the main results concluded from the research and providing future research perspectives.

Chapter 9

References.

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CHAPTER 2: Literature review

2.1 Drivers of change in aquatic ecosystem

A number of factors are responsible for changes observed in the aquatic ecosystem. Such factors may be physical (light and temperature), chemical (organic and inorganic, nutrients), environmental or biological. These factors may influence ecosystem health, occurring at different rates. Generally, rivers and streams undergo fairly predictable daily and seasonal changes in physical chemistry and biota (Townsend and Riley, 1999). One of the most disturbing drivers of change in the aquatic ecosystem is human activity. Globally, humans play significant roles in shaping and disturbing stream ecosystems (Resh and Grodhaus, 1983; Petersen et al., 1987; Younger, 2001). These actions vary from construction of dams, to major earth movement or land clearance, deforestation, introduction of exotic species, and pollution from industrial, urban, agricultural and mining sources. These human-related stressors could negatively impact on aquatic ecosystems and modify their 'health'. The ability of the aquatic ecosystem to cope with stressors depends largely on its resilience. It becomes more challenging when the object of stress is anthropogenically induced. Severe disturbances may result in changes that alter the river's biota, thus affecting the entire biological context of the river, which may then become irreversible, when the natural ability of the river to withstand such stress has been exceeded.

When changes are naturally induced and are within an aquatic ecosystem's evolutionary experience, the ecosystem retains its capacity to maintain its self-organizing processes and does not become transposed to an alternative stable state (Holling, 1973; Gunderson et al., 2002). Aquatic ecosystems, nevertheless, display more sensitivity to changes related to human activities (Bornette et al., 1998; Ward, 1998). The degree to which a natural ecosystem is sustainable, as it withstands human impact while maintaining its ecological structure and function over time, is termed "stream health" (Scrimgeour and Wicklum, 1996). Stream health can be determined by measuring the biological condition and assessing if change has occurred and to what extent. Not all contaminants are pollutants, but all pollutants are contaminants, and in order to determine when a contaminant becomes a pollutant, biological measurements are required in addition to chemical measurements (Chapman et al., 2003).

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2.2 Coal mining in South Africa

South Africa is the world's sixth largest coal producer, generating approximately 220 Mt coal per year (DME, 2004). Coal is produced when huge deposits of vegetative material undergo geochemical processes known as coalification. Coal is made up primarily of organic elements (e.g. C, H) and inorganic elements (e.g., Al, Fe, Ca, Mg, Na, K, and S), and it also contains trace elements (including As, Be, Cd, Co, Cr, Hg, Mn, Ni, Pb, Sb, and Se) (U.S. EPA, 1982; U.S. EPA, 1983). Coal may be mined either at the surface or underground. This is determined by the location of the coal relative to the surface. The country's coal mining industry is the second largest mining sector after gold, with coal supplying 55% of the country's total domestic power needs (Tshwete et al., 2006). There is sustained national reliance on coal-based energy production and of the 450 million tons of waste generated annually, the mining industry is responsible for 70%. This takes its toll on land usage, surface and underground water quality (AngloGold Ashanti Annual Report, 2004).

Historically, coal mining has played a significant role within the Upper Olifants River catchment area of Mpumalanga, resulting in the disposal of mine wastewater that is freely discharged into the natural environment which constitutes an ecological disaster. As a country challenged by a water security dilemma, along with an aggressive mining industry, extensive ecological damage seems inevitable.

2.3 Acid mine drainage as a global problem

There is no doubt that mining and its environmental impact has caught global attention, with the continued drive and awareness in research (Warhurst and Noronha, 2000). The exposed surface area of sulfur-bearing rocks is increased during mining, and this causes the generation of excess acid beyond the natural buffering capabilities found in host rock and water resources. AMD has become a universal environmental problem that adversely affects both surface and ground waters. This is characterized by the formation of several soluble iron sulphates, the production of acidity due to the presence of hydrogen ions, and subsequent metal dissolution and leakage, following the oxidation and hydrolysis of metal sulphides (pyrites) in water permeable strata. AMD, as a complex pollutant, has a high concentration of iron and sulphate, a low pH and an assortment of elevated concentrations of a variety of

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metals (Parsons, 1977; Gray, 1997; Kelly, 1988; Heath and Eksteen, 2005). AMD is not only a problem emanating from active, abandoned and liquidated mines: waste rocks and mine tailings are prone to generating more AMD (Younger and Robins, 2002). If not mitigated, the environmental and ecological, human health and economic consequences of AMD have long-lasting effects. A number of factors are responsible for controlling AMD; first is the production of the acids (the oxidation reaction involving the metal sulphides), the second is the control of the products resulting from the oxidation reactions (which may react with other minerals or potentially neutralize the acids) and lastly, factors that are associated with managing the waste generated (Ferguson and Erickson, 1988), as effective waste management is crucial to reducing environmental pollution.

Worldwide, mine effluents affect an estimated 19 300 km of streams and river and 72 000 ha of lakes and reservoirs causing serious damage (Johnson and Hallberg, 2005). Management options in Alaska involve water treatment measures that encompass physical and chemical methods. Neutralization methods using lime or other suitable alkaline agents are the preferred choice of chemical treatment method employed. Other methods incorporate the use of suitable wetlands and bioreactors that aim to reduce surface bacteria, allowing for precipitation of metals as metal sulfides (NSCEP, 2006).

In New Zealand and Australia, effluent water quality is regulated by State and Federal Government legislation and discharged water treated to meet effluent limits using chemical precipitation methods and/or reverse osmosis (NWQMS, 2000).

In South Africa, legislation and policies relevant to water resources and the mining industry is vested in the Water Act of 1956, later amended as the Water Amendment Act, 1984 (Act 96 of 1984). This law broadened water quality management, in that industrial effluent and sources other than effluent were subjected to pollution control regulations.

The DWAF overseers and ensures continued adequate water supplies of acceptable quality for all recognized users defined in the Water Act 1956 (Act 54 of 1956) which are: domestic, industrial, agricultural, environmental and recreational. The DWAF shifted from the Uniform Effluent Standards approach to water pollution control to enforce the General and Special effluent standards and later, due to continuing deterioration water quality, the Receiving Water Quality Objectives approach for non-hazardous substances and the Pollution Prevention approach for hazardous substances were adopted. This embodies water quality

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management policy aspects of the anticipatory or precautionary principle to environmental protection in order to avert danger and minimize risk to the environment (Fulles et al., 1996 Literature review: Policies and legislation applicable to mine water)

2.3.1 Economic importance of AMD

Mining activities and the resultant AMD generation usually have widespread impacts on various aspects of the natural environment. With significant environmental and socio-economic impacts arising from AMD pollution, the complications associated with mining waste are rated second only to global warming, because of the potential irreversible destruction of ecosystems and associated ecological risk (EEB, 2000). The cost of mitigation of environmental damage from AMD is huge and pollution has persisted even from mines established in Europe during the Roman Empire prior to 467 AD (CSS, 2002). In the Canadian mining industry, an estimated $2 to $5 billion dollars' environmental liability cost due to AMD was proposed (MEND, 2001), as the effects of AMD encompass land and water. In the US, negatively impacted streams from an estimated 20 000 to 50 000 mines stretch for about 8 000 to 16 000 km (USDA, Forest Service 1993). It is estimated that over $15 billion will be needed for the reclamation of watersheds affected by AMD in Pennsylvania (Rossman et al., 1997).

In South Africa, AMD constitutes immense environmental liabilities for the South African Government, especially from closed and abandoned coalmines (Adler et al., 2007). The Department of Water Affairs and Forestry (DWAF) has spent over US$20 million in the last decade, investigating and cleaning up historic pollution (Schwab, 2002). The actual damage cost (calculated cost per environmental impact) on the environment is difficult to project as the amount and concentrations of pollution and waste generated by this exploitation may incur external costs to agents other than the mining industry.

2.3.2 Environmental/ecological importance of AMD

Discharge of acid mine water from either ongoing mining activities or from abandoned mining areas perturbs the integrity of freshwater ecosystems, fisheries, human health, and local rural economies (Feasby and Jones, 1994; Pulles et al., 2005). The resulting toxicity is related to a combination of factors often difficult to separate, including pH alone, increased

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bioavailability of metals at low pH and the precipitation of metal hydroxides (Campbell and Stokes, 1985).

In water, fish may become exposed to metals through their gills or by ingesting contaminated sediments and food, causing toxicity and impairing respiration (Hare, 1992). Metals can adversely affect reproduction rates and life spans of aquatic organisms, bringing about the disruption of the food chain in aquatic environments, reducing the quality of water, as well as bio-accumulating in tissues of plants, macro-invertebrates and fish (Lindegaard, 1995; Heath, 2001). Elevated metals can be so toxic to aquatic organisms that streams become barren (Kimmel, 1983). In some cases, tolerant species replace sensitive ones to compensate for biodiversity loss at low pH (Gerhardt et al., 2004). The degree of toxicity arising from AMD observed in aquatic organisms is influenced by metal speciation, pH, hardness, uptake site, previous exposure, and differences between affected species (Gerhardt, 1993). Acidity has been reported to mobilize metals, thereby making the metals more soluble and toxic to aquatic organisms in coalmine drainage (U.S. EPA, 2005). Low pH conditions change gill membranes or the mucous that coats the gills, causing a decrease in oxygen, which can result in death due to hypoxia (Howells, 1983; Playle and Wood, 1989). Levels of pH below 5.0 may impair osmotic and homeostatic electrolyte mechanisms (Fromm, 1980).

Further environmental impacts include coating of the surface of streams and streambeds which destroys habitat, diminishes availability of clean beds for spawning, and reduces fish food items such as benthic macro-invertebrates (Koryak et al, 1972; Scullion and Edwards, 1980). It also diminishes aesthetic value and recreational use (Ahmad, 1991). Severe degradation of soil quality and aquatic habitats resulting from AMD pollution to the environment has been reported (Adler and Rascher, 2007). Environmental damage from AMD pollution can persist for a long time after mine closure, compromising the health and safety of nearby communities. This is because water quality emanating from mine dumps may be harmful, usually far below what national quality standards permit, and may render in-contact surface water constituents such as streams completely sterile (U.S.EPA, 1994; DEAT, 2006). A case in point is the acid mine water seeping into Blesbokspruit catchment in the Witbank Coalfield of South Africa which was reported to have a pH of 2.8 and a sulphate concentration of 1440 mg/L, killing all vegetation in a 3 hectare area (Bell et al., 2002). Younger (1997) referred to AMD as the single greatest source of fresh water pollution. The

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report from Gitari et al. (2009) highlighted the potential danger of drinking water contaminated by AMD in eMalahleni, Mpumalanga Province of South Africa. Acid mine water discharge can also result in localized flooding, foundation weakening, ground deformation, corrosion of pipes and clogging, which could have devastating consequences (Dumpleton et al., 2001).

2.4 Chemistry and formation of AMD

A chain of complex geochemical and biological reactions leads to the formation of AMD. When sulphide minerals in rocks become exposed to oxidizing conditions such as those seen in the process of mining, or where there has been a major earth movement (Durkin and Herrmann, 1994). Of all the sulphide minerals, iron sulphides are the most prominent (Skousen et al, 1993).

1) $FeS_2 + 7/2\ O_2 + H_2O \rightarrow Fe^{+2} + 2\ SO4^{-2} + 2\ H^+$
2) $Fe^{+2} + ¼\ O_2 + H^+ \rightarrow Fe^{+3} + ½\ H_2O$
3) $Fe^{+3} + 3\ H_2O \rightarrow Fe\ (OH)_3 + 3\ H^+$
4) $FeS_2 + 14\ Fe^{+3} + 8\ H_2O \rightarrow 15\ Fe^{+2} + 2\ SO4^{-2} + 16\ H^+$

Figure 2.1: Equations for the formation of acid mine drainage (from Wildeman et al., 1993).

In the first reaction shown in Figure 2.1, the sulphur is oxidized to sulphate and ferrous ion is released. The second equation, which is pH dependent, involves the conversion of ferrous ion to ferric ion. This reaction is thought to progress faster at a pH of nearly 5. In the third reaction, ferric ion undergoes hydrolysis to produce ferric hydroxide; a yellow precipitate referred to as ‘yellow boy’ ensues, which usually lines the streambed and gives it its characteristic orange-yellow color.

The last reaction involves the oxidation of additional pyrites by ferric iron until the pyrite is decreased. This reaction further worsens the acidity and more H^+ is produced. Acidophilic microorganisms such as *Thiobacillus* sp., *Gallionella ferruginea*, *Thiothrix* sp.,

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Metallogenium sp., and *Leptothrix discophora* (Overly, 1997; Lopez et al., 1999) found in AMD waters can act as natural catalysts for many of the oxidation reactions involved in AMD formation, causing AMD formation to progress at a faster rate (Wildeman et al., 1993).

2.5 Treatment technologies for AMD

To minimize or prevent pollution of water resources, mine water is usually treated to a degree that makes it acceptable for release into natural water resources. There are a number of different treatment technologies that may be considered, incorporating passive and active treatment, and this targets elevated metal levels and acidity which are common outcomes of AMD. Factors such as site characteristics and the treatment methods employed have an impact on the effectiveness of the water treatment. The passive treatment system is mostly employed for abandoned or liquidated mines because they are relatively inexpensive to build and require minimal maintenance, making it more cost effective. Passive treatments usually involve wetlands that operate without the need for chemical adjustments or mechanical assistance and are usually designed to perform optimally at lower flow rates. The majority of wetlands support the growth of vascular plants which in turn reduces the rate of flow of water and provides microenvironments within the water column, as well as supplementary sites for establishment of microbial communities (USDA and EPA, 2002). Although this system has proven to be effective at improving water quality, it has to be properly designed for the flow rate and chemistry of the discharge, so that water quality becomes suitable enough for discharge into a receiving stream (Hedin et al, 1994; Milavec, 2000).

On the other hand, active water treatment systems involve highly planned water treatment facilities which involve manipulation of acid mine water in order to achieve water quality standards stipulated in a discharge permit. Active treatment produces better water quality with an improved degree of certainty over passive treatment. According to the Best Practice Guidelines for Water Resources in SA Mining Industry BPG H4: Water treatment (2007), the active system involves chemical or biological processes such as:

- pH adjustment. A base reagent is added to acidic (low pH) mine waste water for neutralization. Lime ($Ca(OH)_2$), caustic soda (NaOH) and limestone ($CaCo_3$) can be added to acidic mine water to raise the pH to 7 or higher (Van Staden, 1979).

- Metal precipitation. A base reagent is added to acidic, metal-bearing mine waste

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waters to precipitate metals as metal hydroxides that are insoluble.

- Biological based treatments (sulphate reduction/removal). This process involves adding chemicals such as barium chloride/hydroxide/oxide to sulphate-rich mine wastewaters to promote the chemical precipitation of sulphate from the water after lime neutralization.

- Ion exchange. This involves a purification process of water or the recovery of a valued component from solution. This process involves exchange of unwanted ions or potentially harmful ones to a harmless ion (i.e. H^+ and OH^-) from the solid to the solution and vice versa.

- Membrane processes (reverse osmosis and electrodialysis reversal). This process aims to separate and remove molecules from solution. This is achieved by driving the solvent/water through a semi-permeable membrane, thereby separating and removing the solutes from the solution e.g. removal of salts through reverse osmosis (RO); desalination.

- Adsorption treatments (used mostly for sludge treatment). The process involves collecting or accumulating molecules at a surface/interface where they can be removed. The constituents are attracted to the surface of the adsorbing substance and held there by weak reversible forces such as van der Waals forces. Other processes involve electrochemical treatment technologies and physical process technology (e.g. gravity settling, filtration, evaporation) etc.

In spite of the fact that the chemical mechanism of AMD is well understood, predicting its ecological impact or accurately assessing the situation still poses some challenges due to the complexity of the processes involved, because environmental factors such as climatic, hydrological, geomorphic, geological and biological conditions influence each outcome.

2.6 South African water resources and water quality

South Africa is a relatively dry country, having a low and variable rainfall below the world average with 66% of the country being semi-arid to arid; it is consequently regarded as a water scarce country (Kriel, 1975; DWAF, 1993, DWAF, 1996). Water is a valuable and limited natural resource that justifies the need for better conservation and monitoring, in

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order to achieve vital water quality levels. South African water resources and quality are experiencing challenging circumstances with respect to mining (Owens and Burke, 2009). This has led to deterioration in the quality of water in many surface streams, as water pollution results from AMD (Thompson, 1980; Koldas, 2000).

The general health of an aquatic environment is usually assessed based on the water quality, which denotes the biological and chemical constituents as well as physical variables in the river. Water may become negatively impacted such that its quality is compromised, thus making it unsuitable for use. The South African Department of Water Affairs and Forestry through the Water Act of 1956, and the Water Amendment Act, 1998 (Act 36 of 1998) embarked on policies aimed at re-evaluating and developing better water quality management. This was done to make available adequate water supplies of acceptable quality for domestic, industrial, agricultural, environmental and recreational use. It is therefore of utmost importance to achieve quality of a natural water resource appropriate to that of its intended use, geared towards protecting all forms of aquatic life and life cycle.

The major issue facing water quality is water utilization by industries, especially the mining industry whose activities result in water pollution (Gouldie, 1993). Grobbelaar et al. (2000) reported deterioration in water quality in streams in South Africa over the last 20 years due to mining, and of particular relevance is the coal mining industry where seepage and discharges arising from coal mines have made their way into water bodies. This has affected the overall state of impacted rivers or streams and underground aquifers because they contain heavy metals, which is one of the primary causes of toxic pollution and serious water quality deterioration. These challenges therefore necessitate effective monitoring to improve and solve the water quality situation. While a number of studies have been conducted on AMD in the developed world, information in literature available for Asia, Africa and South America is still relatively scarce (Johnson et al, 2001; Harris et al, 2003; Sracek et al, 2004).

With regard to pollution, aquatic ecosystems are the most vulnerable as they act as sinks for chemicals from surrounding terrestrial ecosystems. Aquatic organisms become submerged in "various exposure medium" within their habitat (Clements and Newman, 2002). Many investigations of AMD impacted streams and rivers have made use of one or more biological indices (Jarvis and Young, 2000). Currently in South Africa, no specific AMD screening tool

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is favored and none exists which employs both physicochemical and bio-indicator parameters in conjunction.

2.7 Monitoring and biomonitoring of South African aquatic systems

One tool for identifying and assessing biological condition and change in aquatic ecosystems is using biological monitoring. Biomarkers provide a more detailed assessment of pollution, as they demonstrate the effects on abiotic and biotic components (Bayne et al., 1988). Biomarkers give an indication of an ecosystem's health and the robustness of resident organisms (Stageman et al., 1992). They are useful tools for identifying and assessing biological condition and change in aquatic ecosystems, and have been used for quality environmental monitoring through measurement of direct mortalities to detecting sub-cellular injury following exposure to pollutants (Di Giulio and Benson, 2002). Biological monitoring focuses on monitoring the aquatic community rather than the physico-chemical environment and is a more appropriate health indicator of the water system (Cox, 1991), as it provides an opportunity to detect changes in water quality which may have been missed by periodic chemical analyses alone.

Of particular interest in aquatic ecosystem health assessment is evaluating the 'integrity' of the system in comparison with relatively pristine sites (reference sites) that have experienced very little human interference and are shaped mostly by evolutionary and biogeographic forces. This approach uses the least disturbed streams to establish one end of the scale of reference and the highly disturbed sites to establish the other end of a scale (Wright, 1995).

The Department of Water Affairs and Forestry previously known as the Department of Water affairs in its review of water quality management policies, adopted the receiving water quality based approach for its non-hazardous substances and a prevention based approach for its hazardous substances, different from previous regulations where all effluent was only required to meet a general or special effluent standard (Van der Merwe and Gobler 1990). This change in policy allowed for limits in levels of pollutants to be based on the water quality requirements of each user in a particular water system, an example being the aquatic organisms as users in a stream or river.

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Although monitoring of South African aquatic ecosystems has been done by assessment of water chemistry, the general consensus is that measuring the physical and chemical components of water cannot exclusively supply information about the health of an aquatic system (Lawrence and Williams, 1991; Ten Brink and Woudstra, 1991). This is so because measuring the levels of chemical substances in the environment is no indication that chemical pollution has occurred, and neither is the extent and significance of such pollution alone or relative to other stressors determined simply by measuring the levels of chemical substances in the environment (Ludwig and Iannuzzi, 2005). Such measurements furnish information on substances and the concentrations at which they occur, but lack information on their ability to cause adverse biological effects in the natural environment. Therefore, contemporary measures in ecosystem health assessment are directed towards monitoring the biological components of the ecosystem (Breen, 1996), so as to compensate for shortcomings of chemical monitoring that underestimate or even fail to detect some kind of environmental degradation that may impair biological health (Karr, 1991; Planas, 1996). Since biomonitoring is becoming the primary tool used in evaluating compliance to effluent limits (Herricks and Schaeffer, 1985), in order to adequately protect aquatic life, it could be incorporated into management policies (Metcalfe-Smith, 1991).

From the 1970s the DWAF has been collecting data on the physico-chemical characteristics of the country's water resources, but still lacks biological data. For example the physico-chemical water parameters generated by the Department of Water and Sanitation from 2002 to 2014, at one of the collection points in the study site selected for the current research following active treatment (Figure 2.2) showed values that were in most cases above TWQR. An increase in major ions such as Mg, Na, Ca and sulphate were observed, displayed by the muacha key to the left. An average mean value for EC was at 143 mS/m and 968 mg/l for TDS respectively. Variation in pH was also observed, being highest at 8.03 and lowest at 2.96 suggestive of AMD impact. The only metals analyzed by the Department were Al and Si, whose mean average values were 20.3 mg/L and 2.87mg/L respectively.

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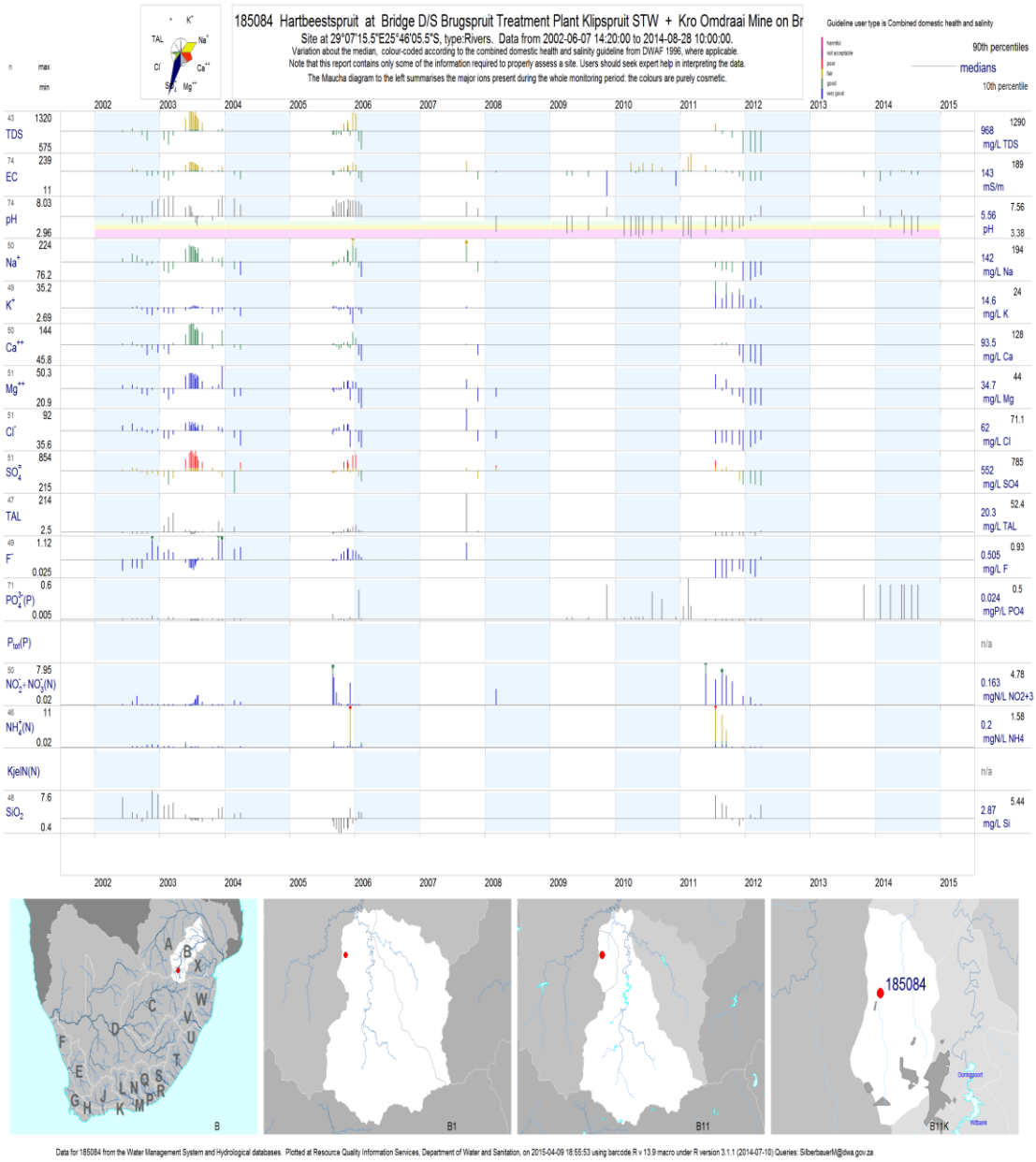
Fig 2.2: Physical and chemical water quality data collected by the Department of Water and Sanitation from 2002 to 2014.



The South African National Water Act (Act 36 of 1998) through its Direct Estimation of Ecological Effect Potential (DEEEP) approach for complex industrial wastewater discharge (DWAF, 2003) established policies that reduce and prevent degradation of water resources and established the National Toxicity Monitoring Program for Surface Waters (NTMP) (DWAF, 2005) to monitor discharges into surface waters. This signified a renewed interest at incorporating biological monitoring into water quality monitoring approaches. The aim of the South African Water Act (Act No. 36 of 1998) is to protect the entire ecosystem provided for

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humans by rivers (e.g. provision of water, disposal of waste, supply of fish, plants, and other biota). In addition to the "basic human needs Reserve" there is provision for an "ecological Reserve" which is the water quantity and quality required to protect ecosystems to secure ecologically sustainable development and use of the relevant water resource (WRC, 2003).

Traditionally, biotas such as macro-invertebrates, fish and algae have been employed for biomonitoring. Benthic macro-invertebrates have been used to a great extent in diverse aquatic stress assessment such as in organic pollution and acidification (Karr and Chu, 1999; Cao et al., 2003; Sandin et al., 2004) and are suitable as indicators for stream hydrology and oxygen depletion (Johnson et al, 2006; Hering et al, 2006). Some others have employed bioaccumulation testing (Du Preez and Steyn, 1992), while others rely on the use of invertebrate species assemblages within communities for biomonitoring (Palmer and O'Keffe 1992). Algae on the other hand have proven useful in assessing nutrient enrichment, salinity and acidity (Round, 1993) on the basis that they are stationary and reflect directly the physicochemical conditions of their natural environment (Stevenson and White, 1995), while fish are reported to be good indicators of habitat degradation and pollution (Bain et al., 1988; Belpaire et al., 2000). Biological indices that focus on macro-invertebrates (Chutter, 1998) and fish (Engelbrecht, 1992) have, however, received the most attention. Fish play a huge role in studies relating to human and ecological health, and are thought to bridge the gap better than any other class of organisms because environmental variables influenced by countless human activities impact the evolution, genetics and adaptation of fish and consequently their use in scientific investigations (Cossins and Crawford, 2005). Since fish have better developed immune systems compared to invertebrates, other lesser organisms may not replace studies on fish because they differ in responses to toxicity.

2.8 *In vitro* techniques

2.8.1 *In vitro* toxicity assessment

The majority of toxicity assessments associated with the aquatic environment employ the use of whole animals (Buikena and Cairns, 1980). For acute toxicity tests, the Environmental Protection Agency (E.P.A, 1996) recommends the use of aquatic species: *Ceriodaphnia dubia* (daphnid), *Daphnia pulex* and *D. magna* (daphnids), *Pimephales promelas* (fathead minnow), *Oncorhynchus mykiss* (rainbow trout) and *Salvelinus fontinalis* (brook trout) but

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the experimental protocols are usually extended, tedious, expensive and require the use of a large number of animals. In the USA alone, an estimated 3 million fishes are used annually for wastewater effluent testing methods (Tanneberger et al, 2013) and there is increased pressure to reduce, replace and refine animal use in research and toxicological studies.

The need for alternative methods for evaluating contaminant risk and hazards in the environment suitable for biomonitoring cannot be over-emphasized. The use of *in vitro* techniques as an alternative system in toxicity assessment is not a new concept in research. The principle behind this technique is that it provides the platform for toxic mechanisms at molecular and cellular levels to be studied using cell cultures in a controlled environment, usually in isolation from multiple physiological systems modulating such cells *in vivo* (Castano et al, 2003; Kilemade and Quin, 2003). This hinges on the fact that functional and structural changes occur between pollutants and cellular components within the organism (Larsson et al, 1985) and consequently, pollutants will exert their effects by interacting or interfering with basic cellular functions.

There is a scientific, technical and ethical convenience to cell culture use in toxicological research. Toxic mechanisms and interactions can be studied at the cellular level due to the absence of complicated toxicokinetics, hence establishing a structure-activity relationship and classifying the toxic action of chemicals or complex environmental samples for their specific effects. Large numbers of samples can also be screened rapidly at a reasonable cost and, most significantly, cell culture use either as cell lines or primary cultures in aquatic toxicology reduces the number of animals sacrificed.

A number of cell lines have been used for toxicity testing (Bols et al, 2005; Castaño et al, 2003; Segner, 2004; Schirmer, 2006). Fish cell lines have been employed in studies involving fish infectious diseases, ecotoxicology, virology and immunology (Kocan et al, 1985; Babich and Borenfreund, 1991; Segner 1998; Fent, 2001; Castano et al, 2003; Schirmer, 2006; Wolf 2007) as an alternative to whole animal tests. The majority of these cell lines are derived from cold water fish, for example the RTG-2 cell line from rainbow trout gonadal cells (Wolf and Quimby, 1962), brown bullhead catfish fibroblast (BB) (Zahn et al, 1995; Clemedson et al, 1996), RGE-2 epithelial cells from Atlantic Salmon (Butler and Nowak, 2004), and RTgill-W1 derived from the gills of the rainbow trout (Bols et al, 1994). Mammalian cell lines have also proved useful in identifying and understanding the potential effects posed to

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humans by chemicals (Rees, 1980). They have been used to estimate the water quality of oil-refinery effluents (Richardson et al, 1977).

Cell lines offer the advantage of the ease of use, reproducibility and the ability to be prepared in large numbers and cryopreserved. The downside is that differentiation from frequent re-culturing could make them become susceptible to changes not characteristic of the tissue from which they were derived (Druker et al, 1989; Castaño et al., 2003). There also exists the likelihood of either overestimating (due to the absence of a compensatory, repair mechanism) or underestimating (due to its volatility and/or poor solubility in the test system) toxicity (Castaño et al., 2003; Mothersill and Austin, 2003).

The use of primary cultures as an alternative to whole animal testing has likewise generated research interest. This is so because primary cultures are reported to be more organotypic, because they maintain the three-dimensional structure of the tissue and cell-to-cell contact, bearing better morphological similarity to the organ of origin (Pärt et al., 1993). An example is primary gill cultures, which communicate through gap junctions (Sandbacka et al., 1999).

2.8.2 *In vitro* cytotoxicity and genotoxicity

Freshney (2001) defined cytotoxicity as "*adverse effect(s) observed in a cell that affects the structural and/or functional processes essential for survival and proliferation following exposure to exogenous chemicals*". Cytotoxic effects are expressed as basal, selective or functional cytotoxicity. Basal cytotoxicity is seen when cellular structures and/or functions are attacked and commonly observed effects affect energy metabolism, plasma membrane and ion regulation (Ekwall, 1983; Ekwall and Ekwall, 1988). Selective cytotoxicity is seen with chemicals exhibiting preferential toxicity to particular cell types; an example is seen in biotransformation potential found in specific cell types (Seibert, 1996). Functional toxicity results in interference with cellular processes, threatening the survival of a particular organ or even the organism as a whole (Mothersill and Austin, 2003).

The reaction of cells challenged with xenobiotics may follow patterns such as sequestration as the cells concentrate the assault in a subcellular compartment (Sheehan et al., 1995). Detoxification of the chemical by non-enzymatic reactions may likewise occur, as is seen with antioxidants conjugation of Reactive Oxygen Species (ROS). Some proteins such as

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metallothioneins actively bind metallic xenobiotics preventing them from binding to other sites, and lastly, xenobiotics may become enzymatically detoxified by specialized systems such as the CYP450 systems for ease of excretion (Mothersill and Austin, 2003).

With *in vitro* cytotoxicity utilizing various endpoints that identify the effective concentration of a chemical that impacts cellular functions by 50%, it is then possible to derive the effective concentration (Fotakis & Timbrell, 2006; Gulden et al., 2005; Schirmer, 2006). Cellular modes of action of contaminants can be interpreted because a number of *in vitro* assay endpoints deal with cellular changes at the molecular, biochemical, histological, or physiological level (Mothersill and Austin, 2003). An ideal endpoint is expected to be relevant, reliable, sensitive, simple, rapid, and cost-effective (O'Brien et al., 2000). With *in vitro* cytotoxicity testing, mechanistic studies can be performed due to the absence of complexities such as bioaccumulation and depuration found *in vivo*. Furthermore, experimental conditions may be modified to suit research interest (Baksi and Frazier, 1990).

The most common endpoint of *in vitro* assays is cell death, measured following exposure to contaminants, usually targeting a single cellular response; cell viability is detected with the use of indicator dye (Schirmer, 2006). Fluorescent indicator dyes such as MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) and neutral red are used for measuring cell proliferation and cytotoxicity (Mosmann, 1983, Clothier, 1990; Cavanaugh et al., 1990). MTT is a yellow water-soluble tetrazolium dye that is reduced by live, but not dead, cells to a purple formazan. The MTT formazan produced in metabolically active cells is proportional to the number of live cells present and can be measured spectrophotometrically, so a decline in readings would indicate a reduction in cell metabolism and a loss of cell membrane integrity (Mosmann, 1983). The neutral red assay provides a measure of lysosomal function and is one of the most used cytotoxicity tests with many biomedical and environmental applications (Babich and Borenfreund 1990; Spielmann and Liebsch, 1990; Repetto and Sanz, 1993). The neutral red (NR) dye accumulates in the lysosomes of viable cells that require the presence of an intact plasma membrane and sufficient energy (ATP) levels, for a functional lysosome to accumulate the NR. The dyes are readily available, relatively easy to use and inexpensive.

Other end points involving *in vitro* assays with regard to pollution related stress involve DNA

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damage assessment because carcinogenesis, teratogenesis and embryotoxicity are possible consequences of genotoxic substances (Klobucar et al., 2003; Kurelec, 1993) which adversely affects ecosystem stability (Mitchelmore and Chipman, 1998). A number of studies using the Comet assay to detect genotoxicity in aquatic environments as a tool for environmental pollution monitoring have been published (Schnurstein and Braunbeck 2001; Avishai et al., 2002; Nehls and Segner 2005). The Comet assay detects DNA damage in individual cells and can be performed on any eukaryotic cell type, usually requiring only a small number of cells, and it is quite sensitive at detecting DNA damage (Shing et al.,1998).
The Ames genotoxicity assay, which utilizes the bacterium *Salmonella typhimurium*, has been used to research the genotoxic potential of substances by assessing the genotoxicity of nuclear DNA effects such as point mutations, gene reversion and gene conversion in specific bacteria following exposure by evaluating the induction of mutation (Maron and Ames, 1983). Both the Ames and Comet assays have been recommended as effective genotoxicity assays (Grummt, 2000; Reifferscheid and Grummt, 2000).

2.8.3 Primary gill culture bioassays

A primary culture is defined as one derived from cells, tissues or organs taken directly from organisms (Schaefer, 1990). This culture system offers a number of advantages such as retaining tissue-specific functions, and their capacity for biotransformation. Moreover, they are considered to be more sensitive as they possess a higher metabolic capacity compared to cell lines (McKim et al., 1985).

Primary gill cell culture is being put to use more frequently in fundamental and applied research to examine the effects of heavy metals (Smith et al., 2001, Jönsson et al., 2006, Walker et al., 2008, Leguen et al., 2011) and xenobiotics (Ramos and Cox, 1986; Celander et al., 1997; Huuskonen et al., 1998 Visoottiviseth and Chanwanna, 2001). They are used in toxicity test screening (Sandbacka et al., 1999; Kelly and Wood, 2001; Zhou et al, 2006), the study of pathogenic viruses (Jose et al., 2010), investigating cellular defence mechanisms such as metallothioneins (Walker et al., 2007) and EROD induction (Carlsson and Pärt, 2001; Jönsson et al., 2006; Leguen et al., 2000).

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2.8.3.1 Primary gill cultures as aquatic biological indicators

The fact that aquatic organisms possess an organ as distinct and essential as the gills, motivated the rationale for its choice as the preferred organ for use in assessing contamination in the aquatic environment. The gills have been reported to be important in toxicity risk evaluation in the aquatic system (Wood et al, 2002; Leguen et al, 2007; Craig et al, 2007). Gills are the first targets for toxicants in the aquatic environment and the prime uptake site of water-borne contaminants into fish (Spry and Wiener, 1991; Wood, 2001). Therefore, the absorption and responses of the gills towards toxic chemicals is usually rapid (Evans, 2005; Mallatt, 1985; Mallatt, 1995). The primary cause of death in acute fish toxicity testing is damage to the gills (Mothersill and Austin, 2003). The gills perform gas exchange, ion transport, acid–base regulation and osmoregulation which are a critical functions for survival (Evans et al., 2005). Other functions, such as nitrogenous waste excretion and the excretion of metabolites from xenobiotic biotransformation, explain their high sensitivity to waterborne toxicants relative to mammalian systems (Wood, 2001). On that account, damage to gills may therefore indicate a compromised functioning of the organism and eventually possible death.

Alternate gill model systems to traditional *in vivo* approaches exist in gill function research (Fernandes et al, 1995, Wood and Pärt 1997, Sandbacka et al., 1999; Fletcher et al, 2000; Chasiotis et al. 2010; Chasiotis and Wood, 2011a; Chasiotis and Wood, 2011b; Kelly and Chasiotis, 2011) and primary gill cultures have been used successfully to assess aquatic toxicants (Lilius et al., 1995; Part 1995; Sandbacka et al., 1999; Wood et al., 2000; Zhou et al., 2006; Galvez et al., 2008; Farkas et al., 2011; Leguen et al., 2011).
In vitro preparations of gill tissue from fish result in monolayers of flat, irregular, polygonal shaped cells bearing similarities to respiratory epithelial cells. The gill epithelium is made up of respiratory epithelial cells, chloride cells and mucous cells (Part et al., 1993). The gill epithelial cells can be cultured on permeable membranes that are able to tolerate water exposure on the apical surface. The cells appeared to be mainly respiratory epithelial cells and a considerable amount of chloride cells may be present (Wood and Part, 1997).

The use of primary gill cultures for detection of dioxin-like substances in complex environmental matrices has also been of research interest because the gill tissue in aquatic

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species serves as a barrier to the surrounding environment, acting as an effective defense against incoming xenobiotic compounds (Wood, 2001). The cytochrome P450 (CYP) monooxygenase system are involved in the metabolism of polycyclic aromatic hydrocarbons (PAHs) and their planar, halogenated counter- parts (PHAHs) such as polychlorinated dibenzo-*p*-dioxins. These are a common source of contaminants present in the aquatic environment that tend to bio-concentrate in fish and other aquatic animals due to their lipophilic and persistent nature (Hahn et al., 1992). The 7-ethoxyresorufin-*O*-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH) are readily induced by PAHs and PHAHs, and are responsible for the biotransformation of many lipophilic xenobiotics (Poland and Knutson, 1982).

2.9 Effects of coal derived PAHs on fishes

Another important environmental concern associated with coal combustion is the production of polycyclic aromatic hydrocarbon (PAH) (Ahrens and Morrisey, 2005) and resultant oxidative stress induction in aquatic organisms exposed to PAH (Winston and Di Giulio, 1991; Livingstone, 2001). CYP1A induction represents a sensitive biomarker of exposure to dioxin-like pollutants, and is now extensively used in environmental monitoring (reviewed by Bucheli and Fent, 1995). Consequently, relating the potential uptake of PAHs derived from coal mining as a parameter of water quality, and subsequent AMD effluent generation needs to be considered for environmental surveillance. Controlled laboratory exposure to test responses of primary gill cultures to AMD impacted water along a receiving stream where coal mining and utilization occur are an essential prerequisite for environmental water quality surveillance. Bioassays of fish using primary cultured gill cells could be a vital means of gaining information regarding changes in water quality with respect to potentially carcinogenic organic pollutants such as PAH.

Cytochrome P450 activity (phase I) and phase II metabolites were detected in isolated gill cells from the gulf toadfish (*Opsanus beta)* exposed to B[a]P (Kennedy and Walsh, 1994). Localization of CYP1A to both pillar and epithelial gill cells has been reported for topminnow (*Poeciliopsis* spp.) following exposure to B[a]P in water (Smolowitz et al., 1992). Kennedy and Walsh (1994) reported the induction of EROD activity in microsomes from gill tissue in Scup (*Stenotomus chrysops*) following intra-peritoneal injection of b-naphthoflavone

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(b-NF). It is likewise important to examine the presence and inducibility of CYP1A in primary cultures of respiratory epithelial cells from the gill of Tilapia fish (*O. mossambicus*), an indigenous fish species that is abundant and widely distributed. It is a recreational and ecologically important species within the tropical and subtropical southern Africa region with adequate background information (Skelton, 1993). *O. mossambicus* has been used successfully as a test organism in a number of biomarker studies (Lee et al., 1996; Hwang and Yang, 1997).

Setting in place an environmental water quality surveillance system which employs a controlled laboratory exposure to assess toxicity associated with point source pollution, for example from coal mines resulting in AMD, using *in vitro* techniques (primary cells and cell line cultures could be innovative for assessing anthropogenic changes in aquatic ecosystems for effective environmental assessment and monitoring. This could therefore provide crucial inferences about the health of an AMD-impacted stream. This study has focused on characterizing the relative importance of AMD toxicity on cultured primary gill cells and other cell lines as a means of addressing the significance of toxicity as a monitoring ecotoxicity tool for AMD contaminants.

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CHAPTER 3: Characteristics of the study sites and water quality

3.1 The Witbank Coalfield

South Africa's available coal resources are contained in 19 coalfields (Erasmus et al., 1981) with the Springs-Witbank Coalfield and the Highveld (Eastern Transvaal) Coalfield being the most important ones (Fig 3.1). Coal mining has been ongoing in the Mpumalanga area since 1870, for the purpose of power generation and the chemical industry. The Kromdraai or Landrau colliery is located in the Mpumalanga Province, northwest of eMalahleni. It forms part of the Witbank coalfields (Erasmus et al, 1981) and lies in the Olifants River catchment area. The bulk of the mining conducted in the region is open cast operations, which causes water from old underground workings as well as rain and ground water inflow to accumulate in pits.

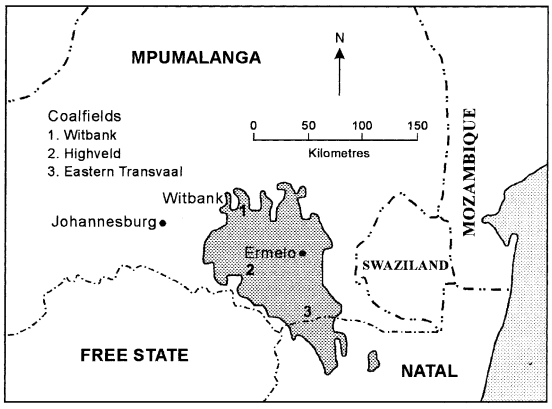

Fig 3.1: Location of the Witbank Coalfield

(Illustration courtesy of S. E. T. Bullock and F. G. Bell. Department of Geology and Applied Geology, University of Natal, Durban, South Africa)

3.1.1 Topography of the area

The topography of this region is relatively high; there are several gently undulating surfaces with numerous local streams, larger rivers and pans shaping the landscape (Fig 3.4). Elevation surfaces are around 1540 meters above sea levels (Azzie, 1999).

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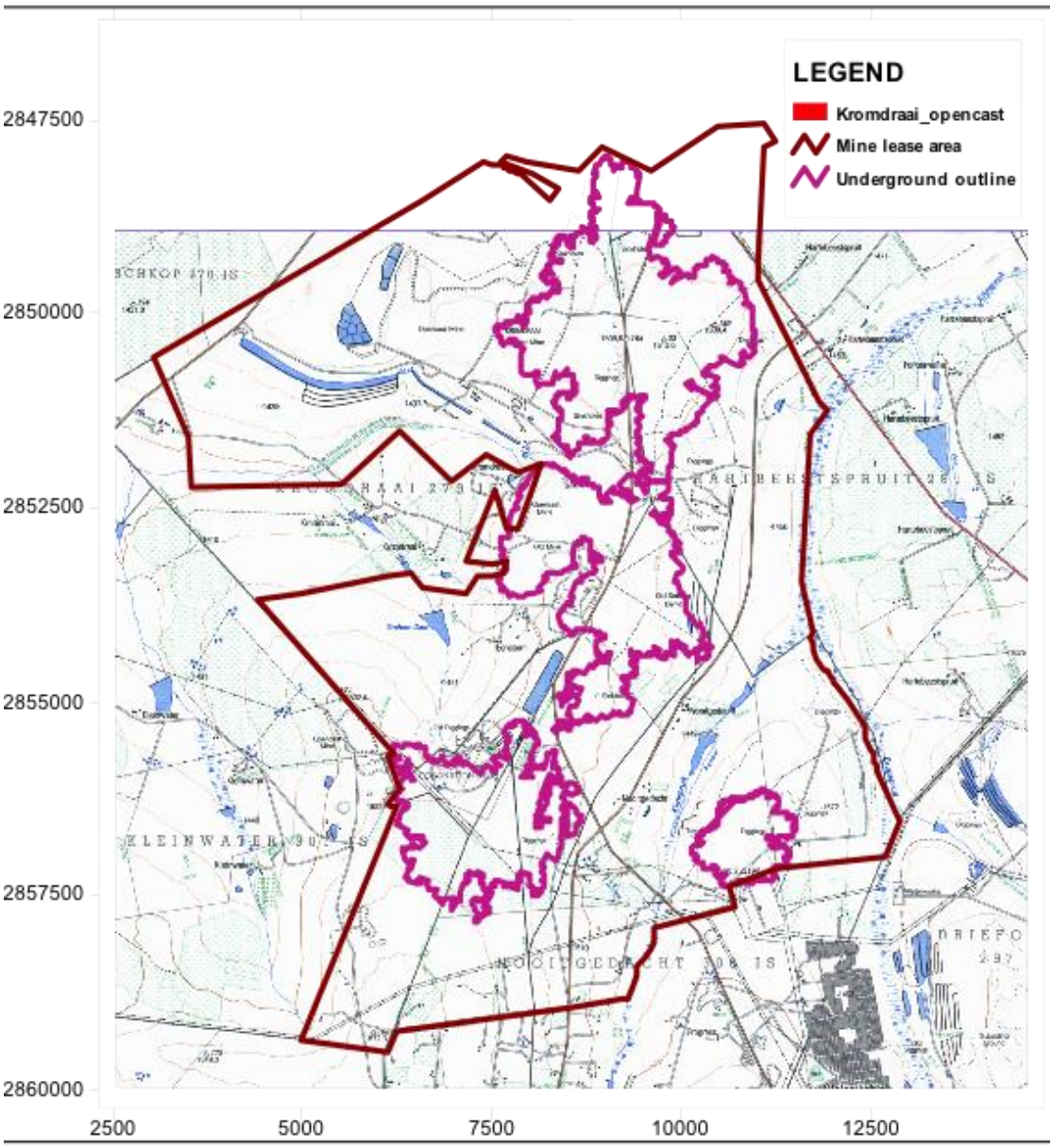

Fig 3.2: Topographic map of Kromdraai area, showing the mine outline and open cast area. (Illustration courtesy of WRC report NO.1263/1/07 ISBN 1-77005-416-2, 2007).

3.1.2 Surface hydrology

The average annual rainfall in the study site is about 684 mm (South Africa weather service, 2007), and the mean annual evaporation is 1700 mm. Kromdraai has a relatively high topographic area, and run-off usually drains from mining areas and main streams all flow away from mining areas. The run–off of the area is 6.7% of the total rainfall (Midgley, 1994).

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3.1.3 Water quality

A large volume of mine-water stored and generated from a number of active mines in the Witbank coalfields (Mpumalanga Province, South Africa) has great potential for acidification because of the low natural base potential in the coal. Water from the evaporation dam in the colliery discharges into the streams. The water qualities follow trends that are characteristic of chemical oxidation of sulphides and resulting acidification. The average sulphate production rate of 5 - 10 kg/ha of spoil per day at sulphate concentrations of between 1 000 - 2 000 mg/L has been reported (Hodgson and Krantz, 1998). The consequences of elevated sulphate levels are such that there is a greater potential for algal blooms, disruption of the riverine ecosystems and impairment of human health. An overall decrease in the aesthetic value of the water is apparent, and when consumed, the water may produce adverse health effects (DWAF, 2002).

Approximately 50 ML/day of water is discharged into the Olifants River Catchment per day (Maree et al, 2004) and estimated post-closure decant from defunct coalmines in 2004 was put at approximately 62 ML/d (DWAF, 2004). Grobbelaar et al. (2002) predicted decant of 360 ML per day would occur following closure of the entire Mpumalanga coalfields.

Potential human health risks and ecological injury could arise chiefly from elevated metals (Adler and Rascher, 2007) as coal mining impacts on the aquatic environment, which consequently affects the socio-economic situation of areas downstream of the river (DWAF, 2004). A study carried out by Coetzee et al. (2002), on the upper Olifants River to determine metal bioaccumulation in fish identified aluminium (Al) as a toxic agent of AMD, and reported that Zn, Cu, Mn, Pb, Cr, Ni, Al and Fe accumulated in the skin, muscle, liver and gill tissues of fish.

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3.2 Site selection within the Witbank Coalfield

3.2.1 Location and description of the study area

The study was conducted where AMD water discharges into a receiving stream located approximately 13 km downstream of an active mine, Kromdraai (25°46' 05.5"S; 29° 07' 15.5"E) in the Highveld region close to Witbank (Mpumalanga Province, South Africa). The study site was selected based on the stream order, stream depth, its accessibility and stream characteristics (outflow from a wetland), and the fact that it is mainly impacted by AMD and relatively free of other influences such as agricultural run offs and human sewage. The water collection site is located several kilometers away from the coal mine and impacted stream flows away from the mine. Effluent discharge outflowing from the mine undergoes passive treatment, as effluent is passed through a wetland (Fig 3.3A-D). The sole aim is to ameliorate water pollution because wetlands act as sieves removing heavy metals and sulphates. The water outflow then passes into the free flowing stream where it is subjected to further treatment to reduce acidity and heavy metal content of the AMD effluent (Zurbuch, 1996**,** Brown, 2005). An in-stream neutralizing agent (such as caustic soda, hydrated lime or lime stone) continuously dosed into the stream to increase the pH of the water (active treatment), in order to further precipitate metals as hydroxides and improve the water quality (Fig 3.3E-H). The in-stream neutralization method uses a fairly sophisticated system where the pH, conductivity and flow measurements are taken. An automated continuous flow reading is taken by the monitoring system, which calculates the amount of discharge, given the recorded flow. The information is then relayed telemetrically to the monitoring station. Two water collection points were selected along the course of the river (25°46' 05.5"S; 29° 07' 15.5"E), the first being immediately following the wetland and the other point post–alkali treatment (Fig 3.3I).

An ecologically similar reference site was selected. This site was a relatively pristine site (reference site) outside of the influence of a colliery and located at 25°47'6"S; 28°28'52"E. The water source was from a natural flowing spring and the water samples collected here were referred to as "reference site water sample" (R).

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Fig 3.3A-I: Kromdraai stream in Mpumalanga province of South Africa. (Photograph courtesy of Liesl Hill, CSIR, South Africa) showing; (A-C) wetland D) streambed E) water treatment plant (F-H) in –stream neutralization I) flowing stream post alkali treatment.

A  B  C 

D  E  F 

G  H  I 

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3.2.2 Water sampling and water quality analysis

The term water quality describes all the factors that control the fitness of water for its uses, be it physical, chemical, biological and aesthetic properties of water such that there is protection of the health and integrity of aquatic ecosystems (DWAF, 1996). Most of these properties are controlled or influenced by constituents that are either dissolved or suspended in water.

3.3 Materials and Methods

3.3.1 Selection and measurement of environmental variables

In this study, water sample collection was done during the low flow rate seasons and samples were collected three times a year (between June and September 2014) over a period of two years. The general climatic condition on sampling days were mild, relatively cool weather with no rainfall; average temperature was between 20-23ºC. As water flow and water quality are intimately linked, variation was reduced by limiting the water collection period to a particular period. Two water collection points were chosen; a water point immediately flowing from the wetland (termed untreated) and another beyond the dosing point of the in-stream neutralizing (termed the treated).

Physico-chemical data of water samples from the impacted stream were measured and analyzed. A total of 24 water quality parameters were measured at each site. The water quality parameters selected for analysis were those known to influence the overall health of the aquatic ecosystem in South Africa (Pegram et al., 1997) and are suggestive of AMD impact (FWPCA, 1968).

3.3.1.1 *In situ* measurements

In situ measurements of temperature, dissolved oxygen and pH were conducted at the water collection points along the course of the stream. Temperature, dissolved oxygen and pH were measured using a portable multimeter (HACH HQd, USA).

Dissolved states of these parameters were analyzed because pollutants in dissolved forms are more bioavailable to aquatic biota and are key to observed detrimental changes in the aquatic

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environment (Chapman, 1988; Luoma and Rainbow, 2005). The pollutants of interest in AMD are acidity, metals, solids and increased conductivity (Heath and Eksteen, 2005).

The electrical conductivity of water (EC) provides an estimate of the total dissolved salts (TDS) in aquatic systems and is measured as an indicator pollutant of the TDS. The total dissolved solids (TDS) in acid mine water includes bicarbonate, individual ions such as calcium and magnesium, sulfate and conductivity (U.S. EPA, 1982). The TDS concentration is related to the Electrical Conductivity (EC) of the water (DWAF, 1996). Salinization occurs when the concentration of dissolved salts (salinity) increases (Williams, 1987), which is characterized by a concurrent increase in total TDS levels.

Dissolved oxygen (DO) concentration provides a useful measure of the health of an aquatic ecosystem, because DO concentration is critical for the survival and functioning of the aquatic biota as it is required for the respiration of all aerobic organisms. When DO becomes depleted, this could potentially lead to a compounded stress response in aquatic organisms. Under such conditions, increased toxicity of zinc, lead, copper, cyanide, sulphide and ammonia have been observed with juveniles of many aquatic organisms showing more sensitivity, such as increased vulnerability to predation and disease (Walmsley and Butty 1980).

Chemical Oxygen Demand (COD) provides a measure of the oxygen requirement of organic material present in the water (DWAF, 1996). When COD levels are high, it is indicative of the presence of a large amount of organic material in the water sample.

Acidity is a measure of the concentration of available hydrogen ions. This is expressed as pH or the logarithmic concentration of hydrogen ion concentration in water (U.S. EPA, 1986). Industrial activities generally cause acidification either from low-pH point-source effluents or from mine drainage and acid precipitation resulting largely from atmospheric pollution caused by the burning of coal and the exhausts of combustion engines (Campbell and Tessier, 1987). Factors such as temperature, the concentrations of inorganic and organic ions, and biological activity affect water pH. The pH may also affect the availability and toxicity of constituents such as trace metals. Acidity in receiving waters can alter gill membranes or change gill mucus resulting in death due to hypoxia (Grim and Hill, 1974). The toxic effects

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of acid pH values on fish increase as the concentrations of calcium, chloride and sodium decrease.

3.3.1.2 Laboratory investigation

Water samples were collected as subsurface grab samples. Before sampling, the collection bottles were rinsed twice with water at the point of collection and then filled to capacity. The bottle mouth was faced upstream and submerged to a depth of about 20 cm. The plastic bottles were placed on ice immediately for transport to the Consulting and Analytical Services Laboratory (Environmental Laboratory) of the CSIR in Pretoria. Laboratory quality assurance measures were taken to avoid microbial contamination by filtering water samples. The quality of the reagents and materials were of research grade.

Heavy metal loading is considered to be one of the most toxic side effects of AMD (Johnson and Thornton 1987). AMD usually contains an effluent laden with heavy metals such as Mn, Zn, Pb, Cu, Al, As, Cr, Ni, Cd, Hg and Fe. Elevated metal concentrations can be toxic to aquatic organisms (Kimmel, 1983).

Aluminium is one of the principal particulates emitted from the combustion of coal and is present in soluble forms at low pH values (pH < 4.0), mainly in AMD waters and in natural waters affected by acid rain. Al toxicity in fish appears to be related to interference with ionic and osmotic balance and with respiratory problems resulting from coagulation of mucus on the gills (Genter and Amyot, 1994).

Arsenic (Ar) is regarded as "very toxic and relatively accessible" to aquatic organisms (U.S. EPA, 1986). Fish and invertebrate populations experience reduction in growth and reproduction when exposed to arsenic, resulting in behavioral changes such as reduced migration in fish. Ar is lipid-soluble and could therefore accumulate in fatty tissue; it can bio-concentrate in aquatic organisms because it has a high affinity for organic substances (Mance, 1987).

Chromium exerts a toxic effect at different concentrations in different groups of aquatic organisms. Toxicity results in a temporarily reduced growth phase for young fish at low chromium concentrations. Fish are more resistant than aquatic invertebrates (Dallinger and Rainbow, 1993).

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Copper (Cu) is one of the world's most widely used metals, and anthropogenic sources of copper in the aquatic environment include liquid effluents and atmospheric fallout from industrial sources such as mining, smelting and refining industries, coal-burning, and iron and steel producing industries (U.S. EPA, 1986). Cuis a micronutrient and an essential component of enzymes involved in redox reactions. It is toxic at low concentrations in water and is known to cause brain damage in mammals (Dallinger and Rainbow, 1993).

Iron occurs most commonly in water as ferrous (Fe^{2+}) and the oxidized ferric (Fe^{3+}) states. In oxygenated waters, most iron occurs as ferric hydroxide in both particulate and colloidal forms and readily forms complexes with organic compounds. At high concentrations, iron may become toxic, inhibiting various enzymes, but is usually found at low concentrations in water columns. Iron release into the environment occurs by human activities, such as burning of coke and coal, acid mine drainage, mineral processing, sewage, landfill leachates and the corrosion of iron and steel (Förstner and Wittmann, 1981).

Manganese does not occur naturally as a metal in aquatic ecosystems but is found in various salts and minerals, frequently in association with iron compounds (U.S. EPA 1986). It is similar to iron in behavior and its concentration in water, as with dissolved Mn, is influenced by changes in redox potential, dissolved oxygen, pH and organic matter (Van der Merwe, 1990).

Mercury (Hg) concentration in the environment is normally very low. In aquatic environments, Hg and Hg-organic complexes are of interest because of their extreme toxicity to aquatic organisms and the potential to bio-accumulate in the food chain. Hg is severely poisonous to mammals, exerting both neural and renal toxicity (W.H.O., 1984).

Ammonia and ammonium salt toxicity to aquatic organisms is directly related to the amount of free ammonia in solution. As pH increases, the NH_4^+ion is converted to NH_3^+, which is considerably more toxic to aquatic organisms (USEPA, 1986). Un-ionized NH_3^+, affects the respiratory systems of many animals, either by inhibiting cellular metabolism or by decreasing oxygen permeability of cell membranes (Gammeter and Frutiger, 1990).

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Nitrites and nitrates are the dissolved forms of inorganic nitrogen. The mode of entry of inorganic nitrogen into aquatic systems is through surface runoff, the discharge of effluent streams containing human and animal excrement, agricultural fertilizers and organic industrial wastes. Their presence in the aquatic environment plays a critical role in eutrophication but this is usually not considered important enough for setting water quality guideline limits for protection of aquatic ecosystems (Ashton, 1981).

Phosphorus concentrations are usually determined as orthophosphates, total inorganic phosphate or total dissolved phosphorus. The most significant effect of elevated phosphorus concentrations is the stimulation of growth of aquatic plants (Chutter, 1998) which results in changes in trophic status usually accompanied by the growth of algae and other aquatic plants in rivers, lakes and reservoirs.

3.3.2 Chemical analysis

The analysis of the metals: Al, Cu, Mn, Si, Fe, Zn, Ar and Hg were measured using inductively coupled plasma atomic emission spectrometry (ICPAES) using standard methods (CMP 1, CMP 26a, CMP 2 (Ar), CMP 3(Hg) at the CSIR Environmental Laboratory, Pretoria.

Nutrients were analyzed using automated flow injection analysis. Nitrate and Nitrite (N) were analyzed using the No CMP 26c method, ammonia nitrogen using method No CMP 26b, and orthophosphate and phosphate using method No CMP 26f. Chloride and sulphate analysis involved automated flow injection analysis method No CMP 26h and CMP 26i. Individual ions such as Ca, Mg, K, and Na were measured using the ICPAES method No CMP 1, CMP 26a. Alkalinity was determined using the potentiometric titration method No CMP17 and chemical oxygen demand was measured calorimetrically using method No CMP 24. All values were measured according to the standard methods for water and wastewater examination (APHA, 1992).

3.4 Results

Acid mine drainage had a negative impact on the water quality at the study site, Kromdraai.

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The mean pH value of water samples immediately flowing from the wetland throughout the sampling period was 3.65 at the impacted site. The process of in-stream neutralization increased water pH to a mean value of 8 following intervention (active treatment with caustic soda). The mean conductivity of 300 mS/m at both collection points was well above values obtained for the reference site and TWQR range (Table 3.1). The metal contaminants likewise exceeded the TWQR, with total dissolved metals following passive treatment (wetland) analyzed as 0.36 mg/L for Fe; Mn was high at 65 mg/L; Zn 3.4 mg/L; Si 9 mg/L and Al 28 mg/L (Table 3.2). In order of ranking, Al, Zn and Mn ranked as the greatest metals of potential concern, and this was calculated by determining the hazard quotient as maximum concentration / TWQR. Metals are considered to be of potential concern when values > 1. For example, the average concentration of Al after passive treatment was 5600 times greater than the guideline levels. The South Africa's TWQR guideline level for Al in aquatic systems (95% protection level) is 0.005 mg/L. The trigger value (guideline) concentration is 95% for each metal applying to moderately disturbed systems to protect 95% of species. There are no guideline values for Si and Fe, while Ar, Cr, Cu, and Hg were within guideline limits. There is evidence of AMD impact with parameters exceeding TWQR for effluents discharged into receiving water bodies, which is indicative of AMD and is potentially harmful for aquatic life (Table 3.3).

Inorganic constituents of water samples from AMD impacted sites exceeded values of that from the reference sites as Na, Mg, K and Ca levels were higher by several orders of magnitude (Table 1). Significant mean difference in values for inorganic constituents (Fig 3.4) was observed for the analyzed water samples for the reference site compared with the AMD impacted site. An increase in these inorganic constituents was observed in effluent post-alkali treatment especially for Na and Ca, an indication of some level of salt precipitation, although the differences pre and post-alkali treatment was not found to be significantly different.

Metal analysis results comparing the reference site and the AMD impacted site showed significant difference likewise in metal burden for Mn, Si, Al and Zn (Fig 3.5). These metals were also found to reduce post-alkali treatment, but only Zn was found to be significantly different in concentration following alkali neutralization, when associating untreated water vs. treated AMD water effluent.

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Concerning nutrient levels, ammonia and nitrate levels were above values obtained in the reference site, but orthophosphate and phosphate levels were comparable in both sites.

The reference site presented with good water quality in this study: metals, conductivity, sulphates and inorganic constituents were significantly lower than those of the impacted AMD site, although the site is considerably smaller in terms of its width and depth compared to the impacted site. A significant difference in EC and sulphate levels between the reference site and impacted site was observed. These values can serve as indicators for the level of AMD impact, with values obtained from the impacted study site; it could be categorized as 'highly impacted' (Table 3.1).

The pH of effluent water emanating from the wetland was also highly acidic, a feature commonly observed in AMD effluent. A number of AMD parameters exceeded TWQR, which raises possible environmental and public health concerns.

3.5 Discussion

In this study, the overall water quality declined due to acid mine drainage. Data on water quality parameters by the Department of Water and Sanitation, as well as sampling carried out throughout the 3 years' study showed similar declines due to acid mine drainage as previously reported (Soucek et al., 2000; Cherry et al., 2001) which are characteristic of acid precipitation (Rosemond et al., 1992), and/or the presence of heavy metals (Kiffney and Clements 1994, Clements et al., 2000). The site could be regarded as being highly impacted due to the low acidity, low alkalinity and the presence of heavy metals and sulphates using the AMD impact criteria (FWPCA, 1968). The presence of metals especially the naturally occurring metals like Fe and Al along with added acidity (H^+) could have resulted in increased metal bioavailability and consequent mobilization of metals into solution (U.S.EPA, 2005). Al, Zn and Mn were of the greatest concern at the site, as they were found at levels several orders of magnitude beyond the TWQR, and if bioavailable, these metals may harm the fresh water ecosystem. When metals are present as free metal ions, they become more bioavailable than metals bound as strong complexes or adsorbed to colloidal and/or particulate matter (Batley, 2002). The presence of heavy metals such as aluminium,

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cadmium, uranium, zinc, cobalt, nickel, lead, manganese and copper are regarded as priority metals of potential ecotoxicological concern in aquatic systems largely as an effect from mining activities (Markich et al, 1997). Coetzee (2002), identified aluminium as a toxic agent of AMD within the upper Olifants River of South Africa.

AMD from surface mines usually contains higher concentrations of manganese than those from deep mines (ERG, 2006). An increase in Mn is mostly related to AMD because it is not a naturally abundant metal; hence it is a good indicator for AMD impacted waters. The reason for the high concentration of Mn post-alkali treatment may be because optimal precipitation of Mn occurs at a higher pH, usually between 9 and 10 (mean) and treatment of AMD by neutralization may not fully precipitate out the Mn.

Another important feature of the study site was the presence of sulphates and high levels of specific ions such as Ca, Na, K and Mg contributing to increase in EC. Mining activity is a major source of salts entering the rivers. Salinization poses a risk to biodiversity and compromises the ecosystems and is a major stressor for fresh water ecosystems (Millennium Ecosystem Assessment, 2005), in addition to the socio-economic consequence to downstream end users. Coal mining provides favourable conditions for leaching of sulphates from coal wastes into surface waters (Bernhardt and Palmer, 2011; Fritz et al., 2010). An indication of the nutrient levels present show that the stream was oligotrophic, characterized by a low accumulation of dissolved nutrient salts which supports only a sparse growth of algae and other organisms.

3.6 Conclusion

The water quality results measured in this study demonstrate that both metals and sulphates were present in inappropriate concentrations that may pose a challenge to some biota within the ecosystem with the concomitant possibility of acid-stress affecting these organisms with resultant possible loss in biodiversity. Metals and acid water components of mine water may cause alterations in the biota of aquatic organisms. This study further highlights the inefficiency of the wetland at effectively removing some metals of potential concern as they were found at levels higher than TWQR. Active treatment with caustic soda reduced metal burden, but affected the stream's aesthetics as water became colloidal with visible salt

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precipitates, thereby reducing dissolved oxygen and light penetration, as well as further increasing the presence of major inorganic salts like Na, Mg, K and Ca and sulphate levels, which increases TDS levels resulting in increased EC.

The complexity of AMD as a pollutant is not yet fully understood, as the presence of pollutants makes it prone to generating more AMD (Younger and Robins, 2002) and the disturbing environmental, ecological, human health and economic consequences of AMD have long lasting effects.

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Table 3.1: Mean values for each of three [redacted] stations defined in the study showing physical and chemical water quality data (mg/l, n = 2 determinations) and target water quality range (TWQR).

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

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Table 3.2: Mean values for each of three sample collection stations defined in the study showing metal concentration (mg/l, n = 2 determinations) and target water quality range (TWQR).

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

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Table 3.3: Water quality parameters that suggest AMD impact (FWPCA, 1968).

AMD Impact Group Criteria				
Measurement	**AMD Criteria**	**No AMD**	**Moderate**	**High**
pH	<6	6.2-7.5	5-6.2	3-5
Alkalinity	<20mg/L	≥30	4-30	0-4
Iron	> 0.5 mg/L	-	-	-
Manganese	> 0.5 mg/L	-	-	-
Sulfates	> 75 mg/L	< 75	75-300	300-450
Aluminium	> 0.3 mg/L	-	-	-
Conductivity	**> 350 µS/cm**	-	-	-
Zinc	**> 5 mg/L**	-	-	-

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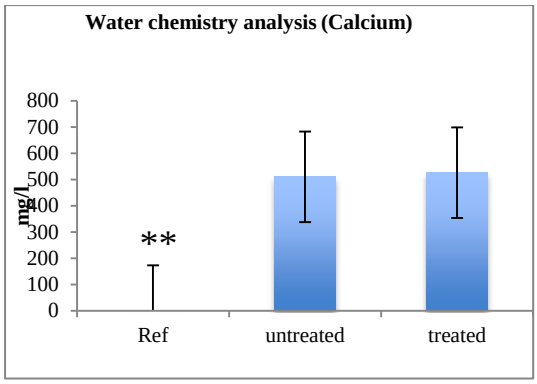
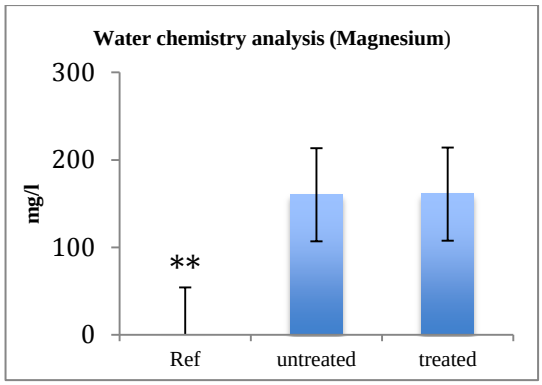
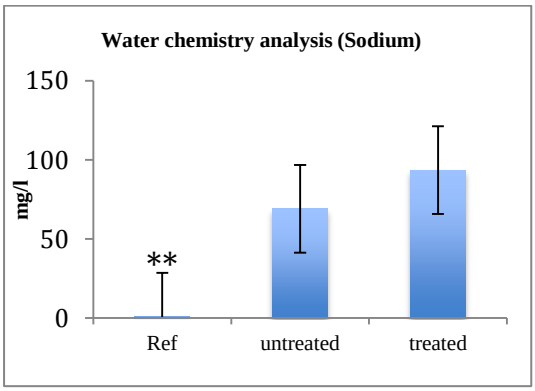
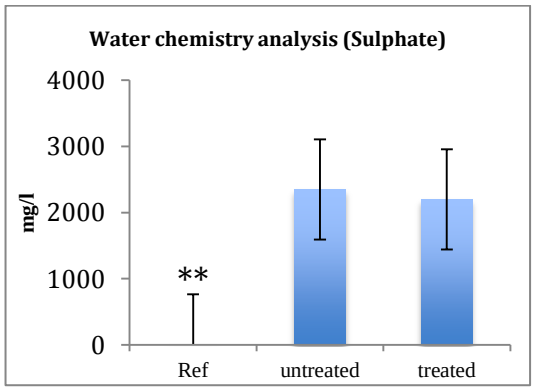
Fig 3.4. Comparison of the mean and SD of inorganic constituents of water samples from the reference, untreated and treated water collection points.









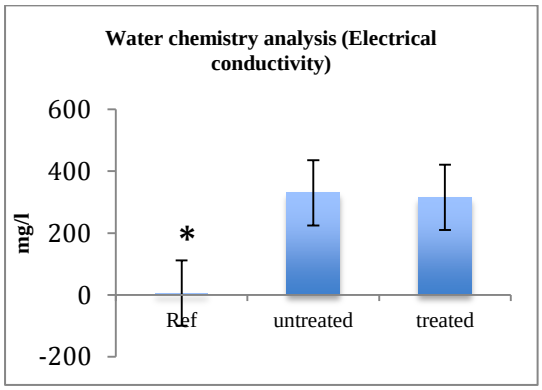


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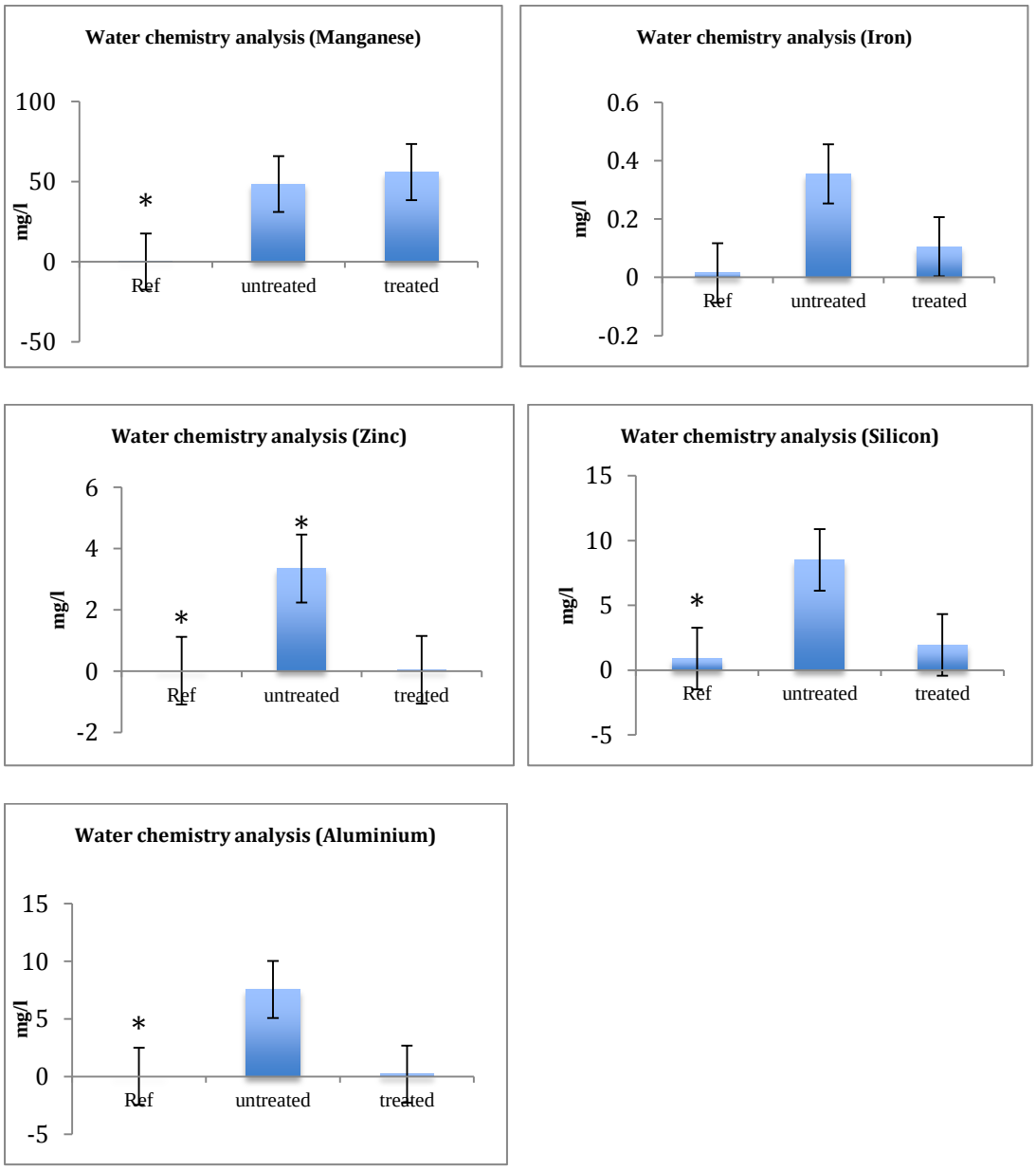
Fig 3.5: Comparison of the mean and SD of metals present in water samples from the reference, untreated and treated water collection points.



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CHAPTER 4: Generation of reactive oxygen species in species relevant cell lines as a bio-indicator of the safety of treated acid mine water

Preface

This chapter was submitted to the ISI-rated journal Water SA.

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Abstract

Reactive oxygen species (ROS) production and resultant oxidative stress (OS) has been implicated as a pathway of toxicity in animal species exposed to pollutants. The gills of aquatic animals and the liver and kidneys of mammalian species are specific cellular sites of toxicity. Although the levels of pollutants such as heavy metals found in acid mine drainage (AMD) water can be quantified following treatment, it is unknown whether this is associated with an equivalent reduction in cellular toxicity.

ROS production by AMD untreated (U) and after treatment (T) was quantified in a fish gill cell line (RTgill-W1) and in two mammalian cell lines (C3A human liver and Vero monkey kidney). ROS production was determined using the oxidant sensitive fluorogenic probe, 2', 7'-dichlorofluorescein diacetate (DCFH-DA) following exposure to U and T, AMD water.

Treatment of AMD water resulted in a reduction in the levels of Al, Zn and Fe while the levels of Mn remained unchanged. For U and T, AMD water a dose-dependent decrease in ROS production was observed.

The percentage of ROS formation decreased from 14% to 4.5%. 16.4% to 7.2% and 25.3% to 17.7% in the RTgill-W1, C3A, and Vero cell lines respectively exposed to 100% AMD water, U and T. The presence of Mn and subsequent ROS formation indicates that this water is still toxic to cells and further processing may be required.

This study shows that the DCFH-DA assay in several cell lines can be used to rapidly bio-monitor the quality of treated AMD water related to the formation of ROS and subsequent cellular effects. However, cutoff levels for cellular toxicity need to be established to ensure the safety of this water for aquatic animals and for animal and human consumption.

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

4.1 Introduction

Coal mining is a major industry in South Africa as coal is the principal energy source for the country (Mangena and Brent 2006). A number of the coalmines are located in the Mpumalanga Province, which hosts more than 10 000 km^2 of hydraulically interlinked mines, supplying 83% of the total produced coal and accommodating eight of the country's ten operational coal-fired power stations (Heath et al. 2010). Most of the rivers have been negatively impacted by the extensive industrial and mining activities in the Mpumalanga Province and the aquatic ecosystems are threatened (De Villiers and Mkwelo 2009). Coal mining is the most important source of acid mine drainage (AMD) contamination affecting the streams and rivers in the upper catchment of the Olifants River (Adler et al. 2007; Driescher 2008).

AMD polluted water contains heavy metals which contaminate the aquatic ecosystem and some notable effects of AMD on the lentic system could be chemical (increased acidity resulting in an increased soluble, bioavailable metal concentration), biological (acute and chronic toxicity) and ecological (loss of habitat and elimination of sensitive species) among others (Gray 1997). In addition human exposure to heavy metals is mostly through the ingestion of contaminated water through drinking, preparation of food or irrigation of crops (Awofolu et al. 2005).

In order to improve the acid mine water quality, the use of neutralization agents such as hydrated lime is employed as an active treatment method so as to precipitate hydroxides of metals such as Fe and Al (Thompson 1980), hence reducing the metal burden and correcting pH (Madeira et al. 2005). Such treatment methodologies give the appearance that the effluents are non- toxic.

The general quality of a water body can be assessed by measuring the physicochemical characteristics of the water, namely the pH, temperature, dissolved oxygen (DO), total dissolved solids and specific inorganic elements (Simpi et al. 2011). However, environmental monitoring by means of biomarkers provides a more detailed assessment of pollution, as it demonstrates the effects on biota, and is preferably applied in conjunction with chemical measurements (Bayne et al. 1988). Biomarkers give an indication of an ecosystem's health and the condition of organisms inhabiting it, and have been used for quality environmental monitoring (Stegeman et al. 1992). Biomarkers are applied as sensitive

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indicators of pollution to detect underlying stressful conditions (Stegeman et al. 1992).

The use of *in vitro* techniques to determine possible toxicological effects of effluent compounds at the sub-cellular level versus whole or intact animal testing offers the advantage of rapid, reproducible and more cost effective research (Bols et al. 2005; Castaño et al. 2003). These *in vitro* systems have been extremely useful in the following studies: cytotoxicity (Environment Canada 1990), drug metabolism and toxicity (LeCluyse 2001), molecular toxicity (Blaauboer et al. 1998) as well as in predicting the biological reactivity of potential toxic compounds (Barratt 2000). *In vitro* systems can be used to study cellular responses to toxicants for example OS, glutathione homeostasis, cellular stress responses, changes in enzyme activities and cytokine responses which, in conjunction with molecular studies, become useful as biomarkers of toxic effects and for the prediction of potential toxic effects in living organisms (Harries et al. 2001).

Cell line use in toxicity testing involving environmental contaminants has been widely reported (Maruoka 1978; Kfir et al. 1981; Van Doren et al. 1984; Mochida, 1986; Bols et al. 2005). An advantage of using cell lines is that the whole water samples containing pollutants can be directly applied and this ensures that little or no toxicant is lost to extraction processes and further sample processing (Dayeh et al. 2002). In addition cell types that represent specific cellular targets can be used such as cell lines derived from fish gills. Fish cell lines are used as potential alternatives to whole fish in testing environmental samples for toxicity. The RTgill-W1 and RTL-W1 liver epithelial fish cell lines derived from rainbow trout have been commonly used (Dayeh 2005). Heavy metals cause multiple organ toxicity in mammals including human and specific organ sites of toxicity are the brain, liver and kidneys André et al. 1991; Bouquegneau and Joiris, 1992; Dietz et al. 1998).

Mammalian cell lines such as the HepG2 and the Vero kidney cell line that represents the liver and kidney can be used to evaluate mammalian toxicity. Mammalian cell cultures have been used successfully in identifying and understanding the possible effects chemicals pose to humans (reviewed in Rees, 1980) and in studies performed by health laboratories and water quality regulators (reviewed in Hunt et al. 1986). Richardson et al. (1977) used mammalian cell culture assay to evaluate water quality of oil-refinery effluents.

Fe, Cu, Cr and Va are redox active metals and generate ROS by redox cycling. Other metals that do not undergo redox cycling such as Hg, Ni, Pb and Cd impair antioxidant defense systems and may inhibit antioxidant enzymes or deplete GSH via direct binding. Another mechanism is via the Fenton reaction whereby Fe^{2+} is oxidized by hydrogen peroxide to Fe^{3+} with the formation of a hydroxyl anion and radical (Sevcikova et al 2011).

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The process of ROS production is a normal and important physiological process in cellular metabolism. However, excessive ROS causes oxidative stress (OS) resulting in modification of lipids, proteins and nucleic acids (Dat et al. 2003; Livingstone 2001; Mittler 2002).

ROS generation initiated by the presence of contaminants, resulting in OS has been reported in biota exposed to pollutants, therefore suggesting a possible connection between contaminant-stimulated ROS production and resultant OS as an established pathway of toxicity in exposed organisms (Di Giulio et al. 1995; Kelly et al. 1998; Winston and Di Giulio 1991). ROS production and the resultant OS have been associated with the presence of metals, aromatic hydrocarbons, pesticides, polychlorinated biphenyls and dioxins in aquatic ecosystems (Valko et al. 2005; Valko et al. 2007). Xenobiotic factors elevate ROS production either by accumulating reactive intermediates, deactivating antioxidant enzymes, or by depleting radical scavengers (Kelly et al. 1998). Hence, ROS production and resultant OS have been connected to a number of pathological processes (Sies 1999; Djordjevic 2004).

A sustained increase in ROS generation (OH- radical, hydrogen peroxide, and superoxide anion) leads to OS, which causes results in a distress in cellular metabolism and its regulation, membrane and organelle damage and/or cellular death (Kelly et al. 1998). Environmental pollutants that cause OS can easily modify the balance that exists between pro-oxidants and antioxidant forces (Winston and Di Giulio 1991). These forces are present in aquatic animals to prevent ROS-induced damage (Livingstone 2001).

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

AMD is a complex mixture and of specific concern is the presence of heavy metals such as Al, Cd, nickel Ni, Pb and Hg that causes OS by redox cycling, depletion of GSH levels, or catalysis of the Fenton reaction (Halliwell and Gutteridge, 1989; Valko et al. 2005; Lushchak, 2008; Sevcikova et al. 2011).

In this study the potential use of ROS generation in RTgill-W1, C3A and Vero cell

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lines as bio-indicator of the safety of treated water from streams polluted by AMD was investigated. .

4.2 Materials and Methods

4.2.1 Location and description of the study area

The study was conducted at the receiving stream "Kromdraai" in eMalahleni, Mpumalanga Province, South Africa, located between 25°46' 32.38" S and 29° 01' 23.77" E. The AMD from the mine passes through a wetland into a free flowing receiving stream which is continuously dosed with a neutralizing agent, in-stream, whose aim is to increase the pH of the water, in order to precipitate metals as hydroxides and improve the water quality of the AMD.

In situ measurements of temperature, dissolved oxygen and pH were conducted at the two sample collection points along the course of the stream. Temperature, dissolved oxygen and pH were measured using a portable multimeter (HACH HQd, USA). The water samples collected immediately after flowing through the wetland (undergoes passive treatment) were named the "untreated AMD water sample" (U), while the water samples collected downstream beyond the dosing tanks (which continuously supplied the neutralization agent) were termed the "post treated AMD water sample" (T). Samples were collected as subsurface grab samples, and placed on ice immediately for transport back to the laboratory for analysis. Water samples were analyzed by the Analytical Services, Chemistry Department, CSIR, Pretoria for the following elements: ammonia, orthophosphate, phosphate, nitrate and nitrite, aluminium, arsenic, chromium, copper, iron, mercury, silicon and zinc were determined according to the methods of the American Public Health Association (APHA), 1995. Water samples for bioassays were filtered through a 0.22-µm in-line filter within 2h of collection and were stored at 4°C at the Faculty of Veterinary Science, University of Pretoria.

4.2.2 Cell cultivation

The C3A cell line (derivative of HepG2) was purchased from the American Type Culture Collection (ATCC® CRL-10741™) and the Vero monkey kidney cells (sourced from the collection of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science,

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University of Pretoria) were grown in closed, filter-cap flasks at 37°C in Minimal Essential Medium (MEM, Sigma-Aldrich®, USA). Vero monkey kidney cell culture medium was supplemented with 0.1% gentamicin (Virbac, PHENIX, SA) and 5% foetal calf serum (FCS, Highveld Biological® South Africa]), whilst the C3A cells were grown in antibiotic free medium supplemented with 10% FCS and 1mM sodium pyruvate (Sigma-Aldrich®, St. MO, USA). Cells of a sub-confluent culture were harvested and centrifuged at 200 x g for 2 min, and re-suspended in growth medium supplemented as above, and 100 µl (5 x 10^4cells/ml) were plated in each well of columns 2 to 11 of a 96-well microtitre plate. The plates were incubated for 24 h at 37ºC in a 95% air 5% CO_2 humidified environment.

The RTgill-W1 cell line (ATCC® CRL2523™) was cultured in Leibovitz's L15 medium supplemented with 12% fetal bovine serum (FBS, Highveld Biological® South Africa) in atmospheric air at 20ºC. Cells from a sub-confluent culture were re-suspended in cell culture medium supplemented with 10% FBS and 2% penicillin-streptomycin (5000 U/l penicillin, 5000 µg/ml streptomycin, Gibco® Life Technologies, USA).

4.2.3 DCFH-DA assay for ROS generation

On the day of the assay, 50 µl of 75 µM DCFH-DA was added to 100 µl of medium per well to give a final concentration of 25 µM DCFH-DA. After the cells were exposed for 45 min in 95% air 5% CO_2 at 37°C for the C3A and Vero monkey kidney cells, and at room temperature in atmospheric air for the RTgill-W1 cells, the excess extracellular DCFH-DA was removed by two washes of phosphate buffered saline (PBS).

In order to assess ROS formation, the adherent cells with intracellular DCFH-DA were exposed to varying concentrations of AMD from the untreated and treated AMD samples. Dilutions of 100% (whole effluent); i.e. 75%, 50%, 25%, 12.5% 6.25%, 3.125% 1.562% and 0.781% were prepared using ultra-pure Milli-Q water that also served as the negative control. For the positive control, 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) was used at 4 mg/ml dissolved in Milli-Q water. AAPH is a water-soluble azo compound, which is used extensively as a free radical generator; it is used often in the study of lipid peroxidation and the characterization of antioxidants (Liégeois et al. 2000). Following the breakdown of AAPH, molecular nitrogen and two carbon radicals are produced which may then combine to produce stable products or react with molecular oxygen to give peroxyl radicals. The fluorescence was measured using a FLUOstar fluorescence plate

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reader (BMG, LABTECH, Germany) with temperature maintained at 37°C using a 485/520 nm excitation/emission filter. The fluorescence from each well was captured, digitized, and stored on a computer using OPTIMA software. Readings were taken at intervals of 120 s in a selected 30-cycle period up to 1h and the data were exported to Excel (Microsoft, Seattle, WA, USA) spread sheet software for analysis.

4.3 Data analysis

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

ROS formation was calculated using the formula:

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

The net change in fluorescence per well was calculated by taking an average of the change in fluorescence over time. The advantage of this method of analysis is that it calculates the net changes in fluorescence, such that the calculated data directly reflects the percentage changes of fluorescence over time from the cells in the same well. This method further cancels out the background fluorescence in each well, and therefore, does not require a “no cell” control. Statistical analyses were performed with StatViewR (SAS Institute, Cary, NC, USA) software, using analysis of variance (ANOVA) followed by Fisher’s Protected Least Squares Difference post hoc test for individual comparisons.

4.4 Results

The pH measured at the two sample collection stations varied considerably (Table 4.1), revealing significant acidification of the water emanating from the wetland i.e. U (passive; natural treatment) into the receiving stream T (active; physical and chemical treatments).

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Conductivity values observed in the U and T (315 and 317 mS/m) water samples were much higher than the recommended guidelines (250 mS/m). The inorganic constituents of the water U and T water had similar levels of total phosphorus, orthophosphate and ammonia.

The AMD water contained metals, Al, Cr, Cu, Fe, Zn, As, Hg, Mn and Si ions Table 4.1). Metal analyses performed on the water samples from the site showed values for Fe, Zn and Mn to be much higher than the maximum recommended values for water to be used for industrial purposes (Government Gazette 1984; Department of Water Affairs and Forestry 2004). Guideline values for wastewater or effluent produced by or resulting from the use of water for industrial purposes for rivers and dams and suitable for protection of aquatic life are also presented in Table 1 (Government Gazette 1984, Department of Water Affairs, Forestry and Environmental Conservation 1980). However this water is often used for drinking, food preparation and therefore the guidelines related the safety of water for domestic use and human consumption is also presented in Table 4.1.

No data were available for Al and Si. The concentrations of As, Hg and Cr were below the recommended guideline levels and with treatment the levels of these metals remained unchanged. The in-stream neutralization process effectively removed Fe, Al, Zn and Si from the water and the levels of Fe and Zn were within the recommended guidelines. Only a slight reduction in Mn and sulphate following neutralization was observed. However, total dissolved solids (TDS) values of Na, Ca, and Mg increased downstream when comparing T and U water samples.

In general, U and T water samples presented higher metal levels than recommended guidelines and this was noticeable for Mn, Al, and Zn.

In this study three cell lines were exposed to serial dilutions of U and T water (Fig 4.2-4.4). For all cell lines an increase in ROS production was observed following exposure to increasing concentrations of AMD water (U and T).

Using whole effluents, the maximal amount of ROS generated was for the untreated AMD water was 14%, 16.3% and 26.5% for the RTgill-W1, C3A and Vero cells respectively. A direct correlation was found between % AMD concentration and % ROS formation for U with a R^2 correlation of 0.912, 0.985 and 0.945 for the RTgill-W1, C3 and Vero cell lines respectively. The rate of decrease in fluorescence with decreasing % AMD concentration was 0.1044, 0.144 and 0.212 for the RTgill-W1, C3A and Vero cell lines respectively. The Vero cell line was the most sensitive to the effects of U water due to the high fluorescence obtained for 100% AMD concentration and gradient.

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Following treatment the maximal amount of ROS generated was for the untreated AMD water was 4.56%, 7.22% and 17.71% for the RTgill-W1, C3A and Vero cells respectively. A direct correlation was found between % AMD concentration and % ROS formation for U with a R^2 correlation of 0.983, 0.9016 and 0.969 for the RTgill-W1, C3A and Vero cell lines respectively. The rate of decrease in fluorescence with decreasing % AMD concentration was 0.0358, 0.0601 and 0.156 for the RTgill-W1, C3 and Vero cell lines respectively. Fold change in gradient U vs T was 2.916, 2.39 and 1.36 for each respective cell line indicating that treatment of AMD water resulted in a decrease in ROS.

4.5 Discussion

Effluent discharge from mining activity impacts water bodies negatively causing a drastic fall in pH, and a resultant increase in the presence of dissolved metals, such as Al, Fe, Zn, Mn, Cu, or Cd (Grippo and Dunson 1996). A number of water parameters fell short of the recommended standards; the South African guideline for conductivity in effluent that could be discharged into the receiving water bodies is 250 µ/Scm (Government Gazette 1984). The observed increase in conductivity indicates the level of pollution (U.S. Environmental Protection Agency 1982). This raises a possible question of compliance, toxicity to water life and suitability for downstream end users.

Aquatic pollutants have the potential to induce the formation of ROS in organisms resulting in OS (Lesser 2006) that can lead to cellular dysfunction and death. The presence of transition redox-active transition metals can initiate a reaction with hydrogen peroxide (H_2O_2) to produce a highly reactive oxygen species (ROS), the hydroxyl radical ($OH^{\cdot-}$) via the Fenton reaction (Walling 1975). Synergistic effects between different metals can cause increased levels of oxidative damage. The concentrations of Al, Fe, Zn, and Mn levels are very high in the effluent water from U (Table 4.1). Of the metals present in the river effluent Fe, Cu, Cr, Ar can generate ROS by redox recycling while Hg depletes GSH and thiol containing antioxidant enzymes. Both processes result in an increase in production of ROS (see review Ercal et al. 2001). Interestingly Zn retards oxidative damage (Gibbs et al. 1985).

With post alkali in-stream treatment there was considerable reduction in heavy metals. Optimal precipitation of Mn has been reported to occur at a higher pH between 9 and 10 (Means 2004). High levels of Mn are associated with ROS induced neurotoxicity (Gavin et al. 1992) because glial cells form *in vitro* findings reveal a high affinity transport mechanism for manganese (Aschner et al. 1992).

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An interesting observation in the AMD was its richness in inorganic salts and sulphate, which may influence bioavailability and toxicity. Higher concentrations of $K^{2+\ (K+)}$ mono ionic, Ca^{2+}, Na^{+} and Mg^{2+} were observed to occur in the AMD, with concentrations increasing downstream after the neutralization treatment.

McCulloch et al. (1993) reported that elevated TDS could also be toxic to freshwater animals by causing osmotic stress and affecting the osmoregulatory capability of the organisms. In the present study following treatment of the AMD water, heavy metal levels were reduced but the levels of TDS increased because conductivity is an index for TDS pollutants (U.S. EPA 1982).

The ability of U and T AMD water to induce oxidative damage in the RTgill-W1 cell line was evaluated. Two mammalian cells lines that represent specific cellular sites of oxidative damage in mammalian species including humans were also included. For all cell lines with treatment, there was a significant reduction in the measured levels of oxidative damage. Contreras et al. (1995) reported a significant increase in ROS and lipo-peroxide levels in seaweeds around the discharges of copper mines while Bopp et al. (2008) reported a 25-35 fold ROS induction in rainbow trout gill cells exposed to copper (total Cu 100 µM, pH 7). However of concern in this study is that although the concentrations of As, Hg and Cr are below the recommended guideline levels, these metals may still cause oxidative damage leading to cellular dysfunction and death.

Since a clear positive correlation has been reported between the number of viable cells and consequent increase in DCF fluorescence (Bopp et al. 2007), it was necessary in parallel to ROS measurements, to investigate cytotoxicity (results not shown) so that, exposed cells do not become damaged from cytotoxic effects and as such underestimate ROS formation.

The rise in pH of the stream achieved using in-stream alkali neutralization process results in complexation phenomena and consequent metal hydroxide precipitates (Madeira et al. 2005). A decrease in toxicity of effluent from an abandoned uranium mine was reported to be concurrent with an increase in pH of the effluent (Franklin et al. 2000). This may account for the reduced toxicity in effluent water T, as % ROS generated using whole effluent showed a decrease from 26.5% to 17.7% in Vero monkey kidney cells, in C3A cells from 16.3% to 7.2% in C3A and 14% to 4.5% in RTgill-W1 cell lines. Water chemistry values for metals such as Al, Fe, Zn and Si reduced by 21, 19, 146 and 4.5 fold respectively in effluent water T (Table 4.1).

Consistent with these findings were those reported by Moore (1992) where increased

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ROS production was seen in isolated hepatocytes of dab (*Limanda limanda*) from polluted sites compared to cleaner sites in the German Bight, North Sea. Schlezinger et al (2000) and Choi and Oris (2000) likewise reported increased ROS production involving a number of water borne contaminants like duroquinone (component of pulp mill effluent) and benzo[a]pyrene (BaP) diones.

The use of *in vitro* contaminant-stimulated ROS production has been demonstrated in a number of species for a number of contaminants (Livingstone 2001). In this study the RTgill-W1, C3A and Vero cells lines were used and represented fish, human and monkey species as well as being representative of specific organ systems. From this study, the Vero cell line appeared to be the most sensitive to AMD water as it generated greater ROS especially at higher effluent concentrations (Table 4.2) and this may be that the kidney is an important target organ related to the toxicity of heavy metals such as cadmium (Gardner et al. 2006; Barbier et al. 2009). In addition, the RTgill-W1 cell line showed the greatest change, a 3.48 fold change in gradient when the effect of U on ROS formation is compared to T. The RTgill-W1 cell line is derived from the epithelium of the fish gills, which is the first barrier against toxicants entering the fish body (Wood 2001). Pandey et al. (2008) observed that exposure to a mixture of heavy metals that included Cu, Cd, Fe and Ni caused a decrease in the levels of antioxidant enzymes, GSH as well as an increase in lipid peroxidation, which is associated with radical damage in fish gills. Koutsogiannaki (2006) also reported that a decrease in GSH occurs following multi-metal exposure on gills, because heavy metal cations have a great propensity for the –SH group of GSH (Viarengo 1993).

This study showed that the water quality parameters of the receiving stream were adversely impacted by AMD discharge and the presence of metals in the water was at environmentally relevant concentrations was detectable at cellular levels in cell lines through transient increases in ROS production for both U and T, AMD.

Since biological diversity and the physiological state are direct indices of water quality, *in vitro* techniques involving cellular ROS generation may serve as an alternative relevant model for evaluating consequences of exposure to environmental pollutants.

This model system using cell lines that represent the target tissue of toxicity can be routinely implemented and is a fast, easy and straightforward manner and to measure the possible consequence of exposure to environmental pollutants. A limitation is that there is no standard cutoff level for ROS formation, which indicates the degree of safety.

Together with other assays related to environment health research, may provide a plethora of interesting research opportunities. Although this technique in determining ROS

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levels in multicellular organisms, particularly aquatic animals, is seldom used, we propose that this method could be applied in conjunction with water chemistry analysis of polluted streams as early onset detection of OS.

4.6 Acknowledgements

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Table 4.1: Water chemistry analytes of AMD water samples collected at Kromdraai, Mpumalanga, South Africa compared to guideline values (Government Gazette 1984; DWAF 1996, 2002, 2007) of effluent produced by or resulting from the use of water for industrial purposes and South African water guidelines for domestic use.

Metals present as ions dissolved in water					
Analysis	**Unit**	**Untreated AMD**	**Treated AMD**	**Guidelines for industrial use**	**Guidelines for domestic use**
Potassium (K^+)	mg/l	12	13	0.1	N/A
Sodium (Na^+)	mg/l	57	130	N/A	100
Calcium (Ca^{2+})	mg/l	500	552	N/A	N/A
Magnesium (Mg^{2+})	mg/l	153	169	N/A	170
Ammonia (NH_4^+)	mg/l	3.6	3.6	1	N/A
Sulphate (SO_4^{2-})	mg/l	2329	2072	1400	200
Chloride (Cl^-)	mg/l	5	5	N/A	250
Alkalinity ($CaCO_3$ levels)	mg/l	5	25	20	20-300
Nitrate & nitrite (NO_3^-)	mg/l	4	6.6	1.5	6
Orthophosphate (PO_4^{3-})	mg/l	0.2	0.2	1	N/A
Total phosphorus (PO_4^{3-})	mg/l	0.2	0.2	N/A	N/A
Aluminium (Al^{3+})	mg/l	6.9	0.32	N/A	N/A
Chromium ($Cr^{2+/3+}$)	mg/l	0.01	0.01	0.05	N/A

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Copper ($Cu^{+/2+}$)	mg/l	0.01	0.01	0.02	N/A
Analysis	**Unit**	**Untreated AMD**	**Treated AMD**	**Guidelines Industrial**	**Guidelines Domestic**
Iron ($Fe^{2+/3+}$)	mg/l	0.38	0.02	0.03	0.1
Manganese ($Mn^{2+/3+}$)	mg/l	75	34	0.1	0.05
Zinc (Zn^{2+})	mg/l	4.1	0.028	0.3	1
Arsenic (As^{3+})	mg/l	0.001	0.001	0.1	0.1
Mercury (Hg^{2+})	mg/l	0.001	0.001	0.02	0.005
Silica ($Si^{4+/4-}$)	mg/l	11	2.4	N/A	N/A
Electrical conductivity	mS/m 25˚C	314	317	250	70
pH	at 20˚C	3.67	8.41	5.5 – 7.5	6-9
Chemical oxygen demand (COD)	mg/l	10	10	30	N/A
Dissolved oxygen (DO)	mg/l	6.05	2.70	5.8	N/A

* N/A = not available

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Table 4.2: Cell line with highest sensitivity to untreated and treated AMD

% AMD water	Untreated	Treated
100	Vero	Vero
75	C3A /Vero/RTgill-W1	Vero
50	Vero	Vero
25	Vero/C3A	Vero/C3A
12.5	C3A /Vero/RTgill-W1	Vero
6.25	RTgill-W1	Vero
3.125	RTgill-W1	Vero/C3A
1.562	C3A /Vero/RTgill-W1	Vero/C3A
0.781	C3A /Vero/RTgill-W1	Vero
*Statistical significance determined using one way ANOVA with $p < 0.05$		

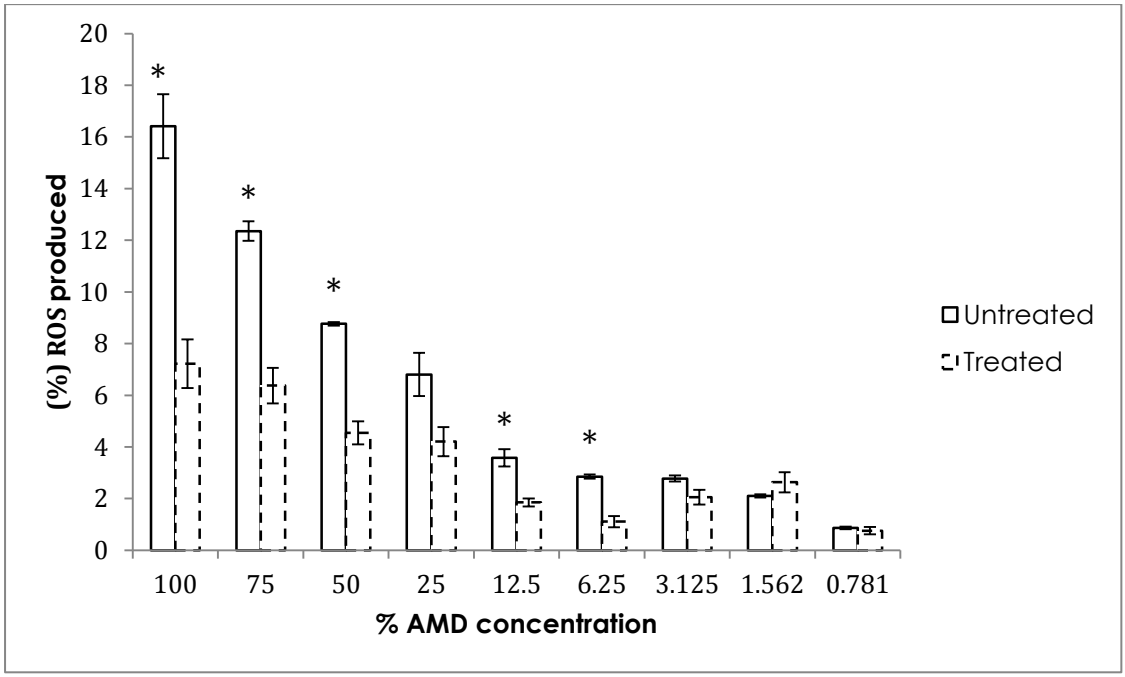

Figure 4.1: Percent (%) ROS produced in C3A cells. Data is an average of 9 data points ±SEM

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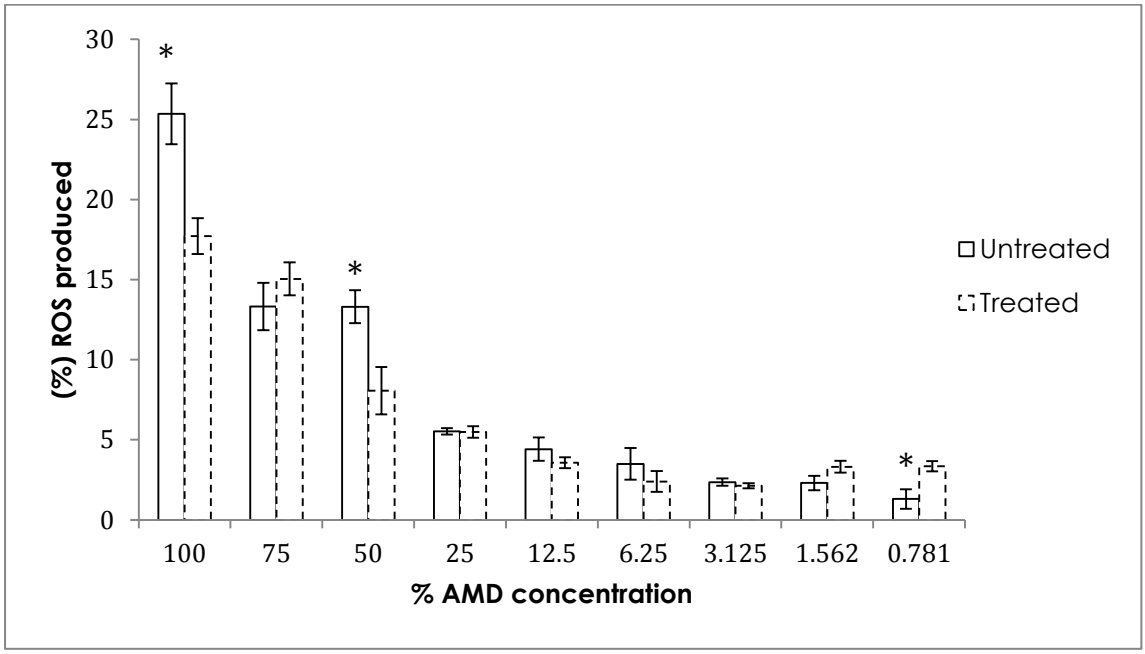

Figure 4.2: Percent (%) ROS produced in Vero cells. Data is an average of 9 data points ±SEM.

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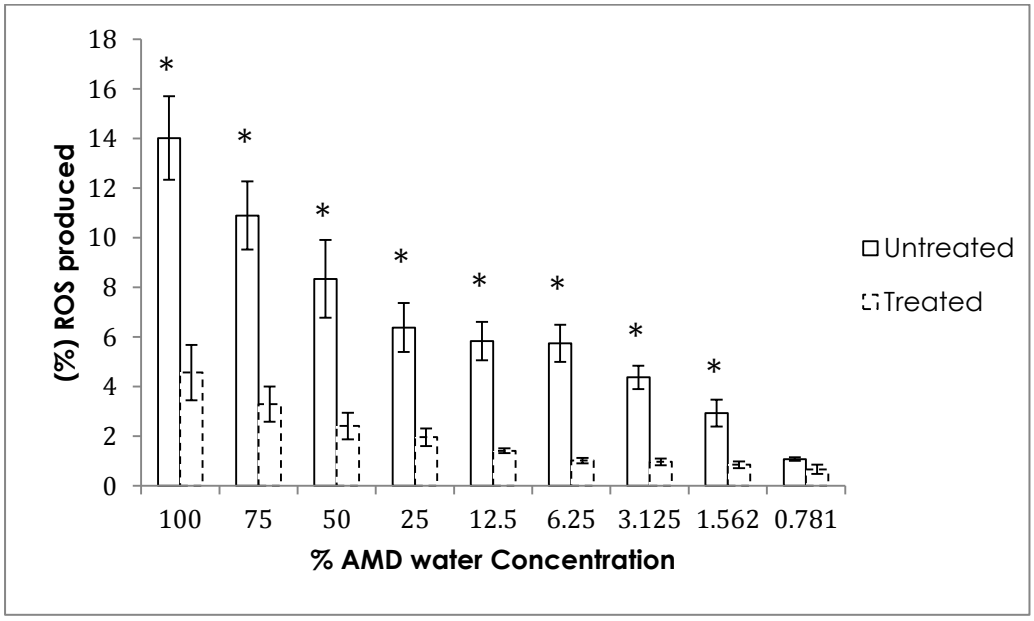

Figure 4.3: Percent (%) ROS produced in RTgill-W1 cells. Data is an average of 9 data ±SEM.

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CHAPTER 5: Assessing the potential of cell lines as tools for the cytotoxicity testing of acid mine drainage effluent impacting a natural water resource

Preface

This chapter was prepared for submission to the ISI-rated journal Environmental pollution and Research.

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Abstract

The present study focused on a water compartment impacted by acid mine effluent from a coalmine. Effluent toxicity was evaluated for *in vitro* cytotoxicity against mammalian (Vero) and fish (RTgill-W1) cell lines, while conducting a parallel physical and chemical characterization of the stream. Metal analyses from water samples showed that values in some cases, especially regarding Al, Mn, Fe and sulphates, were much higher than the maximum recommended values established by South African legislation regarding effluent in receiving streams. Two water collection points were selected in the characterization of the impacted water column: one immediately following a wetland (U) and another downstream (T) of the mixing zone where alkali is used to increase water pH to reduce acidity and precipitate metals. Another unaffected site served as the reference site (Ref).

Cell viability assays were employed to detect effects of water samples on cells following exposure for 24h, 48h and 72h, namely the neutral red (NR), tetrazolium-based (MTT) colorimetric assays and the lactate dehydrogenase assay (LDH) which assess lysosomal, mitochondrial functions and cellular necrosis respectively. Toxicity was detected in U and T water samples against both cell lines using the NR and MTT techniques, being maximal at 72h with IC_{50} values of 23.9% and 20.5% (MTT), and 32.2% and 49.4% (NR) for Vero cell lines (U vs. T). IC_{50} values for the RTgill-W1 cell lines were 8.4% and 7.19% (MTT), and 10.5% and 35.3% (NR) for U and T respectively, but no cytotoxicity was recorded for the water collected from the

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reference site. These assays were complemented with fluorescence microscopy and transmission electron microscopy for visualization of effects of the water samples against the cell lines. However, we observed no effects on viability expressed as LDH leakage in the cells.

Results indicated differences in the cytotoxic potential between both cell lines with the different assay techniques employed, and the MTT assay was more sensitive than the NR. The loss of cell viability resulting in damage to cellular functions gives rise to concerns regarding the potential risks for aquatic animals, as worldwide increases in AMD are a threat to aquatic animal health. The same holds for downstream end users exposed to point source discharges of the untreated and treated effluent from the coal mine.

5.1 Introduction

In assessing the ecological risk of contaminated sites, a sequence of events involving data collection is recorded in order to identify chemicals of potential concern, their concentrations in different environmental compartments, and the risk associated with such chemicals, so as to reduce uncertainties regarding toxicity (Weeks et al., 2004). In the context of pollution, aquatic ecosystems are the most unprotected because they serve as reservoirs for chemicals from surrounding terrestrial ecosystems, and organisms within this ecosystem are usually submerged in "various exposure medium" within their habitat (Clements and Newman, 2002). One of the most disturbing drivers of change in the aquatic ecosystem is human activity. Globally, humans play huge roles in shaping and disturbing stream ecosystems (Resh and Grodhaus 1983; Younger, 2001). Mining activity has been identified as one contributing to severe water pollution because of the generation of acidic waters (acid mine drainage or AMD) resulting from mining activities and, most importantly, the presence of heavy metals in such waters (Rahn et al., 1996; Bleise et al., 2003; Chen et al., 2004). The presence of heavy metals in high concentration and acidic pH values negatively affect aquatic ecosystems (Soucek, 2000). Usually in the aquatic environment, toxicity of pollutants does not occur due to individual compounds but from mixtures (e.g. AMD complex), and the combined effects of mixtures of compounds could result in mixture toxicity (Backhaus et al., 2000; Faust et al., 2001).

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Coal mining is a huge industry in South Africa (Mangena and Brent, 2006), with approximately 220 Mt coal produced per year (DME, 2004). A large proportion of coal mines is located in the Mpumalanga Province, where coal mining operations (existing and historic over the past 100 years) comprise over 10 000 km^2 of hydraulically interlinked coal mines hosting eight of the ten operational coal-fired power stations, and supplying 83% of the total produced coal in the country (Vureen, 2009). Extensive mining activities in the catchment of Mpumalanga Province have been reported to alter the hydrodynamics of most rivers, and threaten the aquatic ecosystems due to acid mine water production (de Villiers and Mkwelo, 2009). In the upper Olifants River, there are a number of reports of contamination of water through pollution of AMD (Adler et al., 2007; Driescher, 2008), with resultant fish and crocodile die-offs (Paton, 2008).

AMD is usually highly acidic (pH between 2.0 and 4.5) and high in metal concentrations. It contains elevated sulphate levels and excessive suspended solids and/or siltation, all of which cause toxicity (Campbell and Stokes 1985). During mining processes, the host rock undergoes sulphide weathering and the acidity causes dissolution and release of acidic metal-laden water into environmental compartments, causing mortality of aquatic organisms in streams impacted by mine drainage (USEPA, 2006). The US Environmental Protection Agency's Clean Water Act considers heavy metals such as zinc, mercury, lead, cadmium, magnesium, aluminum, copper, thallium, as well as other primary pollutants (PPE) to surge in concentration at sites situated close to mining areas (Alberta and Badillo, 1991; Debenest et al., 2012; Kelly et al., 2010).

Natural treatment processes involving wetlands have been utilized to improve water quality in point and non-point sources of water pollution. The USDA and EPA (2002) reported over 500 such systems operating in Appalachia alone used to manage AMD from active and abandoned coal mines. The wetlands provide a dense growth of vascular plants that soak up and regulate water flow, create microenvironments within the water column, and provide attachment sites for microbial communities. Other active treatment process for AMD abound with the most common being neutralization, which corrects pH and precipitates metal, giving the appearance that

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the effluents are non-toxic (Madeira et al., 2005). Such effluents are subsequently often discharged into receiving streams (Akcil and Koldas, 2006). In some instances, treatment technologies may become insufficient or uneconomical to maintain, and as such, a considerable amount of untreated AMD is left (Diz, 1997). One way to evaluate the efficacy of such systems is by physical and chemical monitoring of the quality of the effluent, alongside the use of biological indicators because they respond to environmental changes on a short-term, as well as on a long-term basis (Burgman and Lindemayer 1998; Mota Marques et al., 2000; Ji et al., 2004).

A number of test species such as algae, crustaceans, insect larvae and fish are commonly used in aquatic ecotoxicology (OECD, 1998, 2000; Taylor et al, 1991; Environment Canada, 1992; USEPA, 1994; ASTM, 1997, 2000). All of these employ the use of whole animals (Buikena and Cairns, 1980), involving procedures in which experimental protocols are extended, tedious, expensive, and require the use of a large number of animals. There is an increased pressure to reduce, replace and refine animal use in research and toxicological studies (Castaño et al., 2003) and the need for alternative suitable biomonitoring methods for evaluating contaminant risk and hazards in the environment cannot be over-emphasized. *In vitro* techniques as alternative systems in toxicity assessment are not new concepts in research with the use of mammalian cell lines to monitor water quality being previously reported (Mochida, 1986; Kfir and Prozesky, 1981; Schirmer et al., 2001). Cell cultures, derived from fish, have been effectively used as a biological substitute to the use of whole animals (Segner, 1998; Strmac et al., 2000; Schirmer, 2004; Leguen et al., 2011 Chasiotis and Kelly, 2011; Chasiotis and Kelly, 2011b; Chow and Wong, 2011; Chasiotis et al., 2012; Kolosov and Kelly, 2013). The principle behind this is that it provides a platform to evaluate responses to toxicants at molecular and cellular levels, and these can be monitored in a controlled environment, usually in isolation from multiple physiological systems modulating such cells *in vivo* (Castano et al., 2003). This technique hinges on the fact that functional and structural changes are caused by pollutants at cellular levels within the organism (Larsson et al, 1985) and consequently, pollutants will exert their effects by interacting/ interfering with basic cellular functions.

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In this study, to evaluate the cellular responses of cell lines following exposure to toxicants present in effluent from a coalmine, subjected to passive or active treatment, toxicity assays were carried out on mammalian Vero and fish RTgill-W1 cell lines. This study addresses an ecotoxicological evaluation of a natural flowing stream impacted by AMD effluent from a coal mine, whose effluent was subjected to both passive (wetland) and active treatment (neutralization) methods. Since biomonitoring is regarded as the prime tool needed to adequately protect aquatic life (Herricks and Schaeffer 1985), *in vitro* cytotoxicity assays, involving multiple endpoint measurements of cellular responses to contaminants was used to detect cytotoxicity of AMD water to both Vero and RTgill-W1 cell lines. Lysosomal function was determined using the Neutral Red (NR) assay, while mitochondrial function was assessed with the use of the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromides) reduction assay, and necrosis through the LDH assay. A parallel physical and chemical characterization of the stream was done in conjunction with the cytotoxicity assay to assist in interpreting any observed cytotoxicity.

5.2 Materials and Methods

5.2.1 Location and description of the study area

The study site chosen was a receiving stream established downstream of a colliery, Kromdraai (25°46’ 05.5” S; 29° 07’ 15.5” E) in the Highveld region, close to eMalahleni (previously known as Witbank) in Mpumalanga Province. The acid effluent from the mine passes through a wetland into a free flowing receiving stream and two water collection points were chosen along the course of the stream. The water sample collected prior to treating with a neutralizing agent (caustic soda) immediately flowing from the wetland, was termed the **untreated (U),** whilst the one collected downstream, beyond the point of an in-stream, neutralizing agent which is connected to a dosing tank and monitored telemetrically, was termed **treated (T**).

A reference site characterized by slightly acidic pH (6.5), with absence of any metal or other anthropogenic pollution and most importantly, outside the influence or

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

5.2.2 Water chemistry

Water samples were analyzed by the Analytical Services Chemistry Department, CSIR, Pretoria, for their physicochemical parameters. Nitrate, orthophosphate, chloride, ammonia, nitrogen and sulphate were measured using automated flow injection analysis. Heavy metals in their dissolved states were analyzed (Al, Cu, Mn, As, Si, Fe and Zn) using the inductively coupled plasma atomic emission spectrometry (ICPAES). Calcium, magnesium, potassium, phosphate and sodium were measured likewise using the ICPAES methods. Alkalinity was determined using the potentiometric titration and chemical oxygen demand calorimetrically, all according to standard methods (APHA, 1995; APHA et al., 2005).

5.2.3 Collection and preparation of samples

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

In situ measurements of temperature, dissolved oxygen and pH were conducted at the collection points along the course of the stream. Temperature, dissolved oxygen and pH were measured using a portable multimeter (HACH HQd, USA).

Powdered media was reconstituted with whole water samples before being exposed to cell cultures. Powdered Minimum Essential Medium (MEM) (Gibco® Life Technologies ™, UK) was used for Vero cells and Leibovitz medium (L-15M Sigma-Aldrich, St. Louis, MO, USA) was employed for RTgill-W1 cell lines. Water samples were pH-adjusted using a pH meter (Thermo Fisher Scientific Inc.) and samples filtered using a 0.22-µm-membrane filter (Acrodisc®syringe filter, Life Sciences,

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USA). The osmolality of water samples measured following reconstitution by powdered media (Model 3320 Osmometer, Advanced instrument INC (Massachusetts, USA) were found to be within the range conducive for cell viability. Control media reconstituted with sterile tissue culture water (Milli-Q^{50} France) was supplemented to ensure the availability of essential nutrients using 5% fetal calf serum (FCS, Highveld Biological, South Africa). Cell cultures from the Vero cell line and the RTgill-W1 cell lines were exposed to a range of concentrations (3.75%, 7.5%, 15%, 30%, 45%, 60%, 75%, 90% and 100%) using control medium as diluent. For comparison for each assay, the known cytotoxic compound doxorubicin hydrochloride (Pfizer Laboratories Pty Ltd, SA) reconstituted to 100 µM stock solution served as the positive control, using concentration ranges (0.07%, 0.15%, 0.30%, 0.625%, 1.26%, 2.5%, 5%, 10% and 25% (v/v)). Cells of a sub-confluent culture were plated in a 96-well microtitre plate at a density of 100 µl of 1 x 10^5 cells /ml / well for Vero cell and 100 µl of 2 x 10^5 cells /ml / well for the RTgill-W1 cell lines were incubated for 24h at 37ºC in a 5% CO_2 humidified environment and 20ºC in an air incubator respectively before exposure to water samples. The duration of exposure for the cells were 24h, 48h, and 72h after which cell viability was determined.

5.2.4 Cell viability assay

Two fluorescent indicator dyes, namely MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) and neutral red (Sigma Chemical Co) were used to evaluate cell viability in Vero and RTgill-W1 cultures. The dyes are readily available, relatively easy to use and inexpensive. The intensity of colour (measured spectrophotometrically) of the MTT formazan produced by living, metabolically active cells is proportional to the number of live cells present, therefore, a decline in readings indicates a reduction in cell metabolism and a loss of cell membrane integrity (Mosmann, 1983). The MTT assay was performed as described by Mosmann (1983). The neutral red assay provides a measure of lysosomal function, and was performed as described by Repetto et al. (2008). Absorbance was measured using a microplate reader (BioTek®Synergy HT-BioTek Instruments, Winooski, USA). Wavelengths were 570 and 690 nm for MTT, and 530 and 645 nm for neutral red. Experiments were conducted in quadruplicate and were repeated at least four

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times.

5.2.5 Lactate dehydrogenase activity

The lactate dehydrogenase (LDH) kit purchased from Roche® was used to assess cytotoxicity following exposure to effluent for 24h, 48h and 72h exposure. Leakage into the culture medium was assayed by collecting the culture supernatant and incubating with the reaction mixture. The LDH activity was determined as an enzymatic assay that involved NAD^+ being reduced to NADH/H+ by conversion of lactate to pyruvate. The H/H+ from NADH/H+ is then transferred to the tetrazolium salt INT that is reduced to formazan and absorbance is measured at 490 nm.

5.2.6 Transmission electron microscopy

Samples of RTgill-W1 cells incubated for 72h after each treatment (Media control, Ref, U and T) were fixed at 4°C according to Iger et al. (1995) for electron microscopy. The cells were dehydrated and embedded in Araldite 6005 resin, after which ultrathin sections were contrasted with uranyl acetate and lead citrate. The samples were examined in a Philips transmission electron microscope at an accelerated voltage of 80 kV.

5.2.7 Phase contrast microscopy analyses

Cells incubated for 72h in different water sample treatments were observed for mitochondrial activity. Rhodamine 123® (Sigma-Aldrich, USA) was used as a probe for identifying viable mitochondrial cells. Rhodamine 123 is a cationic lipophilic dye that compartmentalizes into the low electrochemical potential of mitochondrial membranes. Active mitochondria in viable cells stain bright green while non-viable cells result in loss of the dye due to loss of gradients within the cells. Similarly, using MitoTracker®Green FM (Molecular Probes, Cedarlane Laboratories, Hornby, Ontario, Canada), selected preparations were examined for the presence of mitochondria-rich cells. Procedures for the preparation of MitoTracker® Green FM and staining of cultured preparations have previously been described (Kelly and Wood, 2001).

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5.2.8 Statistical analysis

The raw data from cytotoxicity assays were collated and analyzed using Microsoft Excel. Average absorbance readings of the treated plates (four wells per concentration) were then expressed as a percentage of the average readings of the untreated control. To determine cytotoxicity, the mean percentage inhibition relative to the unexposed control ± standard deviation (SD) was calculated using the formula [100 ((Mean Experimental data/Mean Control data) * 100)]. Control values were set at 0% cytotoxicity and 100% viability. Statistical analyses were carried out using one-way analysis of variance (ANOVA) followed by Hartley's f-test for equal variance. Inter- and intra-test variations were assessed using a two-way ANOVA or the appropriate Students T-test. This data analysis was performed using OpenEpi, open source calculator Version 3. Cytotoxicity data was fitted with an appropriate model and IC_{50} values were calculated using linear regression. To test for significance in IC_{50} values between the two cell lines, an unpaired *t*-test was used ($p \leq 0.05$). Significance was set at $p \leq 0.05$. If significance was found, the data were further examined with post-hoc T-tests.

5.3 Results

Water quality parameters of the studied sites and TWQR values are presented in Tables 3.1 and 3.2. Physico-chemical parameters of Ref were generally more or less similar to target water quality range (TWQR) and as such, it is not surprising that no adverse cytotoxic effects were observed in cells exposed to water samples from this site. Water chemistry results (Table 3.1) showed that possible potential contaminants such as metals, salts and sulfates were not present at levels that could be of environmental concern and were lower than suggested TWQR values.

The electrical conductivity values observed for U and T were much higher (around 320 mS/M)) than those recorded in Ref (5 mS/m), and the pH of U was acidic (pH = 3.65). In terms of their inorganic constituents, parameters such as ammonia, orthophosphate, chloride, total phosphorus, nitrate / nitrite showed values that were more or less similar for U and T, marginally increased values were seen for Mg and K (U vs. T), while Na, Ca and sulfate levels increased in T compared to U (~ 2.79X; ~

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1.18X and~ 1.03X) respectively. These values were several folds higher than those of the Ref and the TWQR values. Metal analyses performed in the water samples from the sites showed values that, in some cases, were much higher than the TWQR for aquatic ecosystems (DWAF, 1996) (Table 3.2). In general, U and T presented higher metal levels than were observed for Ref and the recommended TWQR for effluents in natural water resources. However, a noticeable decrease in metal concentration was observed for water samples following in-stream neutralization, and this difference was seen for metals such as Al ~(127X), Mn (1.66X), Fe (36X), Si (4.5X) and Zn (72X), in effluent T compared to U.

No cytotoxicity (% loss in cell viability) was found for Ref site water samples against both cell lines (Vero and RTgill-W1) at all concentrations tested (100% to 3.75%) within the different exposure periods; 24h, 48h and 72h employing both the neutral red (NR) and tetrazolium-based (MTT) colorimetric assays (Fig A). As such, it was not possible to calculate the IC_{50} for the reference site water samples because cell viability was close to 100% at all concentrations tested.

Water samples from impacted receiving streams, however, resulted in loss of cellular viability in both cell lines exposed to U and T AMD water samples ($p< 0.05$) at the 48 and 72h exposure periods respectively. AMD water samples resulted in a dose–dependent decline in cell viability in Vero and RTgill-W1 cell lines. Effluent water was acutely toxic to both Vero and RTgill-W1 cell lines as evidenced by decreased cell viability, which varied with duration of exposure and was maximal at 72h (Fig 5B-C). Exposure to U and T decreased cellular viability in Vero cell lines and RTgill-W1 cells within 24h, but did not result in a 50% loss in cell viability.

Significant linear relationships, with slopes close to 1, were found when the IC_{50} values were derived. For Vero cells exposed to U, obtained IC_{50} values were 48h IC_{50}, = (43.7%; 52.9%), and 72h IC_{50}, = (23,9%; 32,2%) for MTT and NR assays respectively. The IC_{50} values obtained for RTgill-W1 cells exposed to U were 48h IC_{50}, = (25.2%; 51.4%), and 72h IC_{50}, = (8.4%; 10.5%) for the MTT and the NR assays respectively (Table 3.3). Cellular toxicity was detected likewise within 48h and 72h exposure periods in (T) effluent exposed to Vero and RTgill-W1 cell lines and similarly, not within a 24h exposure period because IC_{50} remained higher than 50% at

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whole effluent concentration tested. From the water chemistry analysis (Tables 3.1 and 3.2), T contained lower metal concentrations but higher total dissolved solids (Ca, Mg, K, Na and sulphates). Obtained IC_{50} values were numerically lower, but not significantly different across all concentrations tested in both cell lines when comparing U and T effluent. Obtained IC_{50} values for Vero cells exposed to T using MTT and NR assays respectively were 48h Vero IC_{50}, = (28.8%; 64%) and 72h EC_{50}, = (20.5%; 49.4%), while results for RTgill-W1 were 48h EC_{50}, = (19,8%; 43,1%), 72h EC_{50}, = (7.2%; 35.3%).

Comparing cell viability in cells exposed to U and T (Fig D) after similar exposure periods, either 48h or 72h, showed significant differences ($P<0.05$) in cell viability between Vero and RTgill-W1 cell lines within most of the concentration range tested. To determine the role that duration of exposure has on either cell line while comparing similar assay techniques (i.e. either MTT or NR assays) (Fig 5E), showed that IC_{50} values were significantly lower ($P<0.05$) after 72h exposure compared to 48h, leading to assumptions that cell viability reduced with time following exposure to effluent. A difference in sensitivity of cells was likewise observed (Fig 5F) when comparing both assay techniques within the same exposure period. The RTgill-W1 cell line exhibited greater sensitivity (reduced cell viability and enhanced cytotoxicity) to effluent U and T detected using the MTT assay technique when compared to the Vero cell lines. When comparing the effectiveness / interacter agreement between the two colorimetric techniques employed, both techniques detected loss in cell viability in a dose-response fashion, but the MTT IC_{50} values in Vero and RTgill-W1cells showed values that were significantly lower than those obtained for the NR assays after the same duration of exposure ($P<0.05$). Significant differences in cell viability were observed in both Vero and RTgill-W1 cell lines within exposure periods of 48h and 72h and between the two assay techniques employed, i.e. MTT vs NR.

Cytotoxicity determined with the LDH leakage assay (data not shown) in contrast to the MTT or NR did not result in apparent cytotoxicity when used for the same incubation periods, as no significant differences in LDH activity were detected among any of the treatments compared to the control.

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The photomicrograph of RTgill-W1 cells showing mitochondrial fluorescence using Mitotracker® presented with lower cell density and fewer fluorescing mitochondria in cells exposed to U and T compared to controls; Ref (Fig 5G). Similarly,, a difference in cell density was observed (not shown) in both cell lines exposed to U and T using Rhodamine® for mitochondrial florescence and photomicrograph of exposed cells showing cell density with the NR dye.

Transmission emission microscopy (TEM) images of cells (RTgill-W1) exposed to U and T for 72h (Fig 5H) showed that mitochondria played a role in observed toxicity. Ultrastructurally, mitochondria of cells exposed to U and T displayed morphological alterations, as they became progressively more swollen and in some cases, rupture of outer mitochondrial membranes was observed.

5.4 Discussion

In this study, Vero monkey kidney and RTgill-W1 (derived from fresh water fish) cell lines were used as experimental models for the i*n vitro* assessment of AMD effluent from a coalmine impacting a natural water resource, by assessing its potential cytotoxic effects on these cell lines. These cell lines can respond to certain contaminants in water and display a dose-dependent relationship to toxicants present in polluted AMD water. AMD is known to contain a complex mixture of potentially toxic metals, salts and sulfates, usually at concentrations that pose a risk to the environment and aquatic organisms (Campbell and Stokes 1985; Madeira et al, 2005). The results obtained from the cytotoxicity assays indicate that exposure of RTgill-W1 and Vero cultures to increasing concentrations of the effluent brought about a progressive loss of cell viability as assessed by indicator dyes. The MTT and neutral red assays commonly used in *in vitro* cytotoxicity assays for screening of chemical test agents revealed profound cellular damage in mitochondrial membrane integrity and reduced lysosomal activity on exposure to AMD water. Associating the cytotoxicity of industrial effluent to cell lines is of eco-toxicological importance as it offers alternative monitoring strategies to the use of whole animals (Environment Canada, 1990, Castaño et al., 1994; Hollert et al., 2000; Dayeh et al., 2002) because they are fast, rapid and cost effective and align with the principles of replacement, reduction and refinement in research (Hutchinson et al, 2003). They also satisfy

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ethical concerns associated with use of animals as a conservative estimate of 1 million fish in the EU Member States and about 3 million in the US each year are killed for research and regulatory purposes (Castaño et al., 2003; Tanneberger et al., 2013).

In this study, chemical and cytotoxicity tests were used to obtain information from polluted AMD sites compared with a cleaner site. Test sites had differences in water column (Tables 3.1 and 3.2) in terms of pH, metal load, sulphates and presence of salt. A dose effect relation was observed for the concentrations tested (except for the reference site) and 50% inhibitory concentrations were calculated for each of the cell lines exposed to AMD effluent. The cell lines showed differences in cytotoxic responses based on potential contaminants present in the water samples. Using the water sample, Ref, from the cleaner site indicated there were no losses in cell viability in both cell lines at all concentrations tested (Fig 5A). Physicochemical water analyses showed that a wide range of contaminants usually present in polluted AMD waters were absent in Ref samples (Tables 3.1 and 3.2). However, an indication that toxic contaminants may have a localized effect within the AMD sites was reinforced by the results obtained in the bioassays. For the Vero and RTgill-W1 cells**,** cytotoxicity assays showed substantial deleterious effects of the water column on cell viability, reducing cell viability by at least 50% within a 48h exposure period (MTT, NR). This indicates that heavy metals and/or other contaminants measured were bioavailable in the water samples at concentrations sufficient to cause toxicity. The presence of Mn, Fe, Al, Si and Zn at such concentrations observed in our study site suggested that these metals may present a risk to aquatic freshwater organisms, because bioaccumulation of toxic compounds (such as metals) and their direct exposure in the water column can result in abnormalities in aquatic organisms (Carls et al, 1999; Heinz et al, 1999). A report by the Water Research Commission confirmed Al, Fe and Mn were present at elevated concentrations in many surface waters within and around our study site due to coal mining activities (WRC, 2009). The metals were analyzed in their dissolved states and concentration of metals in their free ionic state were reported to express direct toxic effects (Stumm and Morgan, 1996) as metals occurring in high concentrations are toxic to aquatic animals (Kimmel, 1983). The presence of metals at the observed concentrations in U implied the inefficiency of the passive treatment (wetland) at removing the metal load to such levels that meet the TWQR. With measured water pH at 3.4, the acidity provides an

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environment in which metals become mobilized in acid mine drainage, and consequently, metals become more soluble and toxic to aquatic organisms (USEPA, 2005). An outcome of a previous study within the catchment area of our chosen site, confirmed that Zn, Cu, Mn, Pb, Cr, Ni, Al and Fe accumulated in the skin, muscle, liver and gill tissues of fish (Coetzee et al., 2002). Discharges from mining activities in affected streams in other studies presented with reduction in biodiversity and alteration in species composition (Courtney and Clements, 2000; Soucek et al., 2000). Loss of cell viability and consequent cytotoxicity was detected and reported in some fish cell lines (Babich and Borenfreund, 1991; Segner, 1998). A 96h Zn exposure to the Fathead minnow cell line (FHM) showed that the cell line was significantly more sensitive compared to Fathead minnow fish (Rachlin and Perlmutter, 1968). Babich et al. (1986) and Magwood and George (1996) reported a strong correlation for cationic metals comparing *in vitro*/*in vivo* 24h exposure to metals assayed using the NR dye. Using primary rainbow trout gill cells grown as a double-seeded insert epithelium, predicted zinc and silver toxicity in a number of water compositions and were found to exhibit similarity in sensitivity compared to *in vivo* experiments (Walker et al., 2008). Likewise, cytotoxicity to cell lines resulting from exposure to industrial effluents have been reported in the Rainbow trout liver cell line, R1 (Ahne, 1985); Halder and Ahne, 1990; Rusche and Kohlpoth, 1993), RTgill-W1 cell line, (Dayeh et al., 2002) and Rainbow trout gonad cell line, RTG-2 with experiments displaying good correlation in toxicity ranking (Castaño et al., 2000; Castaño et al., 2003). Dayeh et al. (2004) reported a dose-dependent decline in cell viability in both RTgill-W1 and RTL-W1 cells exposed to metals common in mining effluent, measured with alamar blue and 5-carboxyfluorescein diacetate acetoxymethyl ester indicator dyes.

There were differences between the two cell lines concerning their sensitivity to substances present in the water samples. The RTgill-W1 cells appeared to be more sensitive as indicated by the lower IC_{50} values obtained in the assay, bearing in mind that the gill in the fish would be expected to make contact with environmental contaminants first. This difference could be due to different uptake mechanisms by the two cell lines. Kramer et al. (2009) reported that in most cases, mammalian cell assays appear to be less sensitive than fish acute toxicity studies when assessing the cytotoxicity of chemicals. The use of a fish cell line in assessing aquatic contaminants is considered to be superior compared to using mammalian cell lines, because

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toxicants can be applied to fish cells at temperatures more representative of that in their natural habitat, combined with the ease of comparing susceptibility within the same species. In addition, a fish cell line is expected to better reflect the properties of the fish from which they originated than cells of mammalian origin (Bols et al., 2005). In addition, gill cells are thought to be a major path for the uptake of waterborne toxicants and an organ prone to damage by metals (Wood, 2001; Castaño, 2003). Normal gill functions are disrupted on exposure to waterborne pollutants (Wendelaar Bonga, 1997) and direct toxic effects cause distress in the gill structure and function (Sola, 1994; Mallatt et al., 1995). Basic cellular functions that can result in cytotoxicity arise from xenobiotics in pollutants and the gills play an important role in xenobiotic biotransformation in fish (Miller et al., 1989; Ueng and Ueng, 1995).

The cytotoxicity assays employed revealed different profiles, with the MTT assay being the most sensitive, showing statistically significant difference between the treated cells and the controls. This observation can be explained by the nature of each assay; the MTT assay is mainly based on the enzymatic conversion of MTT in the mitochondria, whereas the LDH leakage assay is based on the release of the LDH enzyme into the culture medium after cell membrane damage. The neutral red assay is a colorimetric assay that measures the uptake of the dye by functional lysosomes. The results using both cell lines suggest that mitochondrial activity and cell viability measured with MTT was more sensitive to contaminants present in the effluents compared to lysosomal activity and LDH leakage. This may point to a specific mechanism of toxicity in which the contaminants present in the AMD water samples target the mitochondria rather than plasma membranes. Mitsuyoshi et al. (1999) reported that the XTT (2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]- 2*H*-tetrazolium-5-carboxanilide) assay, which is similar to the MTT assay, showed more sensitivity than the LDH leakage assay. LDH release, which is an indicator of necrosis arising from cell membrane damage, did not seem to play a big role in loss of cellular viability evidenced by LDH assay results and the presence of intact plasma membrane from TEM findings, a possible indication of an energy-dependent cell death (apoptosis) at least preceding necrosis.

Differences in results based on test agent used and the cytotoxicity assay employed have been reported with *in vitro* cytotoxicity (Weyermann et al., 2005). The

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mitochondria may be a more important site in metal induced toxicity (Lund et al., 1993), as respiratory impairment appears to precede the plasma membrane breakdown. It is believed that when mitochondrial respiration is inhibited, a stimulation of dynamic oxygen related cell death might ensue leading to reactive oxygen species being generated within the mitochondria, thus damaging the mitochondrial components (Koizumi et al., 1996). Therefore, exposure to a mitochondrial toxicant would give earlier and enhanced signs of toxicity using assays based on mitochondrial respiratory activity, e.g. cadmium was reported to disrupt mitochondrial function both *in vivo* (Belyaeva et al., 2002) and *in vitro* (Pourahmad and Brien, 2000).

This effect was further substantiated by TEM findings in which the mitochondria of the RTgill-W1 cells exposed to contaminated stream water showed a variety of ultra-structural changes (Fig 5H). The rupture of the outer mitochondrial membrane facilitated by the state of the mitochondrial permeability transition through the mitochondrial pore opening, is reported to be very common in apoptotic cells (Halestrap, 2002; Sesso, 2004) as it is usually observed in the early stages of apoptosis, in which the mitochondria become increasingly more swollen (Petit et al., 1998). Mitochondrial pore opening as a consequence of mitochondrial swelling is widely documented as promoting apoptosis (Halestrap et al, 1998; Halestrap et al., 2002). Apoptosis has been detected in the absence of mitochondrial swelling and collapse of the inner mitochondrial transmembrane potential (Zhuang, 1998; Liu, 1996; Finucane and Waterhouse, 1999). A previous experiment confirmed increased ROS generation in cell lines exposed to water samples from our study site (Iji, unpublished). Mitochondrial damage is known to be closely associated with the generation of intracellular ROS that could lead to structural disruption of the mitochondrion. When ROS are produced in excess, significant alterations in mitochondrial membrane permeability ensues, the respiratory chain becomes disrupted, with the possibility of DNA damage, which affects cell cycle sequence (Pulido, 2003). Therefore, early signs of toxicity following exposure to a mitochondrial toxicant would be detected in an assay based on mitochondrial respiratory. Evaluating the presence of viable fluorescing mitochondria using the Rhodamine dye and Mitoracker®Green FM stain, likewise showed fewer fluorescing cells in U and T compared to Ref, an indication of possible effects on mitochondria

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activity.

In hydrobionts, metals are known inducers of oxidative stress generated either by interference of metal-related processes or production of free radicals (Lushak et al., 2011), which ultimately ends in cell death via apoptosis and/or necrosis. In aquatic biota, dissolved metal ions are reported to enter biota membranes, causing toxicity (Jarvis and Young, 2000; DeNicola and Stapleton, 2002). Of the metals present in the river effluent, Ercal et al. (2001) reported that metals such as Fe, Cu, Cr and As could generate ROS by redox recycling. RTgill-W1 cells exposed to Cu produced a dose-dependent elevation in cytotoxicity and enhanced ROS formation (Bopp et al., 2008). These results demonstrate the prospect of using cell lines for environmental monitoring, as trout gill cells adapted for use in Canada have proven to be predictive of rainbow trout lethality of effluents from pulp and paper industry (Dayeh et al., 2002). For non-specific chemicals that cause acute toxicity and interference of basic cellular functions, relative toxicity ranking between *in vivo* and *in vitro* appears to correlate well **(**Kilemade, 2002). Bioavailability of contaminants to cells might be responsible for the observed loss in cell viability, since metals recurrently have been shown to be cytotoxic to fish cell lines (Babich and Borenfreund, 1991; Segner, 1998). Their presence in elevated concentrations in streams could be so toxic to aquatic organisms such that living organisms could diminish largely in receiving streams (Kimmel, 1983). Observed cytotoxicity in this study could exclude the influence of pH and osmolarity of the effluent, as they were corrected to levels required by the cells in the media. Differences in nutrient levels (ammonia, nitrate, phosphate) in U and T were mostly marginal and not significantly different, and as such are unlikely to be a contributing factor to the observed toxicity in the cell lines.

Mine effluents, in addition to being rich in metals, also contain elevated inorganic compounds that influence metal bioavailability and toxicity (Antunes, 2007). In our study site, metals occurred alongside elevated total dissolved solids (inorganic salts and sulfates). Metals associated with sulfides are reported to be more bioavailable (Griscom et al., 2000), and this could potentially explain the sustained cytotoxicity (MTT assay) observed in T, despite a decrease in metal burden in the water. With post alkali in-stream treatment there was considerable reduction in heavy metals (Table 2), and though lower, levels still quite exceeded TWQR range and those obtained for the

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Ref site. A resultant increase in TDS (Mg, K, Ca, Na and sulfate) was observed for T following alkali neutralization, resulting in an increased EC (U vs. T). Increases in TDS concentration correlate with increased EC of water (DWAF, 1996) and an extensive neutralization can increase TDS levels. Nobergh-King et al. (2005), in a toxicity reduction evaluation case study, reported TDS toxicity in the invertebrate *Ceriodaphnia dubia* even when heavy metals present in discharged effluent met chemical- specific permit limits.

The presence of inorganic compounds such as Mg, Ca, P and Na has been described to affect the mobility and transformation of metals and ionizable chemicals (Williams, 1987). As such, future mitigation measures should take into account the possible ecological risk associated with salt precipitation. In the aquatic organisms, salinity changes affect the buffering capacity of water that consequently affects metabolism in organisms, which may result in stressful conditions associated with enhanced ROS generation and initiation of oxidative damage (Liu et al., 2007).

One advantage of the use of multiple cytotoxic endpoints is that it gives a possible insight into the toxic mechanisms, as an organism's response to a chemical stressor culminates usually in lethality. *In vitro* cytotoxicity assays are useful for general screening of chemicals in order to predict potential human toxicity (Clemedson and Ekwall, 1999; Scheers et al., 2001). Since a correlation between cytotoxic alterations in the cells can be made with the analytic data of water quality, poor water quality will not sustain life, but produce lethal alterations in fishes. It is imperative to identify substances causing an observed effect and their potential source, in which case a combination of both *in vitro* bioassay and chemical analysis is suggested (Brack and Schirmer, 2003; Kinani et al., 2010). In order to use fish as sentinels of environmental and human health a good understanding of the key regulators of the fundamental cellular events is crucial (Van der Schalie et al., 1999). It is therefore proposed to use an alternative technique to predict toxicity for early detection of possible effects to aquatic organisms, in terms of water quality management, for avoidance of potential ecological disturbances. Using fish cell cultures as a substitute to whole fish in assessing industrial effluents for toxicants is a huge research consideration (Ahne, 1985; Castaño et al., 1996; Dayeh et al., 2002) even though there are reports of lower sensitivity of fish cell lines compared to *in vivo* fish exposure (Saito et al., 1991;

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Castano et al., 1996; Schirmer, 2006). Through *in vitro* cytotoxicity, basic cytotoxic effects of a chemical, at concentrations that are at least as low as those that would affect the whole organism, can be predicted (Kocan et al., 1985).

The current experiment suggests that site-specific information on the toxicity of contaminants present in the aquatic system to cell lines can be obtained to assess the threats posed by point source discharges of this nature in local freshwater resources. Further work is required to understand the complex relationship between metals and other inorganic ions and organic ligands in complex mixtures. The lack of dose–response data for individual metals made it difficult to rank the potential contributions of each metal and salts to overall toxicity. It is expected that the response of an organism to a toxic compound would be in relation to its concentration (Escher and Hermens, 2002); hence, target site concentration may play a role in the observed differences in cell sensitivity. Another consideration with *in vitro* cytotoxicity is interference by media components that could influence bioavailability in this test system (Heringa et al., 2004).

The results of this study suggest that *in vitro* cytotoxicity could be helpful for screening industrial effluent for cellular toxicity and perhaps serve as a complement and/or substitute for fish toxicity testing. Selecting tests that have predictive value for the expression of toxicity may, however, pose a challenge. Using cell lines to screen for toxicants is beneficial relative to whole fish because the amount of sample needed for testing is reduced, thus cutting shipping costs and handling, as well as the time required to complete testing, and this can aid decision-making where an effect is observed. Related *in vivo* toxicity assays with fish were not conducted to validate the toxicity of AMD samples due to the above-mentioned constraints.

5.5 Conclusion

Additional findings should be considered to further show the reliability of the RTgill-W1 bioassay as a replacement for the rainbow trout 96-h lethality test and enough data generated to establish better correlations between *in vivo* and *in vitro* toxicity assays. This is necessary so that the application of whole-water samples to cell lines could then be routinely used to screen industrial effluents for the purpose of quickly

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identifying intermittent events of high toxicity, and to make decisions about measures to be taken, with the aim of protecting aquatic organisms and human health. *In vitro* cytotoxicity assays investigating effects on cell-specific functions would better complement baseline cytotoxicity tests for proper risk evaluation.

Studies relating to the understanding of both environmentally induced cytotoxicity/apoptosis and environmentally induced cellular transformation are necessary to recognize consequences to environmental exposures in human health.

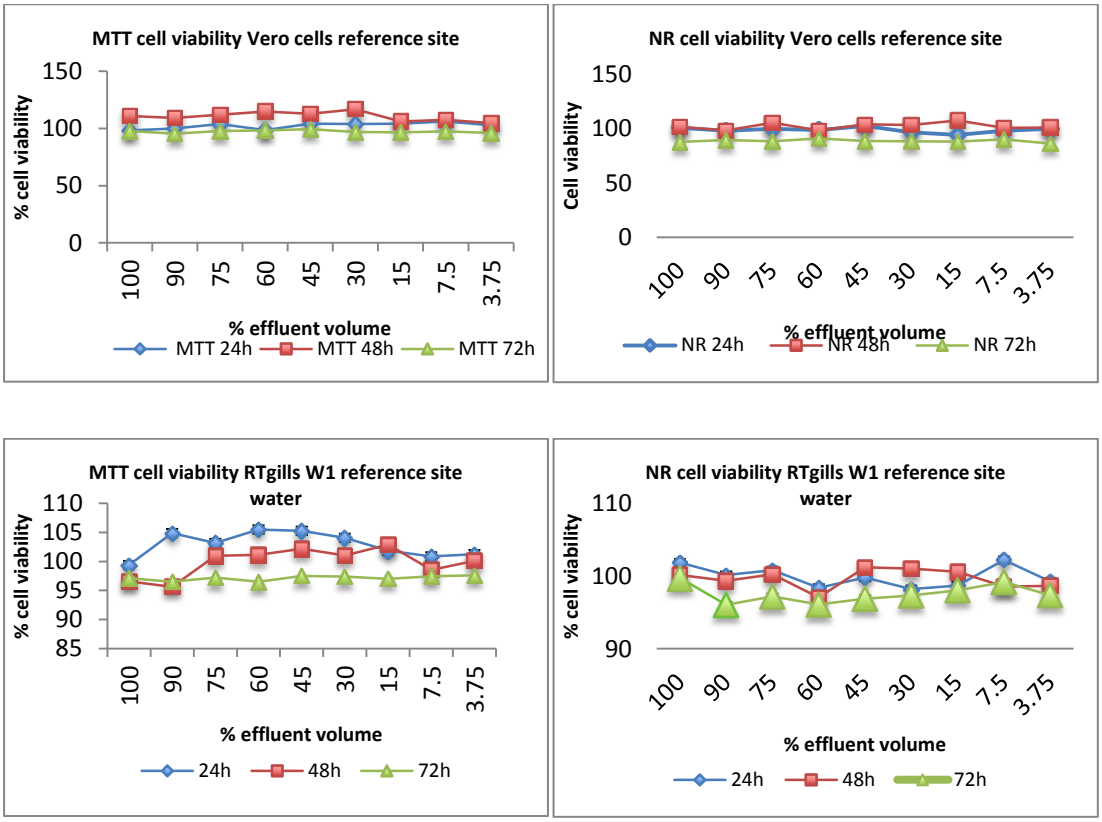

Figure 5A: Cell viability effects of different concentrations of water samples from reference site on Vero and RTgill-W1 cell lines. Data is expressed as a percentage of unexposed controls ± SD of four replicates for each exposure concentration ($P<0.05$) at different concentrations tested exposed for 24, 48 and 72h respectively. Control values were set at 0% cytotoxicity.

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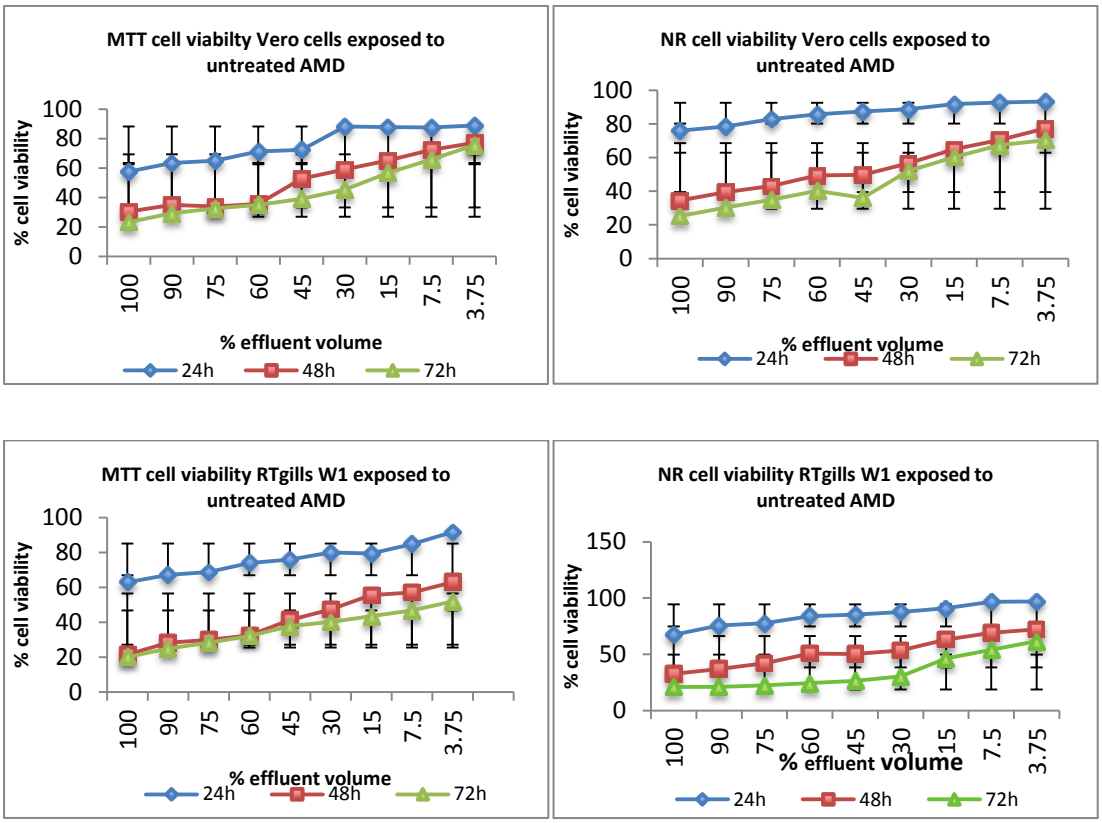

Figure 5B: Cell viability effects of different concentrations of water samples from impacted AMD stream U exposed to Vero and RTgill-W1 cell lines. Data is expressed as a percentage of unexposed controls ± SD of four replicates for each exposure concentration. Control values were set at 0% cytotoxicity. Significant difference in mean values at different concentrations tested at exposure periods of 24, 48 and 72h respectively.

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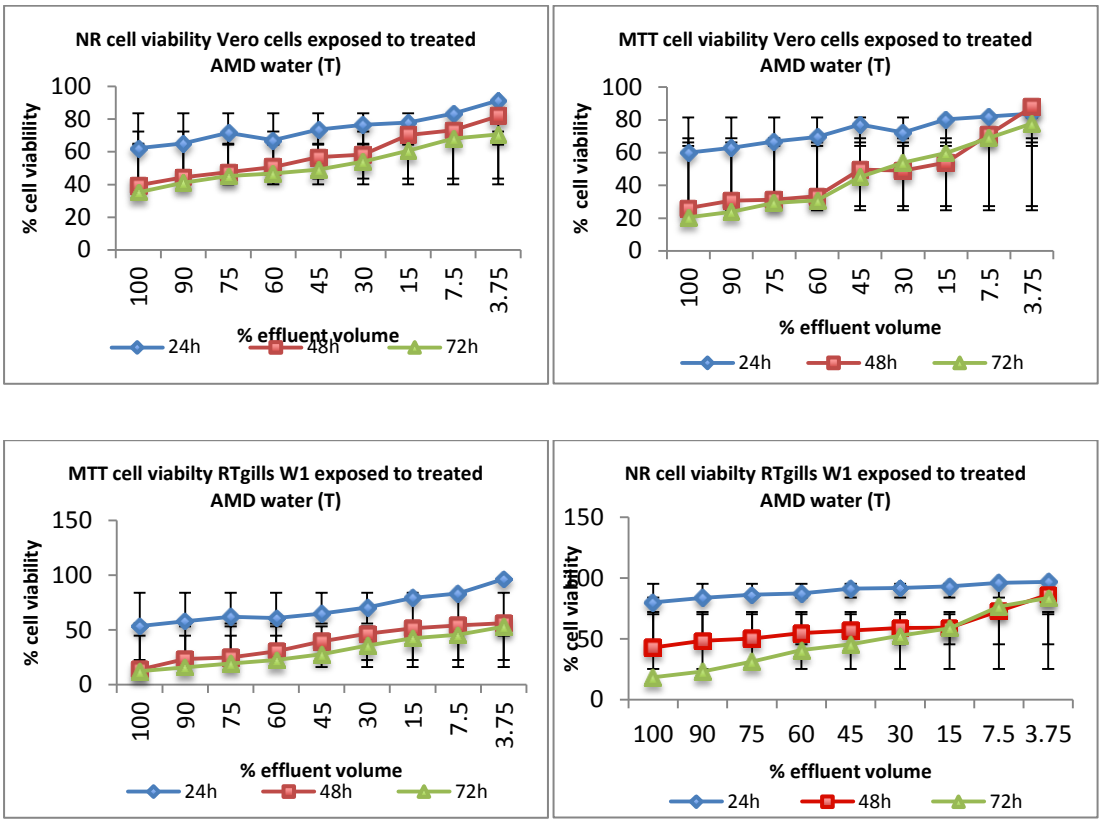

Figure 5C: The cell viability effects of different concentrations of water samples from impacted AMD stream T exposed to Vero and RTgill-W1 cell lines. Data is expressed as a percentage of unexposed controls ± SD of four replicates for each exposure concentration. Control values were set at 0% cytotoxicity. Significant difference in mean values at different concentrations tested at exposure periods of 24, 48 and 72h respectively.

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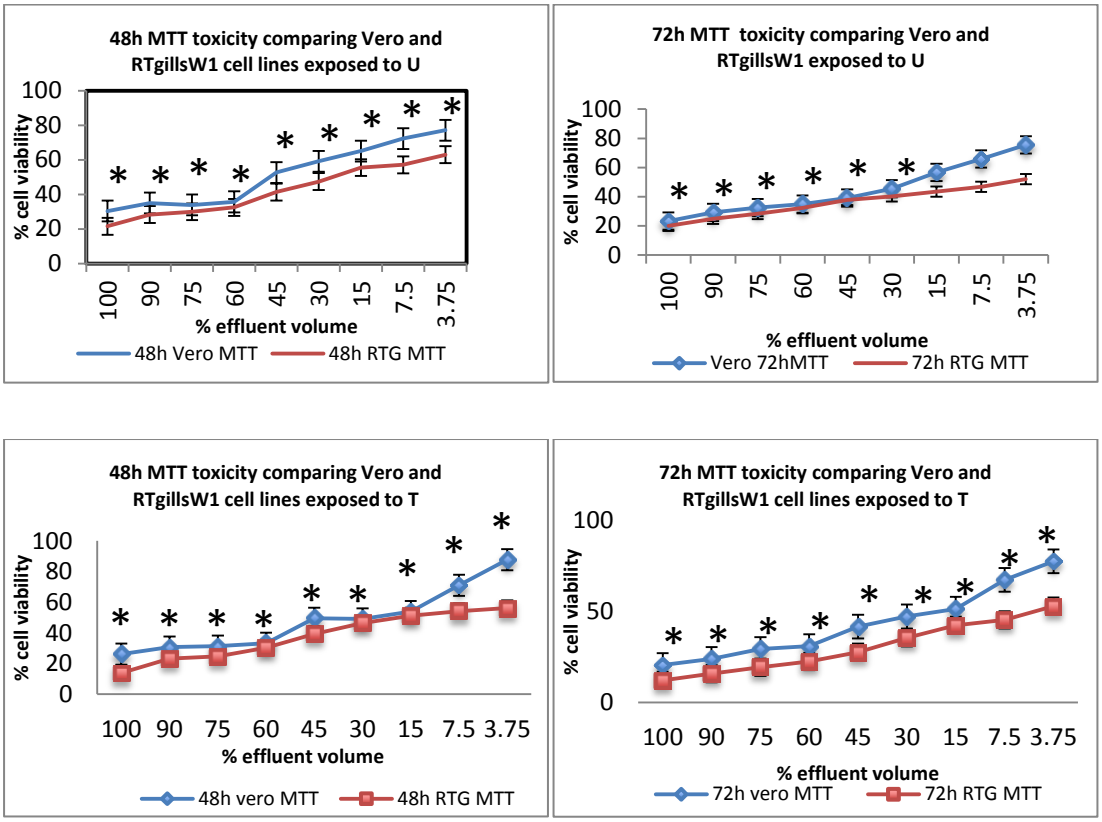

Figure 5D: Cell viability in Vero and RTgill-W1 cells lines resulting from similar exposure period (either 48h or 72h) to U and T for using the MTT assay technique. Significant difference in mean values was set at $p<0.05$.

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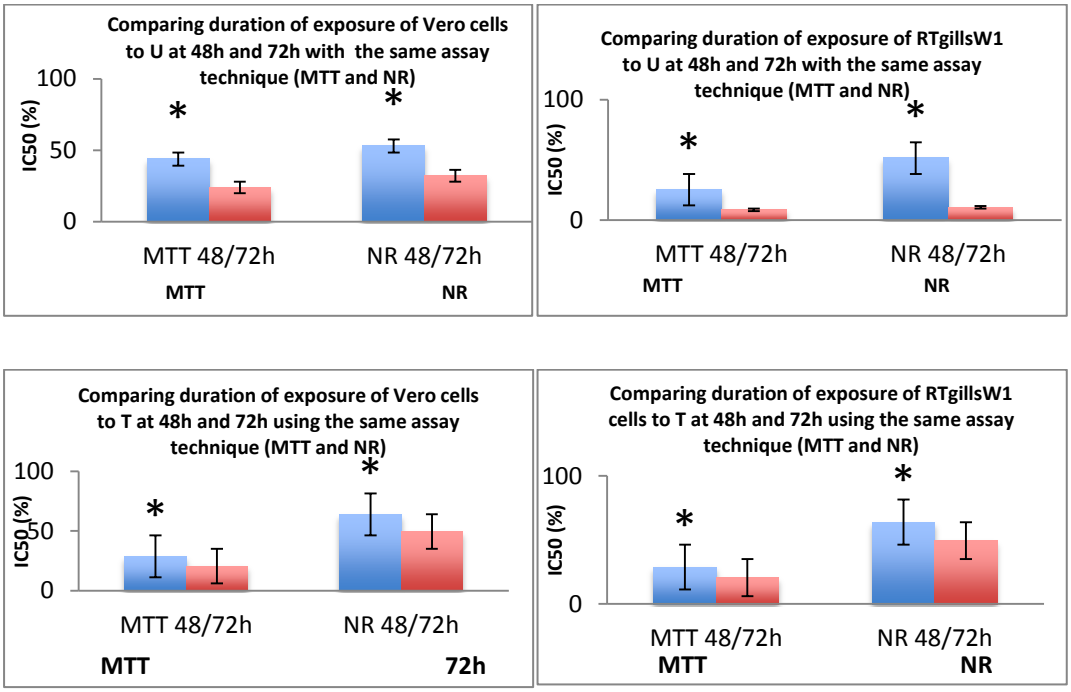

Figure 5E: IC_{50} values obtained with different exposure period (48h and 72h) using either the MTT or NR assay techniques exposed to U and T at $P<0.05$.

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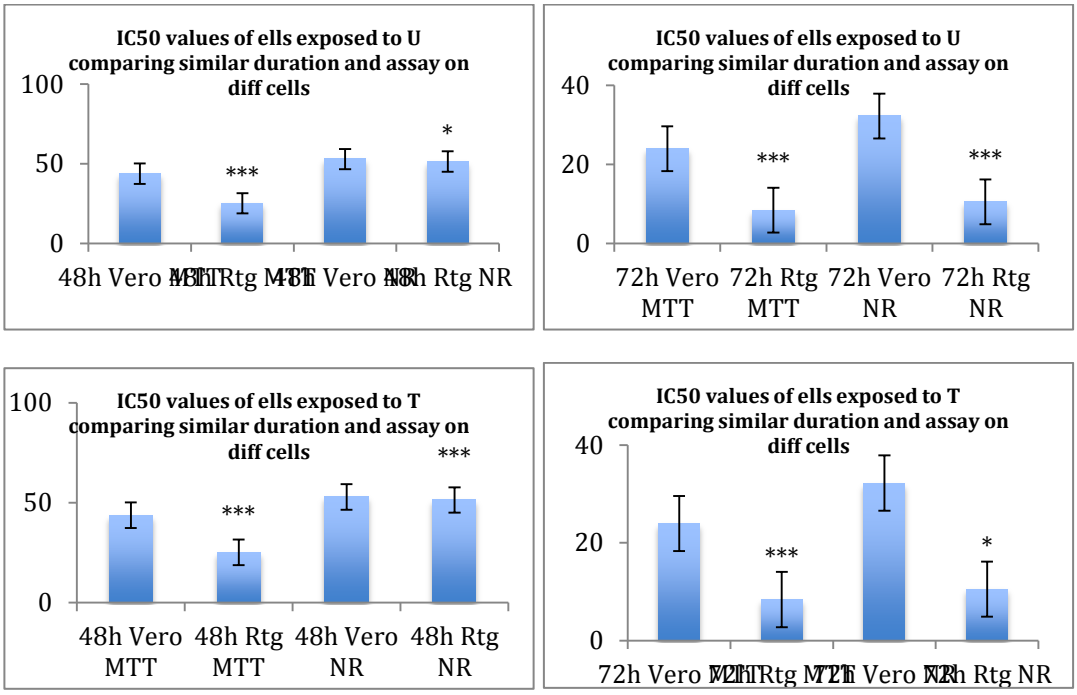

Figure 5F: Compares difference in sensitivity of cells using similar assay techniques within the same exposure period at P<0.05.

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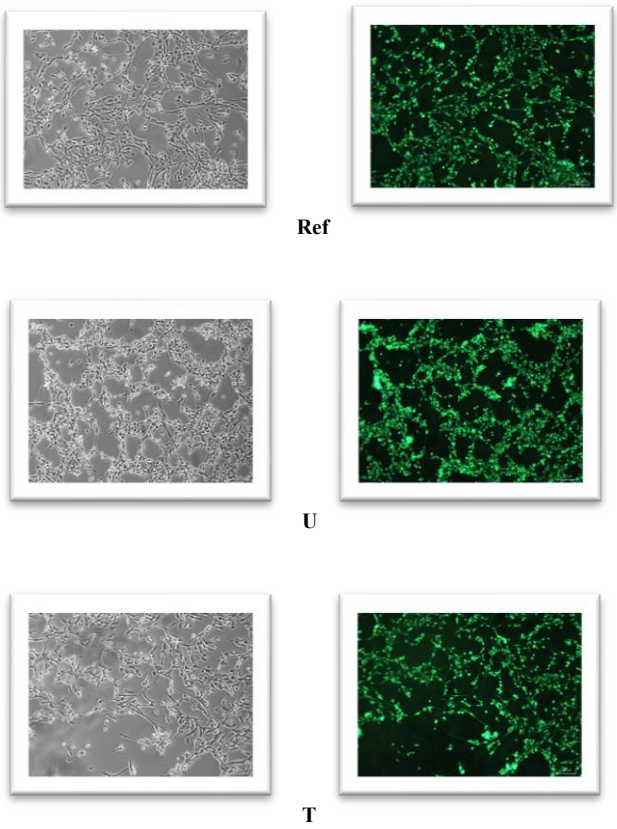

Figure 5G: Phase contrast micrographs and mitochondrial florescence images of RTgill-W1 cells (Mitotracker stain) following 72h exposure to reference site, untreated and treated AMD water samples in RTgill-W1 cell lines.

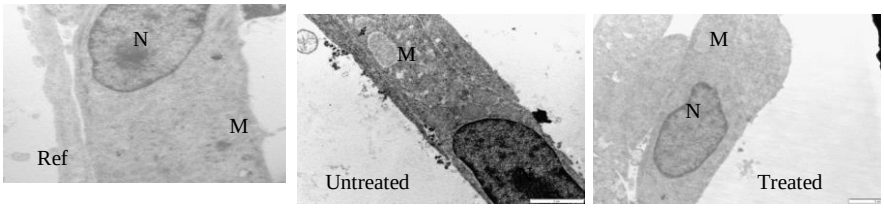

Figure 5H: TEM findings of RTgill-W1cells exposed to media Ref site, untreated and treated AMD water samples (N= nucleus, M= mitochondria). Intact mitochondria observed in Ref but swollen and loss of inner and outer membrane seen in cells exposed to U and T.

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CHAPTER 6: Induction of 7-ethoxyresorufin-*o*-deethylase activity by B[a]P in primary culture of gill epithelial cells from Tilapia (*Oreochromis mossambicus*)

Preface

Manuscript prepared for submission to ISI rated journal Global Veterinaria

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Abstract

Ecotoxicological studies utilizing *in vitro* techniques to detect and measure chemically induced perturbations are fast gaining momentum. With regards to South Africa, toxicity testing is a part of management of water resources. In our study, primary gill epithelial cells established from the gills of indigenous fish (*Oreochromis mossambicus*) were used to assess the CYP1A induction mainly as an alternative to whole fish toxicity testing. Various concentrations of benzo[a]pyrene (B[a]P), a potent aryl hydrocarbon receptor agonist usually found in contaminated water were exposed to primary cultures of gill epithelial cells and a continuous cell line RTgill-W1 cells. The primary gill epithelial cells responded to CYP1A induction, while the commercially available RTgill-W1 cell line showed no activity ($p < 0.001$). Cytotoxicity, determined by the methyl thiazol tetrazolium (MTT) assay, was not observed following a 72 h exposure of the primary gill epithelial cells and the RTgill-W1 cell line to different concentrations of B[a]P. The gill epithelial cells isolated from the gills of Tilapia fish (*Oreochromis mossambicus*) bear close morphological similarities to fish gills *in vivo* and their capacity to respond to the presence of AhR indicates they may serve as a simple, cost-effect screening tool for assessing PAHs and dioxin-like compounds in fresh water.

Keywords: Cultured gill epithelial cells, *Oreochromis mossambicus*, *in vitro*, benzo[a]pyrene.

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6.1 Introduction

A number of industrial wastes and natural chemical compounds find their way into the aquatic ecosystem from time to time, leaving a cumulative and lasting effect on the already stressed hydrosphere (Schwarzenbach et al, 2006). In South Africa, a major challenge to the aquatic ecosystem involves mining. eMalahleni, a city in the Mpumalanga Province of South Africa is home to most of the country's coal reserves, hosting 8 out of the 10 operational coal-fired power stations that supply 83% of the total produced coal in the country (Vureen, 2009). Along the Upper Olifants River catchment area of Mpumalanga, disposal of mine wastewater into natural receiving water systems from coal mining creates an ecological problem. Mining activities are reported to alter the hydrodynamics of most rivers, threatening the aquatic ecosystems due to production of AMD (de Villiers and Mkwelo, 2009) with reported fish and crocodile die-offs (Paton, 2008).

Often associated with coal production is the occurrence of polycyclic aromatic hydrocarbons (PAHs) that may be leached along with other toxic metals, persisting in the environment (Wang et al., 2008). The Department of Water Affairs and Forestry (1996) have established standards for contaminants such as metals present in water compartments but little or no information is available for the occurrence of PAHs in South African waterways, especially in the region where coal is mined. The main source of PAHs is incomplete combustion of organic materials such as is observed with coal utilization, and there are reports of PAHs and other potent inducers of CYP1A contamination in sediments and effluents resulting from point source pollution (Gadagbui and Goksøyr, 1996; Olajire et al., 2005).

Fatoki et al. (2010) examined the levels of PAHs (azulene, pyrene, anthracene, dibenzothiophene and fluoranthene) in water and sediment samples from rivers in the vicinity of Thohoyandou and reported that sediments acted as reservoirs for PAHs. Typically, PAHS are hydrophobic or lipophilic but they may still occur within water compartments, and are known to have an affinity for lipids within organisms with tendencies to bio-accumulate in the food chain (Jones and Voogt, 1999). Additional concerns regarding PAHs is that they act as endocrine disruptors in humans and wildlife (Harrison *et al.*, 1995; Kavlock, 1996), impair the immune systems of top

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predator species (Safe, 1994), increase susceptibility to disease (Leonards, 1997) and are potentially carcinogenic or mutagenic (Conney 1982; Xue and Warshawsky 2005).

A sensitive biomarker of exposure to dioxin-like pollutants is measured as the induction of CYP1A, which is used extensively in environmental monitoring (Bucheli and Fent, 1995). These enzymes are involved in biotransformation of both endogenous compounds and xenobiotics. Certain xenobiotics induce CYP1A (Hahn, 2002), mediated via the aryl hydrocarbon receptor (Ah-receptor) pathway. This may be detected either by measuring CYP1A mRNA or protein content, or catalytic activity in which the substrate 7-ethoxyresorufin (EROD) is metabolized to fluorescent resorufin. Some Ah-receptor agonistic pollutants include polyhalogenated aromatic hydrocarbons (PHAHs), polychlorinated biphenyls (PCBs) (Behnisch et al., 2003) and polycyclic aromatic hydrocarbons (Lee and Anderson 2005) such as benzo(*a*)pyrene (BaP) which is a well-known CYP1A inducer (Shimada and Guengerich, 2006). Although analytical chemistry techniques can detect dioxin-like compounds, the use of animal cell bioassays offers the advantage of a rapid and cost effective method (Firestone, 1991; Hahn, 2002).

With regard to studies involving human and ecological health, fish are thought to play a massive role; not only are they sensitive to changes in their environment, they have better developed immune systems compared to invertebrates and, as such, lesser organisms may not replace studies on fish because they differ in responses to toxicity (Van der Oost et al., 2003; Siroka and Drastichova, 2004). Tilapia (*Oreochromis mossambicus*), an indigenous fish species that is abundant and widely distributed, is a recreational and ecologically important species within the tropical and subtropical southern Africa region (Skelton, 1993). *O. mossambicus* has been used successfully as test organism in a number of biomarker studies (Hwang and Yang, 1997; Li, 1997; Chen, 2001; Shailaja and D'Silva, 2003).

Fish gills are primary targets for toxicants in the aquatic environment and the principal site for uptake of water-borne contaminants (Wood, 2001). The gills function to excrete metabolites of xenobiotic biotransformation and are sensitive to waterborne toxicants (Wood, 2001). Due to high metabolic rates in hepatic tissue, the

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EROD assay is typically conducted using fish liver. Gill tissue may be even more suitable to detect biomarkers of exposure to waterborne pollutants due to first pass effects in which pollutants absorbed through the gills may be metabolized before reaching the liver (Levine and Oris, 1999; Jönsson et al., 2004).

The use of bioassays involving primary cultured gill cells in fish could be a vital means of gaining information regarding changes in water quality with respect to organic pollutants such as B[a]P. Primary cultures are considered to be more sensitive than continuous cell lines as they possess a higher metabolic capacity compared to cell lines (McKim et al., 1985; Lee et al., 1993) and are more stable during *in vitro* incubation (Segner, 1998). They tend to retain their CYP1A expression capability better than cell lines, which may have little or no ability to express CYP1A (Lee et al., 1993). It has also been established that the metabolic profile of B[a]P in fish is comparable quantitatively either *in vivo* and *in vitro* (Steward et al., 1990; Nishimoto et al., 1992). Since biomarkers serve as early warning signals for exposure to pollutants before observed undesirable effects in organisms or populations are obvious, setting in place an environmental water quality surveillance system which employs a controlled laboratory exposure to assess toxicity using *in vitro* techniques such as primary cells and cell line cultures could be useful for assessing anthropogenic changes in aquatic ecosystems for effective environmental assessment and monitoring.

The main objective of this study was to evaluate the EROD assay in primary gill cultures of Tilapia fish (*O. mossambicus*), an indigenous African freshwater fish, as a tropical model species for detecting CYP1A induction in cultured gill cells following waterborne exposure to B[a]P, a toxicant associated with coal utilization.

6.2 Materials and Methods

6.2.1 Fish

Male Tilapia fish weighing 70–100g were obtained from a local fish farm (De Wildt fisheries, Brits) and were acclimatized in a 1500 l tank containing water with constant aeration and water circulation. The tank was connected to an external filter system that was cleaned once a week and half the water in the tank replaced with fresh

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running tap water weekly. The fish were kept at room temperature following the natural variation over the year. During this time they were fed commercial fish pellets five times a week. The pH of the running water was between 7.6–7.7 and the ionic composition (mg/l): Na^+; 21, Cl^-; 27, Ca^{2+}; 23, Mg^{2+}; 13, HCO_3^-; 100, Alkalinity; 100. Ethical permission for the study was obtained from the Animal Ethics Committee, University of Pretoria (protocol number V027-12).

6.2.2 Chemicals and cell culture medium

Trypsin, Leibovitz L-15 culture medium, penicillin and streptomycin, were purchased from Gibco®(Life Technologies, USA). Fungizone, rhodamine 123, ethylene-diaminetetraacetic acid (EDTA) was obtained from Sigma-Aldrich® (USA), gentamicin 50 mg/ml from Virbac (South Africa) and fetal bovine serum (FBS) from Highveld Biological® (South Africa). Culture dishes (Nunculon) were obtained from Nunc®, Denmark, and 7-ethoxyresorufin and dicumarol were sourced from Sigma-Aldrich®. Other chemicals were commercial chemicals of reagent grade.

6.2.3 Cells isolated for primary cultures

Fish were starved for 3 days and then kept in sterile aerated tap water for 1h prior to preparation of cells. After being stunned by a blow to the head, the fish were decapitated. All procedures for gill cell isolation were conducted using sterile techniques from this point onwards in a biohazard cabinet (ESCO class 11 BSC, Labotec). The gill cell isolation protocol by Kelly et al. (2000) was adopted with a few modifications.

In essence, gill arches were excised into a wash solution made up of phosphate buffered saline (pH 7.7: 136.9 mM NaCl, 8.06 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4) containing the antibiotics penicillin (200 IU/ ml), streptomycin (200 µg/ml), gentamicin (12 µg/ml) and fungizone (2.5 µg/ml). Gill filaments were blotted to remove excess mucus and were cut into approximately 1-mm3 pieces, and washed 3 times in 10 ml of the wash solution for 10 mins with frequent manual agitation.

The washed filaments were then subjected to three consecutive cycles of tryptic digestion until filaments appeared translucent using trypsin (0.05% trypsin in PBS,

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with 5.5 mmol/l EDTA) for 20 min/cycle at 300 rpm on a shaker (microporous quick shaker QB-9001). The tryptic digest was mechanically agitated following each cycle using a transfer pipette in order to release the cells which were then strained through a 80 µm cell strainer into a stop solution (10% FBS + PBS pH 7.7) using a different cell strainer each time. The stop solution containing the cells was then centrifuged for 10 min (260 g at 0-4°C), leaving a pellet, which was washed in cold rinse solution (2.5% FBS + PBS pH 7.7) and centrifuged for 10 min at the same speed.

Cells were then re-suspended in cold L-15 culture medium supplemented with 5% FBS, 100 i.u./ml penicillin, 100 µg/ml streptomycin and 20 µg/ml gentamicin into flasks or 96 well microtitre plates at a density of 1-1.25 x 10^6 cells/cm^2 and kept at 21°C in an air atmosphere incubator. After 24h, non-adherent cells were removed by changing medium with above constituted L15 medium. Media change was repeated after 72 h. After a further 24-48 h in culture, assays were carried out using the 96 well plates.

6.2.4 Culture of RTgill-W1 cell lines

The RTgill-W1 cell line (ATCC® CRL2523™) was cultured in Leibovitz L15 medium supplemented with 5% FBS in an atmospheric air incubator at 20ºC. Cells from a sub-confluent culture were re-suspended in cell culture medium supplemented with 10% FBS at 0.2x10^6 cells/ well in a 96 well plate and allowed to attach for 24h before assays were performed.

6.2.5 Ethoxyresorufin-O-deethylase (EROD) activity

This protocol was adapted from Behnisch et al., (2002). RTgill-W1 cells maintained in whole culture media over 24h were plated at 0.2 x 10^6 cells /well. Primary gill cell cultures at day 6-8 were containing an average of 1.68x10^5±0.25 cells/well were assayed. Exhausted culture medium was removed and cells were exposed to a serial dilution of B[a]P (1x10^{-4} M to 1x10^{-12}M) for 72h. The B[a]P was dissolved in acetone, maintaining a final concentration of 3% v/v in each treatment. Control cells contained no B[a]P but only 3% acetone. Experiments were repeated at least three times and data represent replicates from three separate cell preparations. After 72h

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exposure to B[a]P, the exposure medium was removed and cells rinsed with PBS. HEPES Courtland (HC) was prepared at pH 7.7 using 5 mM potassium chloride, 133 mM sodium chloride, 0.9 mM magnesium sulphate, 2 mM calcium chloride, 3 mM sodium phosphate, 6 mM HEPES and 5 mM glucose. The HC-dicumarol buffer contained 10µM dicumarol, to which 16 µM 7-ethoxyresorufin was added. A 100 µl of HC–dicumarol + 7-ethoxyresorufin solution was added to the cells and the plates incubated at 21°C for 2 h, after which 90 µl of the reaction mixture was transferred to a 96 well black plate (Nunc, AEC Amersham, Kelvin, South Africa) containing 100 µl methanol. Fluorescence was measured at 544 nm excitation and 590 nm emissions using a multiwell fluorescence reader (BioTek®synergy HT-BioTek instrument, Winooski, USA).

6.2.6 Statistical analysis

All assays were performed in three independent experiments and the results are presented as mean ± SD (standard deviation) values. The significance of differences at $p<0.05$ was examined by one-way analysis of variance (ANOVA), Hartley's f test for equal variance. Inter- and intra-test the appropriate Student- T-test. This data analysis was performed using open source calculator Version 3.

6.3 Results

6.3.1 Cell viability of primary fish gill cells

The average yield of cells from approximately 3 g wet mass of gill filaments was 79±13 x 10^6 cells (mean ± S.D, *N*=12), excluding red blood cells (RBC) with about 10% (n=6) being rhodamine positive, indicating the presence of mitochondria-rich (MR) cells (Fig 1a, 1b). These were observed initially in culture, but did not propagate, as they were not present at confluence. The attachment efficiency in culture media, 24h after seeding was 32±5%. A number of RBC and non-viable cells were removed when culture media was changed after 24 h. The single cells appeared elongated while colonies appeared more as round cells (Fig 1b). The cells reached confluence between 6-8 days. Attached cells, 24h after seeding, were incubated with the fluorescent dye rhodamine to detect presence of chloride cells, and Fig 1c and d show bright green fluorescent cells which were not seen at confluence.

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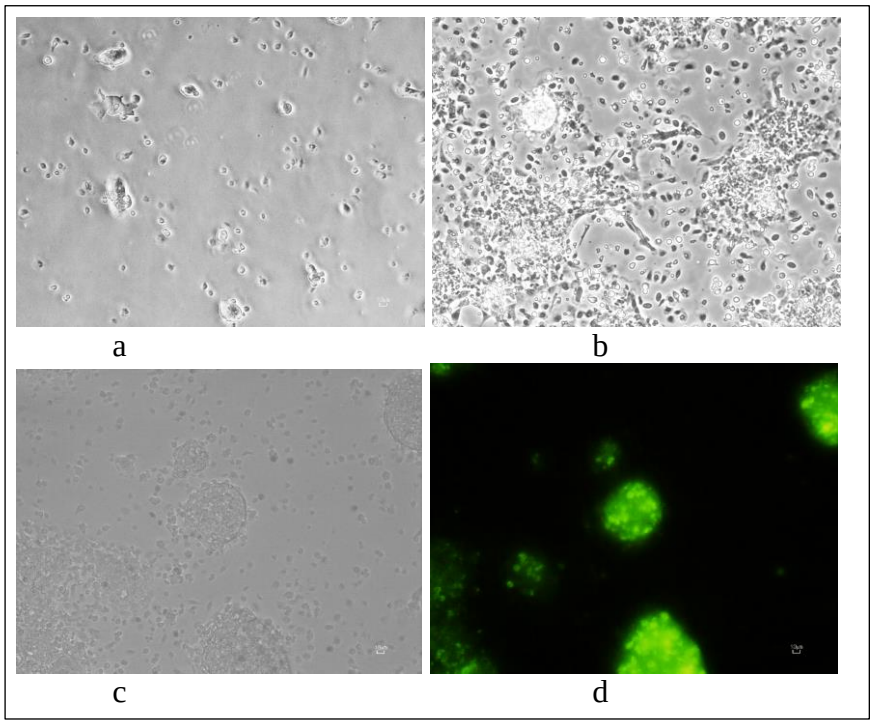

Fig 6.1 Photomicrographs of epithelial cells from fish gills grown in plastic culture dishes (x200) (a) cells at day 1 after seeding (b) 3 days old culture (c &d) show attached cells stained with rhodamine and fluorescing MR cells which were not present at confluence.

6.3.2 EROD induction in cells

Following 72h exposure of the primary cultures of gill epithelial cells to B[a]P at various concentrations, CYP1A induction was evaluated as increased EROD activity. The control epithelia showed very low EROD activity (200 pmol/h/well). EROD induction by B[a]P evidently was initially concentration-dependent, but as the concentration of B[a]P increased from 10^{-6} M, there was a drastic decrease in EROD activity (Fig 6.2). In primary gill cultures exposed to B[a]P**,** the peak EROD activity observed was at $1x10^{-7}$ M which was about 4 times higher than observed for the control. The RTgill-W1 cell lines on the other hand, did not show EROD induction or loss at the tested concentration range. The acetone vehicle used in both cell lines did not interfere with EROD activity induction. A significant difference in EROD activity

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was observed when comparing both cell types ($p < 0.001$).

The MTT assay technique employed following a 72h exposure of the primary gill epithelial cells and the RTgill-W1 cell line to different concentrations of B[a]P did not reveal cytotoxicity, as cell viability was maintained at 80% and above (Table1).

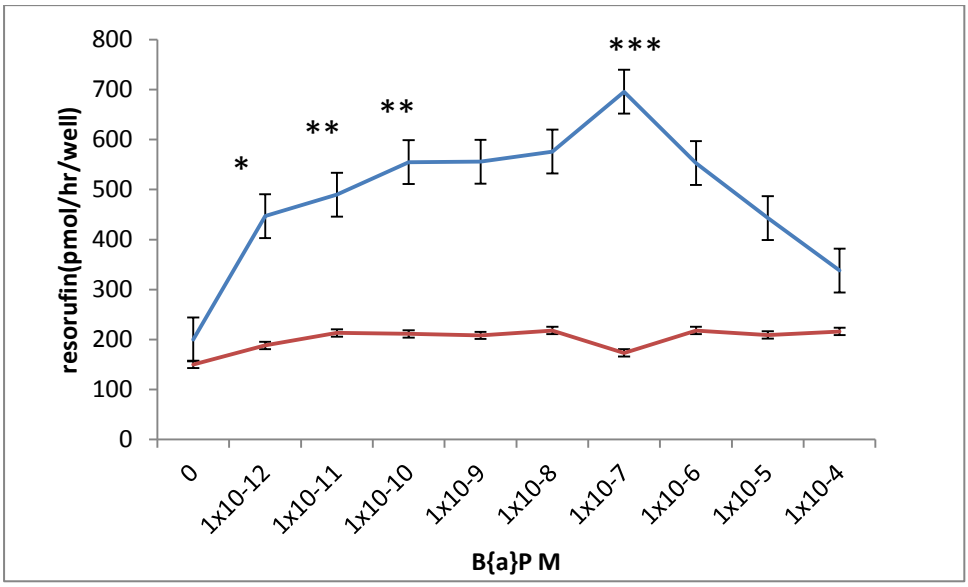

Figure 6.2. Dose dependent effect of B[a]P primary gill epithelial cells (blue line) and the RTgill-W1 cell line EROD activity (red line) after a 72h exposure period. Each data point represents the mean of three independent cultures ±SD. Only the gill epithelial cells induced EROD activity and maximum activity was observed at 10^{-7}M B[a]P. Significant difference established at ($P<0.05$).

6.3.3 Phase contrast microscopy

Growth and proliferation were monitored using a phase contrast microscope (Nikon Eclipse TS100). The primary culture was monitored for growth and, after exposure to B[a]P for 72h, gill epithelial cells were monitored for cellular changes. Cell preparations stained with rhodamine were carried out to examine the presence of mitochondria-rich cells.

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Summary of cell viability (%) of primary gill epithelial cells and RTgill-W1 exposed to B[a]P

	1x10^{-11}M B[a}P	1x10^{-10}M B[a}P	1x10^{-9}M B[a}P	1x10^{-8}M B[a}P	1x10^{-7}M B[a}P	1x10^{-6}M B[a}P	1x10^{-5}M B[a}P	1x10^{-4}M B[a}P
Mean ±SD (Pg)	83±0.6	81±0.3	82±0.7	81±0.7	82±0.8	83±0.6	80±0.6	82±0.4
Mean ± SD RTG	86±0.5	85±0.9	83±0.7	84±0.7	84±0.3	85±1	86±0.4	86±0.5

Table 6.1 Dose dependent effects of B[a]P on primary gill epithelial cells (Pg) and the RTgill-W1 cell lines (RTG) viability after a 72 h exposure period using the MTT assay. Each data point represents the mean of triplicate experiments ±SD.

6.4 Discussion

The functional properties of a number of fish cell culture assays have justified their use in research (Castano et al., 2003). Fish gills most importantly have received much attention for use in assessing basic toxicological responses in contaminated waters because they act as the first barrier for waterborne toxicants (Wood, 2001). Millions of fish are used annually for waste effluent testing and/or bio-monitoring. In the US alone an estimated 3 million fish are sacrificed annually (Tanneberger et al., 2013). This practise does not support the 3 R's (reduce, replace and refine) in research and toxicological studies. Therefore, alternatives to whole animal testing in the form of primary gill culture systems from fish gills are of major importance in assessing the presence of AhR agonists such as B[a]P, a potent CYP450 inducer found in contaminated water. The overall aim was to assess gill EROD assay as a vertebrate-based biomarker in water toxicity assessment.

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Primary gill cultures from Tilapia fish bear morphological similarities to those observed in the epithelium from intact secondary lamellae from Tilapia gills and are referred to as respiratory epithelial cells. Primary cultures are considered to be more organotypic as they have the ability to preserve the three-dimensional structure of the tissue and maintain cell-to-cell contact, bearing resemblance to gills *in vivo* (Wood, 2001; Kelly et al, 2000; Kelly and Wood, 2002). The MR or chloride cells initially found in culture (about 10% of the total cell population) did not seem to survive or propagate in the culture conditions, perhaps because crucial factors necessary for their survival may be lacking, as they were absent at confluence.

A number of studies have shown that PAHs and dioxin-like compounds mediate through AhR, exhibiting toxic and biochemical responses (Giesy et al, 2002; Hahn, 2002). EROD induction in cultured gill epithelial cells due to the presence of B[a]P exposed at environmentally relevant concentrations was observed in this study. This followed a pattern in which higher EROD induction was observed at lower concentrations, alongside reduced responses at higher concentrations, which is suggestive of possible inactivation of the CYP1A enzyme at high concentrations by B[a]P. This reportedly has been attributed to substrate competition of B[a]P and 7-ethoxyresorufin at the catalytic site of the CYP1A enzyme (Smeets et al, 1999). Comparable maximal EROD induction at similar concentration was reported in Indian catfish (*Heteropneustes fossili*) (Ghosh et al., 2001) and in primary hepatocytes of the African sharptooth catfish (*Clarias gariepinus*) (Naicker et al, 2006).

The epithelial gill cells and pillar cells in the topminnow (*Poeciliopsis* spp.) were previously reported as sites of CYP1A induction following exposure to B[a]P in water (Smolowitz et al., 1992). Likewise, the gills of the gulf toadfish (*Opsanus beta)* showed cytochrome P450 activity on exposure to B[a]P in water (Kennedy and Walsh, 1994). Furthermore, the gill filaments and the hepatic microsomes of the African sharptooth catfish induced CYP1A activity after waterborne exposure to B[a]P (Mdegela et al, 2006). Zhou, (2005), likewise confirmed a dose-dependent increase in ethoxyresorufin-O-deethylase (EROD) activity in cultured gill epithelia of Tilapia fish (*Oreochromis niloticus*) following exposure to low concentrations of AhR receptor agonists.

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Gill cell cultures arising from fish within a local geographical zone may be more suitable for toxicity testing of xenobiotics such as PAH than those originating from temperate regions, since factors such as temperature influence cytotoxic responses to xenobiotics (Babich et al, 1999). Determining CYP1A induction in tissues proximate to the ambient water, such as the gills, has proven to be a sensitive, accurate and straightforward biomarker for waterborne, dioxin-like pollutants (Jönsson et al, 2002; Levine and Oris, 1999). This study confirms a previous report by Bury et al. (2014) in which RTgill-W1 cell lines lacked CYP1A enzyme activity, as CYP1A activity measured in RTgill-W1 cell lines did not induce EROD activity.

Since biomarkers serve as early warning signals for exposure to pollutants before observable adverse effects in affected organisms, information collected on the relationship between EROD activity and detrimental effects on primary fish gill cultures may serve as a predictive tool for contaminant risk assessment, because measured biomarker responses, contributes to the overall assessment of contaminated sites (Vander Oost et al, 2003), hence, the successfully documentation of measured EROD activity in fish as sensitive index for pollution in contaminated sites (reviewed by Stegeman and Lech, 1991; Bucheli and Fent, 1995; van der Oost et al., 2003), which provides information mainly based on empirical observations and not on a priori pollutants, when compared to routine chemical analyses in the biota (Brack et al., 2005).

6.5 Conclusion

This study shows that primary gill epithelial cells can be isolated from tropical freshwater fish (*Oreochromis mossambicus*) using the trypsin digest procedure outlined above, and isolated cells may be employed for use in *in vitro* studies involving CYP1A activity following exposure to the polycyclic aromatic hydrocarbon, B[a]P, a potent AhR agonist. This finding supports the use of primary epithelial gill culture systems as an effective aquatic-ecotoxicological tool for assessing the presence of dioxin–like pollutants present in fresh water in Southern Africa that may serve to assist ecosystem health managers to prioritize catchment areas or watersheds for more in-depth studies.

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Overall, analysis of cultured gill EROD activity in Tilapia fish ((*Oreochromis mossambicus*) exposed to waterborne B(a)P provides an appropriate tool for toxicity assessment in aquatic environments.

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CHAPTER 7: Evaluation of the genotoxic potential of water impacted by acid mine drainage from a coal mine in Mpumalanga, South Africa using the Ames test and comet assay

Preface

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Abstract

A number of potential genotoxins found in water samples arise mainly from anthropogenic activities. Acid mine effluent resulting from coal mining poses serious environment concerns all over the world. Treatment systems such as the use of natural wetlands (passive) or conventional physical and chemical processes (active) are mainly employed to meet certain water quality guidelines, nonetheless, potential genotoxic compounds or residues remain which influence the quality of discharged effluent. The objective of this study was to evaluate the genotoxic potential of acid mine drainage (AMD) effluent released into a free flowing natural stream after treatment by passive and active methods, because physico-chemical water analyses do not provide enough information about the biological effects of such pollutants. Two genotoxicity assays were used to determine the toxicity potential of water collected at three different sampling points. The Ames test performed without metabolic activation using *Salmonella typhimurium* TA98 and TA100 strains showed no indication of genotoxic activity in any of the water samples tested. Differing results were however obtained for the comet assay using the African Vero monkey kidney cell line and a fish gill cell line (RTgill-W1), which revealed genotoxicity in all the water samples. A significant reduction in DNA damage was observed following active treatment of the AMD. The results suggest that none of the treatment technology was efficient at removing all potential genotoxins, and further improvements are required. Since only the comet assay proved sensitive enough to detect genotoxicity, we suggest that this *in vitro* bioassay has the potential to be integrated into an environmental monitoring framework.

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Keywords: coal mining, acid mine drainage, *in vitro* cell culture, DNA damage, genotoxicity.

7.1 Introduction

A number of stressful conditions arising from pollution influence the overall health of the aquatic ecosystem, mostly because water bodies end up being the ultimate destination of most pollutants (Zalasiewicz et al., 2010). Contaminated water from mining, industrial and agricultural activities constitute problems for drinking water sources and for the environment in a way that natural water bodies become vulnerable (Žegura et al., 2003).

Over the years, deterioration of stream water quality in South Africa due to mining has been reported (Grobbelaar et al., 2000) and with particular relevance is the coal mining industry where seepage and discharges arising from coal mines have made their way into water bodies. This has affected the overall state of impacted rivers or streams and underground aquifers because of heavy metals content, which is one of the primary causes of toxic pollution and serious water quality deterioration. The country faces major problems with regard to the management and treatment of contaminated mine water in both operational and abandoned mines (Schwab, 2002).

Acid mine drainage (AMD) arises when minerals containing reduced forms of sulphur (pyrites, sulphides) are oxidized upon exposure to water and oxygen (Gray, 1996). The reaction results in increased acidity due to the presence of hydrogen ions and subsequent metal dissolution and leaching. AMD as a complex pollutant has a high concentration of iron and sulphate, a low pH and an assortment of elevated concentrations of a variety of metals (Gray, 1997; Heath et al., 2009). AMD has been identified as the single most significant threat to South Africa's environment, because pollution resulting from it places pressure on fresh water systems and its resources, with possible toxic interactions in aquatic systems (Jarvis and Younger, 2001).
Environmental biomonitoring is time consuming and costly, and monitoring usually employed for AMD evaluates the physical and chemical components of water which cannot exclusively supply information about the health of an aquatic system and

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associated potential environmental and human risk (Lawrence and Williams, 1991). This risk can be evaluated using bioassays which have specific, direct and appropriate measurements, especially in cases where measured pollutants do not exceed maximum allowable limits, but through synergistic interactions with other pollutants, are able to bring about undesirable effects (Hernando et al., 2005). Therefore, such assays that measure genotoxicity in cell lines can identify toxic background pollutants present in complex mixtures like AMD effluent, for a proper holistic ecological risk assessment, because this pollution source is recognized as a potential risk to the environmental and human health (Kamer, 2002; Pulles et al., 2005;). This simple monitoring is particularly essential in developing countries due to the rapidly growing pressure on the aquatic environment from pollution, so as to establish contaminant load and impact and their potential roles in causing toxicity and genotoxicity effects on living organisms.

Testing of polluted water for genotoxicity may become a routine requirement for industrial wastewater discharge permits, because aquatic organisms exposed to wastewater discharges are likely to experience increased risk of genetic damage. Balch et al. (1995) reported prevalence of tumors in fish inhabiting rivers and lakes of industrialized areas in the US and Europe. A survey of Beluga whales inhabiting the St Lawrence estuary in Quebec, Canada, attributed the decline in population to cancers due to discharge of effluents from agricultural and industrial regions (Martineau et al., 2002). Likewise for downstream end users such as humans, the likelihood of a potential risk ensues since epidemiological studies revealed an association between genotoxicity of drinking water and increased cancer risk (Haider et al., 2002; Lu et al., 2002; Komulainen, 2004), not forgetting that aquatic organisms in the wild serve as food sources to a number of rural dwellers in Africa (Chigor et al., 2012).

In order to assess the genotoxicity and mutagenicity of environmental pollutants, *in vitro* models involving cell cultures have been extensively used (Mazzeo et al., 2013). Cell culture models present with numerous advantages such as ease of use, the ability to be prepared in large numbers, ethical convenience, reproducibility and the sensitivity of cell cultures to physical and chemical agents used in toxicological research (Rogero et al., 2003; Freshney, 2005; Cardozo et al., 2006). Fish and

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mammalian cell cultures have been successfully used to understand the possible effects chemicals pose to fish and humans. The RTgill-W1 epithelial fish cell line derived from freshwater rainbow trout has been explored as a potential alternative to whole fish in toxicity testing of environmental samples (Dayeh, 2003), while health laboratories and water quality regulators have used mammalian cells such as Vero cells initiated from the kidney of African green monkey (Richardson, 1977; Rees, 1980; Jos, 2003; Katsuo et al., 2007). These cell lines play a role in metabolizing and detoxifying xenobiotics which are known to react with DNA, inducing possible mutational events (Park and Choi 2007; Tsuboy et al., 2007).

The comet assay, or single cell gel electrophoresis, detects DNA damage induced by alkylating, intercalating or oxidative agents (Cotelle and Férard, 1999). DNA fragments are detected following electrophoresis under alkaline conditions that cause a migration of such DNA fragments from the nuclear core to result in a "comet" formation. Detected DNA damage could be single or double strand breaks, cross-links and DNA alkali-labile sites (Singh et al., 1988; Tice et al., 2000; Collins, 2004). Numerous studies have confirmed the efficacy of the comet assay at detecting genotoxicity even in individual cells within the aquatic environment and it is a reliable tool for environmental pollution monitoring (Schnurstein and Braunbeck 2001; Avishai et al., 2002; Nehls and Segner, 2005). Another advantage is that only a small number of cells are needed to carry out the assay, and assays can be carried out essentially on all eukaryotic cells, since it is precise at detecting DNA damage (Singh et al., 1988).

The *Salmonella* gene mutation assay or Ames test has been used likewise to assess genotoxic activity of wastewater (Stahl, 1991). The Ames test assesses the genotoxicity of nuclear DNA effects such as point mutations, gene reversion and gene conversion in specific bacteria following exposure by evaluating the induction of mutation (Maron and Ames 1983). Both the Ames and comet assays have been recommended as effective genotoxicity assays (Grummt, 2000; Reifferscheid and Grummt, 2000). Bacterial strains such as *Salmonella typhimurium* TA98 and TA100, which detect frameshift mutagens and base-exchange mutations respectively, may be used for determining genotoxicity of wastewater (Mortelmans and Zeiger, 2000).
Investigation of the applicability of genotoxicity bioassays in environmental

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biomonitoring of AMD has come into focus and the aim of this study was to evaluate the use of the comet assay and the Ames test as genotoxicity/mutagenicity tests to characterize the potential genotoxic burden of AMD effluent from a coal mine impacting a receiving water body.

7.2 Methodology

7.2.1 Location and description of the study area

The study site chosen was a receiving stream established downstream of a colliery, Kromdraai (25°46' 05.5" S; 29° 07' 15.5" E) in the Highveld region, close to eMalahleni (previously known as Witbank) in Mpumalanga Province, South Africa. Samples collected undergoing passive treatment immediately following a wetland were termed **untreated** (**U**). The water samples collected after treatment, beyond the point of an in-stream, neutralizing process were termed **treated** (**T**). A reference site with similar geographical parameters outside the influence/ impact of a mine, located at 25°47'6"S; 28° 28' 52"E, served as the control site and water samples collected here were referred to as **(R).**

7.2.2 Effluent collection

Samples of effluent were collected in September 2014. Sampling was performed according to the recommended standard method (ISO 5667-5). Water samples were transported to the laboratory in 500 mL plastic bottles that were placed on ice inside coolers. Water samples were stored at –20°C until further testing.

In situ measurements of temperature, dissolved oxygen and pH were conducted at the water sampling points along the course of the stream using a portable multimeter (HACH HQd, USA). Water samples were analyzed by the Analytical Services, Chemistry Department, CSIR, Pretoria for nutrients (ammonia, orthophosphate, phosphate, nitrate and nitrite), metals (aluminium, arsenic, chromium, copper, iron, mercury, silicon and zinc) and inorganic constituents (Ca, Mg, K, Na, Cl and sulphates). Analyses were all performed according to the methods of the American Public Health Association (APHA, 1995).

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7.2.3 Cell cultivation

Vero cells were grown in closed, 75cm^2 filter-cap flasks at 37°C in Minimal Essential Medium (MEM, Sigma-Aldrich®, USA), supplemented with 0.1% gentamicin (Virbac, South Africa) and 5% fetal calf serum (FCS, Highveld Biological® South Africa). Cells of a sub-confluent culture were harvested and plated in 6 well plates and incubated for 24 hours at 37ºC in a 95% air 5% CO_2 humidified environment.
The RTgill-W1 cell line (ATCC® CRL2523™) was cultured in Leibovitz's L15 medium (Sigma- Aldrich®, USA) supplemented with 10% fetal bovine serum (FBS, Highveld Biological® South Africa) in atmospheric air at 20ºC.

7.2.4 In vitro comet assay

The cell lines (Vero and RTgill-W1) previously maintained for 24 hours for a complete cell cycle were exposed to pH adjusted (7.3), syringe filtered (0.22-µm membrane) water samples in 25cm^2 flasks. Fresh media was prepared from either powdered MEM for Vero cells and powdered L-15M for RTgill-W1 cells using syringe filtered (0.22-µm membrane) to obtain sterile water samples. Either 75% or 50% test water samples were prepared for exposure to the cells. Only the RTgill-W1 cells were exposed at both concentrations, and Vero cells were exposed only at 50% concentration due to cell availability. Milli-Q water served as negative control while 2 mM ethyl methane sulfonate (EMS; Sigma-Aldrich) was used as positive control. The cells were incubated along with the test agents (water samples) for 24 hours at 37°C for Vero and 20°C for RTgill-W1 cell lines, and the assays were performed in triplicate.

For the comet assay, the standard alkaline comet assay method described by Singh et al. (1988) was followed. After culture medium was discarded from the flasks, the cells were washed twice with 5 mL PBS, and trypsinized (0.25% trypsin-EDTA). The trypsinizing process was stopped by adding fresh culture medium supplemented with serum. The content was homogenized and centrifuged at 1 000 rpm for 5 min and re-suspended in phosphate-buffered saline (PBS) to a final concentration of between 10 000 and 15 000 cells/mL. Cell viability was determined using trypan blue, allowing only a minimum of 80% viability for progression of experiments.

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In brief, 10 µL of the cell suspension mixed with 300 µL of 0.8% pre-heated low melting point agarose (in PBS), were spread over coated 1% normal melting agarose (NMA) pre-treated super frosted microscope slides, which were placed on ice for 10 min until the agarose cell mixture solidified. The slides were transferred into lysis buffer (2.5 M NaCl, 100 mM EDTA Titriplex, 10 mM Tris, and NaOH pellets; pH 10). This was placed overnight in the fridge at 4°C and rinsed twice with denaturation buffer. To unwind DNA, freshly prepared denaturing/electrophoresis buffer (0.3 M NaOH and 1 mM EDTA) was added for a further 40 min at 4°C in slides placed within the electrophoresis chamber. Electrophoresis was performed in a pre-cooled (4°C) horizontal electrophoresis tank for 20 min at 25 volts and 300 mA. Following electrophoresis, the slides were placed in neutralization buffer (0.4 M Tris-HCl, pH 7.5), and subsequently in iced cold methanol for 10 min. Dry slides were stained with ethidium bromide (10 µg/mL in water) for 5 min and analyzed for comets.

For each cell line and in some cases different concentration (RTgill-W1) exposed to the effluent from each water sample collection point, 100 nuclei images were selected randomly for analysis (50 nuclei per slide, in duplicate), which were visually classified into one of five classes of damage: zero (no damage) to 4 (maximum damage), according to the methodology proposed by Collins (2004). A calculation of the total damage in the cells was determined as the comet score using the formula

$$\frac{[(\text{Class } 0)\times 0]+ [(\text{Class } 1)\times 1]+ [(\text{Class } 2)\times 2]+ [(\text{Class } 3)\times 3]+ [(\text{Class } 4)\times 4]}{\text{Total number of cells counted per slide}}$$

and the number of damaged cells (damage frequency) analyzed based on the ability of the tail to migrate under electrophoresis, such that its intensity and size are suggestive of the index of fragments generated by the exposure to genotoxic agents.

7.2.5 The Ames test

Mutagenicity of the samples was assessed using the *Salmonella* plate incorporation assay according to the standard test protocol of Maron and Ames (1983) without exogenous metabolic activation (addition of S9 microsomal fraction). *Salmonella typhimurium* test strains TA98 and TA100 were employed to investigate the mutagenic activity of sediment extracts. While strain TA100 is sensitive for detecting base-exchange mutations, strain TA98 is able to detect frame shift mutagens, which

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lead to a mismatch of the amino acid sequence during translation (Mortelmans and Zeiger; 2000). All chemicals used were of research grade along with instruments that were autoclaved to prevent contamination.

Briefly, 100 µL of *S. typhimurium* strains TA98 and TA100 were inoculated into 10 mL Oxoid nutrient broth No.2, and then incubated at 37^{o}C for 16 hours. A volume of 100 µL water test samples (AMD water), negative (sterile distilled water) and positive control (4-nitroquinoline-N-oxide, at a concentration of 2 µg/mL) were dispensed in allocated sterilized culture tubes and 500 µL phosphate buffer was then added, followed by addition of 100 µL of bacterial strain. To 100 mL top agar (Difco®), placed in a water bath at 50^{o}C, 10 mL biotin/histidine mixture was added and 2 mL aspirated into the test tube containing the test samples. Test tubes containing samples were placed in a water bath at 37^{o}C, mixed thoroughly by vortexing, poured onto the agar plates, and then incubated for 48 hours at 37^{o}C. Genotoxicity was accessed through bacterial colony counts after the 48h incubation period using a colony counter, and the results were expressed as the mean (±standard error) number of the revertant colonies per plate. A positive mutagenic response in the Ames test is recorded when there is doubling of the number of revertant colonies at any concentration of the test sample compared to the negative control (Verschaeve and van Staden, 2008).

7.2.6 Statistical analysis

All assays were performed in three independent experiments and the results presented as mean ± SD (standard deviation) values. Statistical analysis was carried out using a one-way analysis of variance (ANOVA) followed by Hartley's *f* test for equal variance. Inter-test variations were assessed using the appropriate Student *t*-test. This data analysis was performed using OpenEpi, open source calculator Version 3. P value was set at $p<0.05$.

7.3 Results

7.3.1 Physico-chemical composition of water samples

The physicochemical parameters of the water samples showed variation in

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constituents with the pH revealing an increase in acidification of the stream immediately following the wetland output and before active in-stream neutralization. Dissolved oxygen content was lowest in post-treated water samples, a possible indication of salt precipitation evidenced by increase in Na, Mg and Ca contents downstream of the AMD impacted water shed. Compared to the reference site, significant higher conductivity and sulphate levels at the impacted site were detected. In relation to the metal contents of the water samples, metals such as Al, Mn and Zn indicated a significant increase in Untreated and Treated compared to Reference (see Table 7.1).

7.3.2 Mutagenic potential of water samples

The results of the Ames test showed that the mutagenic activities expressed as induction factors (multiples of the background levels) using the TA98 and TA100 strains were all less than 2. None of the water samples produced a response that was at least twice as high as that of the negative control. Assays with the strain TA100 and TA98 generally had lower levels of base-substitution mutagens, but displayed variation when comparing induction factors in the two strains as TA98 was more sensitive to the positive control and the water sample T, while TA100 was more sensitive to the water samples R and U (see Table 7.2).

7.3.3 DNA damage in cells treated with water samples

The results of the comet assay with Vero cells showed that all the test water samples (R, U and T) exhibited an increase in DNA damage relative to the negative control, which was statistically significant (Figure 7.1). The damage frequency was highest in U, the water sample with the highest metal content. The distribution of comet cells (Figure 7.2) showed that the negative control had the most cells which fell into the class 0, and the test water sample U produced more damaged cells than the R and T water samples. This trend was observed likewise using the RTgill-W1 cell line where cells exposed to two different water concentrations (75% and 50%) presented with greater damage frequency and higher comet score in U than for other water samples (Figure 7.3).

All three water samples tested showed genotoxic potential compared to the negative control (Milli-Q water) at the two concentrations tested using the RTgill-W1 cell line.

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A statistically significant reduction in damage frequency and/or comet score ($p<0.001$) however, was observed between the two concentrations tested (75% and 50%) in R and T but not for U. When comparing the comet score between water samples obtained from a relatively pristine site R, to U and T, a significantly lower damage in frequency was observed using both cell lines at the concentrations tested. The water sample R however, showed a significant increase in comet score values compared to the negative control, despite the fact that the water chemistry parameters fell within target water quality range. Distribution of comet cells in RTgill-W1 cells following exposure to water samples (Figures 7.4 and 7.5) indicated that comet formation order was N<R<T<U.

Comparing similar concentrations using both cell lines (i.e. at 50%), the Vero cells presented with slightly higher comet score and consequently, greater damage frequency compared to the RTgill-W1 cell lines ($p<0.001$). This is a possible indication for better sensitivity to genotoxic damage. When comparing the treatment technologies used, there was a significant reduction in genotoxicity (U and T) using both cells at the same concentration range (Figure 7.6).

7.4 Discussion

By convention, physical and chemical monitoring of water quality give information about the presence of substances and the concentrations at which they occur, leaving out highly relevant details about the toxicological or ecological effects of such waters. Although effluents such as AMD that are generated during coal mining are required to meet either the general or specific effluent standard before discharge into natural watercourses (Van der Merwe and Grobler, 1990), the presence of pollutants from mines generating AMD which contain sulphates, acidity, salinity and metals (including Al, Fe and Mn) contribute to surface water pollution (Heath et al., 2009). Proper management of surface waters for adequate protection of aquatic ecosystems requires a robust system that integrates chemical, biological and cellular analyses in order to detect the impact of contaminants on aquatic systems (Ohe et al., 2004) especially with regards to pollution related stress, and the presence of potential genotoxic substances in water which causes DNA damage (Klobucar et al., 2003). Ohe et al. (2004) proposed that mutagenicity/genotoxicity assays could be a useful

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water-monitoring tool. Both the Ames and comet assays have been suggested as effective genotoxicity assays (Grummt, 2000; Reifferscheid and Grummt, 2000), with a number of studies (Schnurstein and Braunbeck, 2001; Avishai et al., 2002; Nehls and Segner, 2001) recommending the use of the comet assay as a tool for environmental pollution monitoring of genotoxicity in aquatic environments.

In this study, the physico-chemical analyses (Table 7.1) revealed that effluent collected immediately following the wetland (U) had significantly higher metal levels and acidity compared to R and U, an indication of the possible inefficiency of the natural wetland to remove all potentially toxic pollutants. From our results, it was observed that U and T presented with high values of conductivity, but this was not the case for R. Conductivity, which is an indirect measurement of pollutant concentration, exceeded levels suggestive of environmental impacts (DWAF, 1996). The presence of sulphates and metals such as Al, Mn and Zn which are regarded as pollutants of interest in AMD (Heath et al., 2009) in U and T at significantly higher concentrations than R, further suggests the extent of AMD pollution at this site.

The use of species relevant cell lines such as Vero kidney cells (a mammalian cell line) and the RTgill-W1 cells (a fish gill cell line) as *in vitro* bioassays to assess the environmental quality of mine effluent impacting a natural water downstream of a colliery was utilized in our study. *In vitro* genotoxicity assays employing the use of cell lines have been previously reported. Amaeze et al. (2015) evaluated the cytotoxic and genotoxic responses of RTgill-W1 cells to sediment quality in a polluted lagoon. Other researchers have used the goldfish skin cell line (Vevers and Jha, 2008) and the RT gonad cell line (RTG2) (Reeves, 2008). Likewise, Kienzler et al, (2012) assessed the sensitivity of the RTgill-W1, RTL-W1 and PLHC-1 to a number of environmental pollutants using *in vitro* comet assay. Literature is replete with information on the use of *in vitro* mammalian cell lines such as HepG2 for testing environmental chemicals, pesticides, environmental mixtures, and other agents (Dearfield et al., 1991; Dearfield and Moore, 2005; Lah et al., 2005; Cimino, 2006).

The cell lines used in this investigation were efficient at detecting effluent genotoxicity, submitted to conventional treatments. The active system (in-stream neutralization) significantly reduced DNA damage in T compared to U. This could be

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attributed to a reduction in metal constituents of the water sample, which resulted in a seemingly better water quality due to lower metal load. A significantly higher genotoxicity observed with U, compared to R and T, confirmed the presence of AMD infiltration following passive treatment in the constructed wetland, and the genetic damage observed could be due to the presence of dissolved chemical agents and/or heavy metals not removed by the passive treatment method employed. Godet et al. (1996) reported that metals such as Al and Zn can induce genotoxicity, while Cu is believed to induce bulky lesions and DNA strand breakage (Lloyd et al, 1997). An *in vitro* comet assay in RTgill-W1 cells implicated Cu as the potential genotoxic agent causing ROS formation (Bopp et al., 2008). Synergistic effect of these substances along with other compounds present in the effluents may contribute to observed genotoxicity in the cells.

The presence and persistence of potentially toxic agents was further confirmed with the water chemistry results. The presence of metals along with sulfides increases its bioavailability (Griscom et al., 2000). Water sample U did not produce a significant reduction in DNA damage when tested at 75% and 50% dilutions using the RTgill-W1 cells. This implies that dilution may not play a role in toxicity reduction because toxic agents persisted and as such, metal precipitation through in-stream active water treatment technique will be required immediately following the wetland water flow. However, with R and T, a significant decrease in DNA damage was seen after dilution of water samples. A rise in the pH of the stream achieved using in-stream alkali neutralization process has been reported to result in complexation phenomena and subsequent reduction in metal burden as metals present precipitate as metal hydroxide (Madeira et al. 2005). This could be responsible for the observed significant reduction in DNA damage comparing water samples U (passively treated) and T (actively treated). Franklin et al. (2000) also reported a decrease in toxicity of effluent from an abandoned uranium mine in relation to an increase in pH of the effluent.

Although water chemistry results revealed a significant reduction in metals for Al, Mn and Zn, following active in-stream treatment, the presence of other potentially toxic agents like sulphates and salts may have played a role in the observed DNA damage which persisted despite lower metal burden. The unnecessary presence of sulphates

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and salts in aquatic environments has been reported to result in stress conditions associated with enhanced reactive oxygen species generation (ROS), which is implicated in oxidative damage. ROS are also highly reactive with DNA, proteins, carbohydrates and lipids (Curtin et al., 2002) and have been linked to the presence of metals, aromatic hydrocarbons, pesticides, polychlorinated biphenyls and dioxins in aquatic ecosystems (Valko et al., 2005; Valko et al., 2007).

Although the DNA damage resulting from cells exposed to water samples from the reference site was significantly lower than seen in U and T, there was significant DNA damage in cells compared to those exposed to the negative control. There are no untoward effluent inputs known at this site, and water chemistry parameters analyzed were within good water quality range (DWAF, 1996). Speculatively, it would appear that there are compounds undetected or not analyzed in our study that may have contributed to DNA damage through possible interactions (synergism, additivism) between the individual compounds in the whole water samples, resulting in cumulative response of the water sample to induce the observed genotoxicity.

Results showed that both cell lines are able to detect genotoxic potential in the surface water, but differences were evident with respect to sensitivity as seen with the Vero cell line. Previous work (Iji et al., unpublished) confirmed greater ROS generation in these cell lines compared to the RTgill-W1 and the C3A cell lines. The reason for this is not yet identified, but certain immortalized mammalian cell lines are reported to possess a greater degree of metal sensitivity (Freedland, 1989).

Mutagenicity of the water samples was not observed in the Ames test. A number of mutagens especially those that are important to human exposure are usually biologically inert, requiring metabolic activation to their mutagenic state. This activation can be achieved *in vitro* through the use of rodent liver enzyme (S9 microsome) activation, which was not employed in this study. An observed significant difference in response to the different water samples was seen when comparing both strains of bacteria. This was speculated to be due to certain substances in the effluent having affinity for either base-exchange mutations in TA100, or frame shift mutation in TA98 (Mortelmans and Zeiger 2000). The Ames test uses several *S. typhimurium* strains, such as TA97, TA98, TA100, TA102,

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TA104, TA1535 and TA1537 and each strain is genetically different, consequently the use of several strains in a test increases the likelihood of detecting a mutagenic chemical. While not all genotoxic substances stimulate the establishment of mutations, the Ames test (Maron and Ames 1983) is still widely used to assess genotoxic activity of wastewater (Stahl, 1991).

In this study, the comet assay detected DNA damage in both cell lines exposed to water samples (Ref) from a reference site, a passively treated effluent (U) and effluent that has been actively treated in-stream (T), proving the sensitivity of the comet assay as a quick, simple and effective genotoxicity assay for the detection of DNA damage (single-, double-strand breaks, alkali-labile sites or DNA-DNA and DNA-protein crosslinks) in specific cells (Fairbairn et al., 1995) which could be useful in genetic toxicology studies (Cotelle and Férard, 1999).

7.5 Conclusion

Both the comet assay and the Ames test are appropriate as bioassays for the detection of genotoxic and mutagenic potentials in effluent evaluation for environmental monitoring. The comet assay appeared to be more sensitive, being specific to changes in individual cells, detecting almost any interaction with DNA, while the Ames test is restricted to basic mutagenic events. Moreover, in assessing *in vitro* sensitivity to genotoxicants arising from an AMD effluent, Vero cells would be preferred to RTgill-W1.

As it is not time and cost effective to perform a chemical analysis of all known contaminants within the aquatic environment, a bioassay-directed analysis may be a substitute especially in developing countries where aquatic ecosystems remain vulnerable due to indiscriminate mining activities and rapid industrialization, as well as inadequate legislation designed to fully protect water quality from the impact of mining. Rapid and cost-effective bioassays such as these will help target areas of concern for comprehensive hazard assessment. However, validation of the endpoints for ecological significance may still be required and once this is calibrated, it can be used in a more quantitative way.

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A future perspective for comprehensive assessment of genotoxocity in surface water should involve a combination of the comet assay and a full complement of *S. typhimurium* bacterial strains including exogenous bio-activation of samples with liver metabolizing enzymes.

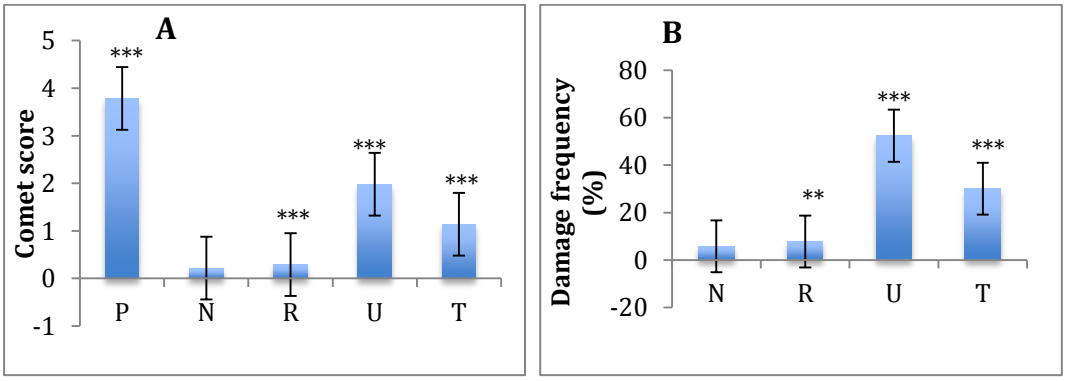

Figure 7.1: Damage (A) and Frequency (B) Indices of DNA damage to Vero cells exposed to 50% water sample impacted by AMD from a coalmine. P is positive control, N, Negative control, R; Reference, U; Untreated and T; treated water samples. ** $p \leq 0.01$ and *** $p \leq 0.001$

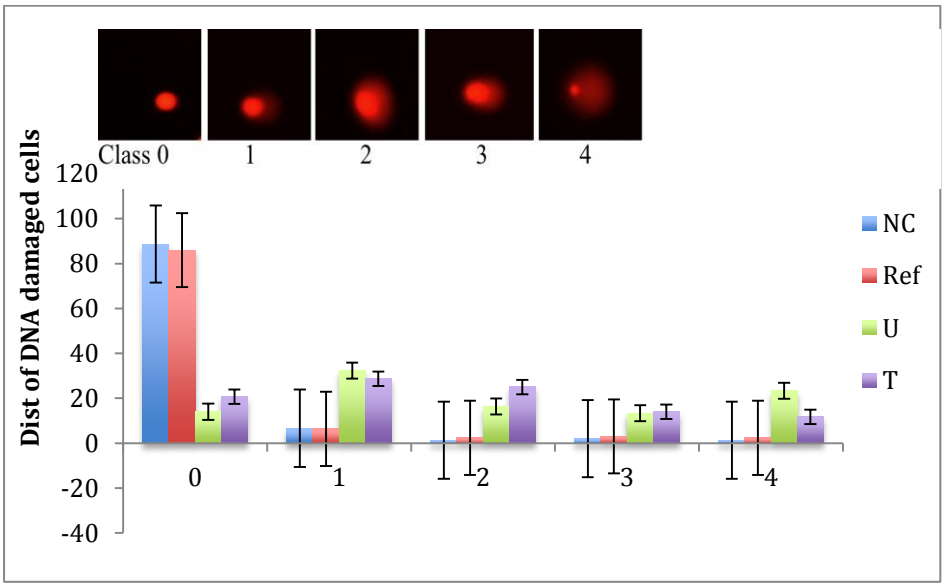

Figure 7.2: The DNA damage was visually classified from class 0 (no damage) to 4 (maximum damage), according to the size and shape of the tail. Mean values of nuclear DNA damage distribution in Vero cells exposed to water samples NC (negative control), Ref (reference site), U (untreated) and T (treated). Results are an average of 3 experiments (n=100).

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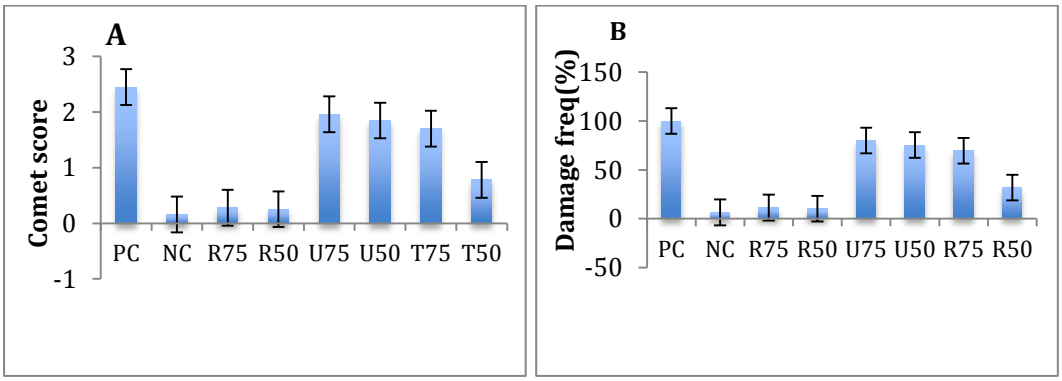

Figure 7.3: Oxidative damage to DNA assessed by the Comet assay in RT-gill W1 cells following 24h exposure. The values represent the average of repeats and the average % DNA damage measured in 100 cells from each repeat. PC is positive control, NC is negative control, R, U and T represents reference, passively treated and actively treated water samples at 75% and 50% respectively.

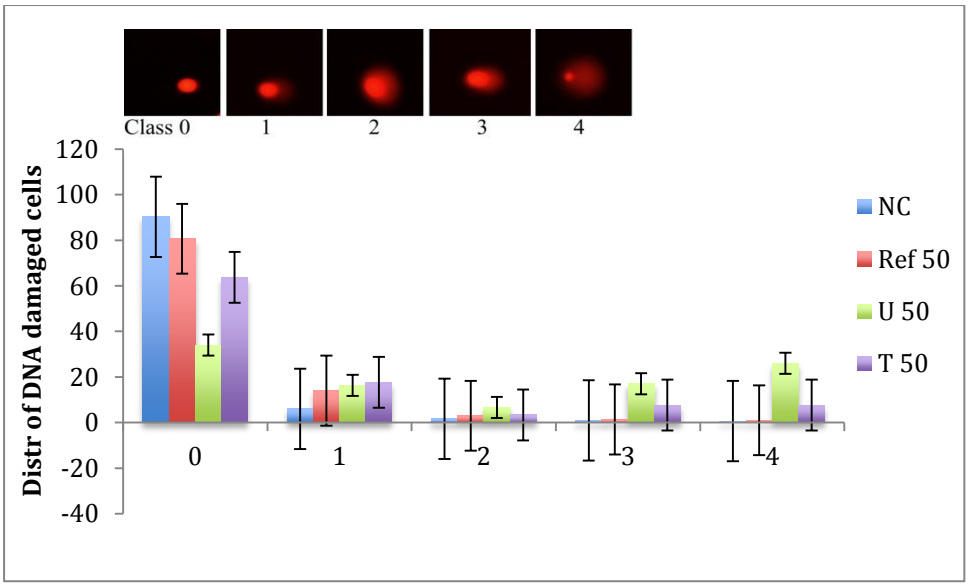

Figure 7.4: The DNA damage was visually classified from class 0 (no damage) to 4 (maximum damage), according to the size and shape of the tail. Mean values of nuclear DNA damage distribution in RTgills-W1 cells exposed to water samples at 50%. NC (negative control), Ref (reference site), U (untreated) and T (treated). Results are an average of 3 experiments.

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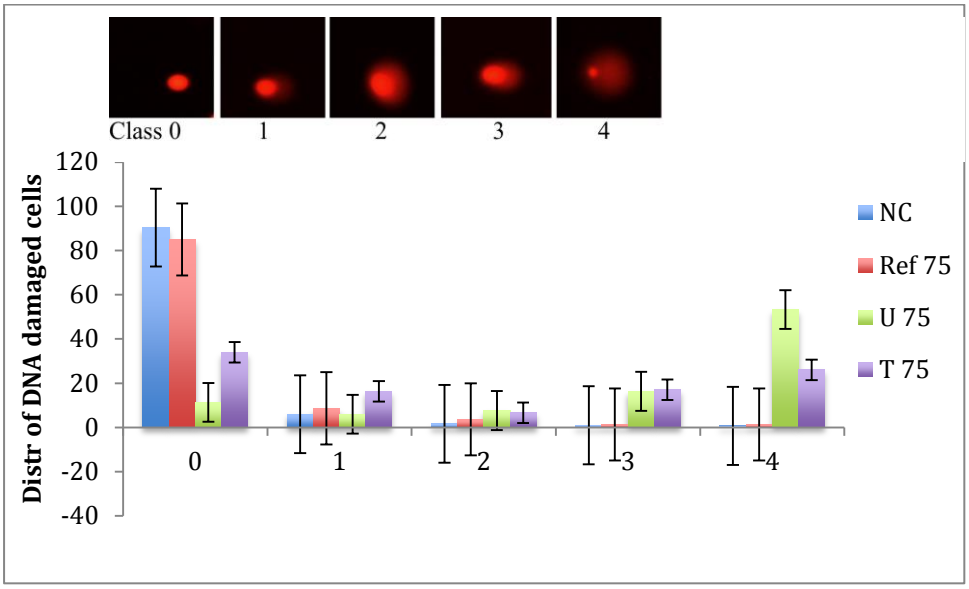

Figure 7.5: The DNA damage was visually classified from class 0 (no damage) to 4 (maximum damage), according to the size and shape of the tail. Mean values of nuclear DNA damage distribution in RTgill-W1 cells exposed to water samples at 75%. NC (negative control), Ref (reference site), U (untreated) and T (treated). Results are an average of 3 experiments.

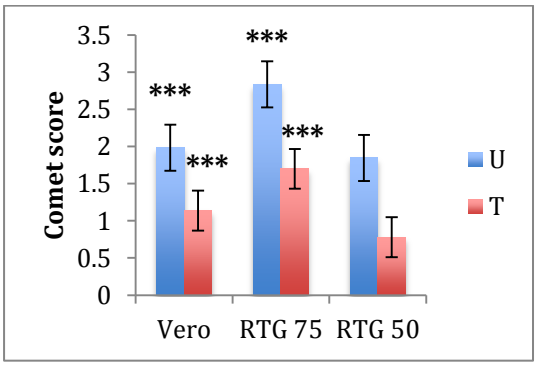

Figure 7.6 Oxidative damage to DNA comparing differences between water samples passively (U) and actively treated (T) in Vero and RTgill-W1 cell lines at the tested concentrations.

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Table 7.1: Physico-chemical analytes of water samples R, U and T at the different water collection points.

	pH	**DO (%)**	**Cond (MS/m)**	**Mg (mg/L)**	**Na (mg/L)**	**Ca (mg/L)**	**Sulp (mg/L)**	**Al (mg/L)**	**Fe (mg/L)**	**Mn (mg/L)**	**Zn (mg/L)**
Ref	6.67±0.30	8.45±0.77	5.73±0.73	0.45±0.07	0.90±0.10	0.15±0.07	4.50±0.70	0.03±0.01	0.01±0.01[a]	0.01±0.01	0.15±0.01
Untreated	3.20±0.70	6.90±0.20	320±7.77	152±2.12	43±0.20	475±0.35	2169±0.98	7.55±0.9	0.36±0.03 [a]	65±0.14	3.35±1.06
Treated	8.00±0.57	4.90±0.10	326±0.12	170±0.70	120±0.14	561±0.12	2336± 3.73	0.15±0.01	0.02±0.01[a]	39±6.36	0.04±0.02

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Table 7.2: Number of revertant colonies of *Salmonella typhimurium* strains TA98 and TA100 induced by untreated and treated AMD water samples from a coalmine in Mpumalanga province, South Africa

Sample		TA98		TA100
	Revertants /plate	Induction factor	Revertants /plate	Induction factor
MilliQwater	17±0	1	159±3	1
	Mean ±SD	IF±SD	Mean ±SD	IF±SD
Ref	18±0	1.06±0.01	182±23	1.13±0.07
Untreated	26±1	1.52±0.02	276±1	1.72±0.002
Treated	27±1	1.58±0.02	163±8	1.10±0.02
4NQO	253±23	18.88±0.71	822±3.06	5.17±0.13

4NQO- 4-Nitroquinoline-N-Oxide (Positive control), SD- Standard deviation, IF- Induction factor

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CHAPTER 8: General Discussion and Conclusions

An important component in the management of watersheds influenced by anthropogenic activities, such as mining, involves assessing and understanding such impacts on the biological, physical and chemical components of the aquatic ecosystems. In order to improve water quality, monitoring is essential for detecting changes in water chemistry that have resulted from pollution, as it evaluates the impact of mining activities. Biological monitoring on one hand offers a more detailed extension of monitoring, because changes are detected relative to the environment. Conventional chemical analysis carried out to estimate the risks of contaminated sites does not provide enough information about the potential hazards to organisms. Conversely, the use of cultured cells and cell lines in *in vitro* bioassays have been shown to be very useful tools for describing the environmental quality of water samples because they provide an estimate of the total biological activity of chemicals present in the water column. A combination of both *in vitro* bioassays and chemical analysis offers the advantage of identifying substances with the potential to cause observable biological effects, and their sources. This is premised on the fact that toxicity is first displayed at the cellular level and, as such, *in vitro* structural and functional changes arising from exposure to substances are indicative of basal toxicity responses that pose a threat to the whole organism.

The advantage of *in vitro* bioassays is that they are alternatives to the use of whole animals, and it offers an understanding of the toxicity of chemicals to aquatic organisms, or even the aquatic environment as a whole, which is essential in the management of contaminated sites since it is cumbersome and unrealistic to identify and measure the concentrations of all toxicants in the water column at all times.

The aim of this study was to develop an *in vitro* based monitoring system for assessing toxicity associated with AMD effluent from coal mines impacting natural water resources.

In order to achieve this aim, the following objectives were set out:

- Investigate the generation of reactive oxygen species (an indicator of oxidative stress) in species relevant cell lines as a bio-indicator of the safety of treated acid mine water.

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- Determine, *in vitro*, cytotoxic endpoints of bioavailable water contaminants in cell lines exposed to AMD effluent, to determine if cytotoxic responses correlate strongly with water chemical parameters in-stream.
- Establish and validate primary fish gill cultures in freshwater fish (*Oreochromis mossambicus*) as an *in vitro* toxicity monitoring system using specific endpoints such as CYP1A induction as a biomarker of exposure to environmental contaminants for potential toxicity evaluation of environmental samples.
- Determine the genotoxic potential of impacted stream by AMD effluent using the Comet assay and the Ames test in human and fish cell lines.

The extent to which these objectives were attained is discussed herein.

In the first objective, results showed that the DCFH-DA assay technique in cell lines could be used to rapidly biomonitor the quality of treated AMD water as it relates to the formation of ROS, an indicator of oxidative stress. This assay revealed that transient increases in ROS production are detectable in cell lines and may be useful in biomonitoring of contaminated water. This is an alternative, relevant model for evaluating consequences of exposure to environmental pollutants involving coal mines, to detect the onset of oxidative stress in the aquatic environment.

The second objective was to determine the cytotoxic endpoints of AMD effluent in cell lines and to correlate these with physico-chemical parameters of water samples. Results indicated dose dependent cytotoxic responses in both mammalian and fish cell lines, with the RTgill-W1 cells showing greater loss of cell viability and thus increased sensitivity compared to Vero cells. It was evident that the presence of contaminants in water (physico-chemical properties) influenced cytotoxic responses, with heavy metals and sulphates playing a major role. The MTT assay technique used to detect cytotoxicity was more sensitive than the NR or the LDH, an indication that the mitochondria perhaps play a role in the mechanism of toxicity. This confirmed TEM findings that showed ultrastructural changes in the mitochondria following exposure of cells to AMD water.

The third objective evaluated the use of primary gill cultures of Tilapia fish (*O. mossambicus*), an indigenous African freshwater fish, as a tropical model species for assessing the biomarker of exposure to environmental toxicants such as B[a]P, a toxicant

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associated with coal utilization, through induction of CYP1A. Results from the study validated the use of primary gill cultures from Tilapia as an *in vitro* toxicity monitoring system. This suggests that the primary gill cells could be a useful tool to screen freshwater systems in Southern Africa for the presence of CYP1A inducers such as polychlorinated di-benzodioxins, dibenzofurans, biphenyls and polycyclic aromatic hydrocarbons.

The final objective was to evaluate the genotoxic potential of AMD effluent on the impacted stream using the comet assay and the Ames test in human (Vero) and fish (Rtgill-W1) cell lines. Significantly higher genotoxicity was observed in the passively treated effluent compared to the actively treated effluent, which was attributed to the presence of dissolved chemical agents and/or heavy metals. A significant reduction in DNA damage was observed following active treatment of the AMD and both cell lines showed evidence of genotoxicity using the comet assay. However, mutagenicity was not observed in cell lines using the Ames test. In assessing *in vitro* sensitivity to genotoxicants arising from AMD effluent, Vero cells showed better sensitivity compared to RTgill-W1. We therefore suggest that *in vitro* genotoxicity bioassay has the potential to be integrated into an environmental monitoring framework.

This study established that deleterious substances present in contaminated water can elicit biological responses in cell lines. Effects noted correlated with physico-chemical water quality monitoring, particular heavy metals, although the lack of dose–response data for individual metals made it difficult to pinpoint the cause of the observed effects. *In vitro* bioassays potentially could be beneficial in detecting the degree and extent of impact of AMD in natural water, as well as the effectiveness of intervention. This could be a rapid and cost-effective tool needed for assessing risk and potential toxic effects of contaminated AMD impacted watersheds, and an early warning tool preceding a more detailed analysis. In general, the use of fish gill cell lines over mammalian cell lines and other cell types could be more advantageous as the relatively low temperatures at which they are cultured and exposed to environmental samples limits the extent to which microbes can proliferate, making exposure to unfiltered surface water samples a possibility.

A future perspective could involve multi organ systems that better mimic the three-dimensional organ structure that further improves *in vitro* screening of environmental contaminants. This would contribute to a better understanding of the ecology and

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biochemistry of many environmental contaminants. Effects of its successful application would eventually allow for designing automated ways for the high-throughput screening of contaminants that harm the environment.

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CHAPTER 9: References

Adler R, Claassen M, Godfrey L, Turton AR (2007). Water, mining and waste: A historical and economic perspective on conflict management in South Africa. Econ Peace Secur J. 2: 32–41.

Adler R, Rascher J (2007). A Strategy for the Management of Acid Mine Drainage from Gold Mines in Gauteng CSIR Report Number CSIR/NRE/PW/ER/2007/0053/C (Pretoria: CSIR).

Ahmad S (1991). Public Attitude Towards Water and Water Reuse. Wat. Sci. Tech 23: 2165-2117.

Ahne W (1985). Use of fish cell cultures for toxicity determination in order to reduce and replace the fish tests. Zentralbl. Bakteriol. Mikrobiol. Hyg. (B) 180:480–504.

Ahrens MJ, Morrisey DJ (2005). Biological effects of unburnt coal in the marine environment. Oceanography and marine biology, 43:69-122.

Akcil A, Koldas S (2006). Acid Mine Drainage (AMD): Causes, treatment and case studies. Journal of Cleaner Production 14:1139-1145.

Alberta LA, Badillo F (1991). Environmental lead in Mexico. Rev Environ Contam Toxicol 117:1-49.

Amaeze NH, Schnell S, Sozeri O, Otitoloju AA, Egonmwan RI, Arlt VM, Bury NR (2015). Cytotoxic and genotoxic responses of the RTgill-W1 cells in combination with the yeast oestrogen screen to determine the sediment quality of Lagos lagoon, Nigeria**.** Mutagenesis 30:117–127.

American Public Health Association (APHA) (1995). Standard Methods for the Examination of Water and Wastewater, In: Eaton, A.D., Clesceri, L.S., Greenburg, A.E. (Eds.), 19th Edition Washington, DC.

American Public Health Association (APHA) (2005) In: Eaton, A.D., Clesceri, L.S., Rice, E.W., Greenberg, A.E. (Eds.), Standard Methods for the Examination of Water and Waste Water. Port City Press, Baltimore.

Andre JM, Boudou A, Ribeyre F, Bernhard M (1991). Comparative study of mercury accumulation in dolphins (*Stenella coeruleoalba*) from French Atlantic and Mediterranean coasts. Sci. Total Environ. 104:191-209.

Anglogold Ashanti Annual Report (2005). http://www.anglogold.com.

Antunes SC, Pereira R, Gonåalves F (2007). Acute and chronic toxicity of effluent water from an abandoned uranium mine. Arch. Environ. Contam. Toxicol. 53:207–213.

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Aschner M, Gannon M, Kimelberg HK (1992). Manganese uptake and efflux in cultured rat astrocytes. J. Neurochem. 58: 730–735.

Ashton PJ (1981). Nitrogen Fixation in a Nitrogen-limited Impoundment. Journal of the Water Pollution Control Federation 51: 570-579.

ASTM (1997). Standard guide for *Daphnia magna* life-cycle toxicity tests. Report E1193-97. American Society for Testing and Materials, Philadelphia.

ASTM (2000). Test method for measuring the toxicity of sediment associated contaminants with freshwater invertebrates. E 1706-00. Annual Book of American Society for Testing and Materials Standards. Philadelphia, USA.

Aust SD, Morehouse LA, Thomas CE (1985). Role of metals in oxygen radical reactions. J Free Radicals Biol. Med. 1: 3-25.

Avishai N, Rabinowitz C, Moiseeva E, Rinkevich B (2002). Genotoxicity of Kishon River, Israel: the application of an *in vitro* cellular assay. Mutat. Res. 518: 21–37.

Azzie BA (1999). *The geochemistry and quality of water from selected collieries on the South African Highveld.* M.Sc thesis, University of Cape Town, South Africa, 181 pp.

Babich H, Borenfreund E (1991). Cytotoxicity and genotoxicity assays with cultured fish cells: A review. Toxicol. In Vitro 5:91-100.

Babich H, Rosenberg DW, Borenfreud E (1991). In vitro cytotoxicity studies with the fish hepatoma cell line, PLHC-1 (Poeciliopsis lucida). Ecotoxicol. Environ. Saf, 21: 327-337.

Babich H, Shopsis C, Borenfreund E (1986). In vitro cytotoxicity testing of aquatic pollutants (cadmium, copper, zinc, nickel) using established fish cell lines. Ecotoxicol. Environ. Saf. 11:91–99.

Backhaus T, Altenburger R, Boedeker W, Faust M, Scholze M, Grimme LH (2000). Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to Vibrio fischeri. Environ. Toxicol. Chem. 19:2348–2356.

Baksi S, Frazier J (1990). Isolated fish hepatocytes - model systems for toxicology research. Aquatic toxicology, 16: 229-256.

Balch GC, Metcalfe CD, Huestis SY (1995). Identification of Potential Fish Carcinogens in Sediment from Hamilton Harbour, Ontario, Canada. Environ Toxicol Chem 14: 79-91.

Barbieri E (2009). Concentration of heavy metals in tissues of green turtles (*Chelonia mydas*) sampled in the Cananeia estuary, Brazil, Brazil. Journ. Oceanogr., 57: 243.

Barratt MD (2000). Prediction of toxicity from chemical structure. Cell Biol Toxicol. 16:1-13.

Bastos ACS, Freitas, AC (2000). Agents and interference processes, degradation and

© University of Pretoria

environmental damage In: Cunha SB; Guerra AJT, editors. Agents and interference processes, degradation and environmental damage. 2nd ed. Rio de Janeiro: Bertrand.

Batley GE, Burton GA Jr, Chapman PM, Forbes VE (2002). Uncertainties in sediment quality weight of evidence (WOE) assessment. Human Ecol Risk Asess 8:1517-1547.

Bayne BL et al. (1980). "Measurement of the responses of individuals to environmental stress and pollution: Studies with bivalve Molluscs." Trans. Royal Philosoph. Soc. (G. B.), 286, 563; WRC Info 1: 80-2500.

Bayne BL, Clarke KR, Gray JS (1988). Background and rationale to a practical workshop on biological effects of pollutants. Mar Ecol Prog Ser. 46: l-5.

Behnisch PA, Hosoe K. Sakai SI (2003). Brominated dioxin-like compounds: in vitro assessment in comparison to classical dioxin-like com- pounds and other polyaromatic compounds. Environ Int 29 : 861- 877.

Bell FG, Halbich TFJ, Bullock SET (2002). The effects of acid mine drainage from an old mine in the Witbank Coalfield, South Africa, Quarterly Journal of Engineering Geology and Hydrogeology 35: 265–278.

Belpaire C, Smolders R, Auweele IV, Ercken D, Breine J, Van Thuyne G, Ollevier F (2000). An index of biotic integrity characterizing fish populations and the ecological quality of Flandrian water bodies. Hydrobiologia 434:17-33.

Belyaeva AE, Glazunov VV, Korotkov MS (2002). Cyclosporin A- sensitive permeability transition pore is involved in Cd2+-indiced dysfunction of isolated rat liver mitochondria: doubts no more. Arch. Biochem. Biophys 405:252–264.

Bernhardt ES, Palmer MA (2011). The environmental costs of mountaintop mining valley fill operations for aquatic ecosystems of the Central Appalachians. Annals of the New York Academy of Sciences 1223:39-57.

Birnbaum, LS. Tuomisto J (2000). Non-carcinogenic effects of TCDD in animals. Food Addit Contam 17: 275–88.

Blaauboer BJ, Balls M, Barratt M, Casati S, Coecke S, Mohamed MK, Moore J, Rall D, Smith KR, Tennant R, Schwetz BA, Stokes WS, Younes M (1998). 13th Meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): alternative testing methodologies and conceptual issues. Environ Health Perspect. 106: 413–415.

Bleise A, Danesi PR, Burkart W (2003). Properties, use and health effects of depleted uranium (DU): A general overview. J Environ Radioactiv 64: 93–112.

Bols NC, Dayeh VR, Lee LEJ, Schirmer K (2005). Use of fish cell lines in the toxicology and

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ecotoxicology of fish. Piscine cell lines in environmental toxicology. In: Moon TW, Mommsen TP, editors. Biochemistry and Molecular Biology of Fishes. Vol. 6. Amsterdam: Elsevier Science.

Bols NC, Lee LEJ (1994). Cell lines: Availability, propagation and isolation. In: Hochachka PW, Mommsen TP, editors. Biochemistry and molecular biology of fishes, vol. 3. Amsterdam: Elsevier Science; p. 145–149.

Bopp SK, Abicht HK, Knauer K (2008) Copper-induced oxidative stress in rainbow trout gill cells. Aquat Toxicol 86:197–20.

Borenfreund E, Babich H, Martin-Alguacil N (1988). Comparison of two *in vitro* cytotoxicity assays: The neutral red (NR) and tetrozolium (MTT) tests. Toxicol in Vitro 2:1–6.

Borenfreund E, Puerner JA (1984). A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR90). J Tissue Cult Methods 9: 7–9.

Bornette G, Amoros, C, Lamouroux N (1998). Aquatic plant diversity in riverine wetlands: the role of connectivity. Freshw Biol 39: 267-283.

Bouquegneau JM, Joiris C (1992). Ecotoxicology of stable pollutants in cetaceans: organochlorines and heavy metals. In: (Symoens J.J., ed.) Whales: Biology - Threats - Conservation. Symposium. Proceedings (Brussels, 5-7 June 1991). Royal Academy of Overseas Sciences (Brussels). pp 247-250.

Brack W (2003). Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures? Anal Bioanal Chem 377:397–407.

Brack W, Schirmer K, Erdinger L, Hollert H (2005). Effect-directed analysis of mutagens and ethoxyresorufin-*O*-deethylase inducers in aquatic sediments. Environ. Toxicol. Chem 24: 2445–2458.

Breen D, Breen B, Laycock P, Hore R (1996). Biological monitoring at the new Quicksilver Connections visitor pontoon, Agincourt Reef No. 3, 1994-96. A report to the Great Barrier Reef Marine Park Authority.

Brown MT, Vivas MB (2005). Landscape development intensity index. Environmental Monitoring and Assessment 101: 289-309.

Bucheli TD, Fent K (1995). Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Crit. Rev. Environ. Sci. Tech, 25: 201- 268.

Buikena AL Jr, Cairns J Jr (1980). Aquatic invertebrate bioassays: ASTM STP 715. Philadelphia.

Burgman MA, Lindenmayer DB (1998). Conservation Biology for the Australian Environment. Surrey Beatty and Sons, Chipping Norton, Sydney, Australia.

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Bury NR, Schnell S, Hogstrand C (2014). Gill cell culture systems as models for aquatic environmental monitoring. J. Exp. Biol 217: 639–650.

Butler R, Nowak BF (2004). A dual enzyme method for the establishment

Cairns J Jr. (1986). The myth of the most sensitive species. Bioscience: 36: 670-672.

Campbell PGC, Stokes PM (1985). Acidification and toxicity of metals to aquatic biota. Can. J. Fish. Aquat. Sci. 42:2034–2049.

Campbell PGC, Tessier A (1987). Metal speciation in natural waters: Influence of environmental acidification. In Sources and Fates O.1. Aquatic Pollutants, ed. R.A. Hites & S.J. Eisenreich, Washington, DC, American Chemical Society, pp. 185 207.

Cardozo TR, Rosa DP, Feiden IR, Rocha JAV, Oliveira NCD, Pereira TS, Pastoriza TF, Marques DM, Lemos CT, Terra NR, Vargas VMF (2006). Genotoxicity and toxicity assessment in urban hydrographic basins. Mutat Res 603:83–96.

Carls MG, Rice SD, Hose JE (1999). Sensitivity of fish embryos to weathered crude oil: I. Low level exposure during incubation causes malformations, genetic damage, and mortality in larval Pacific herring (*Clupea pallasi*). Environ. Toxicol. Chem 18:481–493

Carlsson C, Pärt, P (2001). 7-Ethoxyresorufin *O*-deethylase induction in rainbow trout gill epithelium cultured on permeable supports: asymmetrical distribution of substrate metabolites. Aquatic Toxicology 54: 29–38.

Castano A, Bols N, Braunbeck T, Dierickx P, Halder M, Isomaa, B, Kawahara, K, Lee LEJ, Mothersill P, Pärt P, Repetto G, Sintes JR, Wood C, Segner H (2003). The use of fish cell in ecotoxicology. ATLA 31: 317–351.

Castaño A, Sanchez P, Llorente MT, Carballo M, de la Torre A, Mun˜oz MJ (2000). The use of alternative systems for the ecotoxicological screening of complex mixtures on fish populations. The Sci. Tot Environ 247:337–348.

Castaño A, Vega M, Blazquez T, Tarazona JV (1994). Biological alternatives to chemical identification for the ecotoxicological assessment of industrial effluents: the RTG-2 in vitro cytotoxicity test. Environ Toxicol. Chem 13:1607–1611.

Cavanaugh PF et al (1990). A semi-automated neutral red based chemosensitivity assay for drug screening. Invest. New Drugs 8: 347–354.

Celander M, Bremer J, Hahn ME, Stegeman JJ (1997). Glucocorticoid – xenobiotic interactions: dexamethasone- mediated potentiation of cytochrome P4501A induction by b-naphthoflavone in a fish hepatoma cell line (PLHC-1). Environ. Toxicol. Chem. 5: 900–907.

Center for Streamside Studies (CSS) (2002). Environmental impacts of hardrock mining in

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Eastern Washington. College of Forest Resources and Ocean and Fishery Sciences, University of Washington, Seattle, WA.

Chapman DW (1988). Critical review of variables used to define effects of fines in redds of large salmonids. Tran. Am. Fish. Soc.117: 1-21.

Chapman GA (1981). Do organisms in laboratory toxicity tests respond like organisms in nature? In: Bishop, N.E., Cardwell, R.D. & Heidolph, B.B. (eds) Aquatic toxicology and hazard assessment: sixth symposium, pp. 315- 327. ASTM Philadelphia.

Chapman JC (1995). The role of ecotoxicology testing in assessing water quality. Australian Journal of Ecology 20: 20-27.

Chapman PM (2007). Determining when contamination is pollution – weight of evidence determinations for sediments and effluents. Environ. Int. 33: 492-50.

Chapman PM, Wang F, Janssen CR, et al (2003). Conducting ecological risk assessments of inorganic metals and metalloids: Current status. Hum Ecol Risk Assess 9:641–97.

Chasiotis H, Kelly SP (2011a). Effect of cortisol on permeability and tight junction protein transcript abundance in primary cultured gill epithelia from stenohaline goldfish and euryhaline trout. Gen. Comp. Endocrinol. 172: 494-504.

Chasiotis H, Kelly SP (2011b). Permeability properties and occludin expression in a primary cultured model gill epithelium from the stenohaline freshwater goldfish. J. Comp. Physiol. B 181:487-500.

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

Chen C, Liu MC, Shih ML, Yu S, Yeh C, Lee ST, Yang T, Hung S (2001). Microsomal monooxygenase activity in Tilapia (*Oreochromis mossambicus*) exposed to a bleached kraft mill effluent using different exposure systems. Chemosphere 45: 581–588.

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

Cherry DS, Currie RJ, Soucek DJ, Latimer HA, Trent GC (2001). An integrative assessment of a watershed impacted by abandoned mined land discharges. Environmental Pollution 111: 377-388.

Chigor VN, Umoh VJ, Okuofu CA, Ameh JB, Igbinosa EO, Okoh AI (2012). Water quality assessment: surface water sources used for drinking and irrigation in Zaria, Nigeria are a public health hazard. Environmental Monitoring and Assessment 184: 3389-3400.

Choi J, Oris JT (2000). Evidence for oxidative stress in bluegill sunfish (*Lepomis macrochirus*) liver microsomes simultaneously exposed to solar ultraviolet radiation and

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anthracene. Environ Toxicol Chem. 19: 1795-1799.

Chow SC, Wong CKC (2011). Regulatory function of hyperosmotic stress induced signaling cascades in the expression of transcription factors and osmolyte transporters in freshwater Japanese eel primary gill cell culture. J. Exp. Biol. 214:1264-1270.

Chutter FM (1998) Research on the rapid biological assessment of water quality impacts in streams and rivers. Water Research Commission Report No. 422/1/98. WRC, Pretoria.

Cimino MC (2006). Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes. Environ. Mol. Mutagen. 47:362–390.

Clemedson C, Ekwall B (1999). Overview of the final MEIC results. I. The in vitro–in vitro evaluation. Toxicol. *In Vitro* 13:657–663.

Clemedson C, McFarlane-Abdulla E, Andersson M, Barile F, Calleja M, Chesne C et al (1996). MEIC evaluation of acute systemic toxicity. Part II. *In vitro* results from 68 toxicity assays used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. Atla-alternatives to Laboratory Animals 24: 273-311.

Clements WH and Newman MC (2002). Community Ecotoxicology. John Wiley and Sons, Chichester, United Kingdom, 336 pp.

Clothier R (1990). The FRAME modified neutral red uptake cytotoxicity test. Protocol INVITTOX no. 3. DB-ALM, ECVAM database service on Alternative Methods to Animal Experimentation. http://ecvam-dbalm.jrc.cec.eu.int/.

Coetzee L, Du Preez HH, Van Vuren JHJ (2002). Metal concentrations in Clarias gariepinus and Labeo umbratus from the Olifants and Klein Olifants River, Mpumalanga, South Africa: Zinc, Copper, Manganese, Lead, Chromium, Nickel, Aluminium and Iron. Water SA 28: 433.

Collins AR (2004). The comet assay for DNA damage and repair: principle, applications and limitations. Mol Biotechnol 26:249–261.

Conney AH (1982). Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G.H.A. Clowes memorial lecture. Cancer Res 42: 4875-4917.

Contreras L, Moenne A, Correa JA (2005). Antioxidant responses in *Scytosiphon lomentaria* (Phaeophyceae) inhabiting copper-enriched coastal environment. J Phycol. 41: 1184-1195.

Cossins AR, Crawford DL (2005). Fish as models for environmental genomics. Nat Rev Genet. 6: 324-333.

Cotelle S, Ferard JF (1999). Comet assay in genetic ecotoxicology: a review. Environ Mol

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Mutagen 34: 246-255.

Courtney LA, Clements WH (2000). Sensitivity to acidic pH in benthic invertebrate assemblages with different histories of exposure to metals. J Nam Benthol Soc 19: 112-127.

Cox EJ (1991). What is the basis for using diatoms as monitors of river quality? In Use of Algae for Monitoring Rivers (Whitton, B.A., Rott, E. & Friedrich, G., editors), 33–40. Universita¨t Innsbruck.

Craig PM, Wood CM, McClelland GB (2007). Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*). Am. J. Physiol. Regul. Integr. Comp. Physiol. 293: R1882–R1892.

Curtin JF, Donovan M, Cotter TG (2002). Regulation and measurement of oxidative stress in apoptosis. J Immunol Methods. 265: 49–72.

Dallinger R, Rainbow PS (1993). Ecotoxicology of Metals in Invertebrates. Lewis Publishers, Florida.

Dat JF, Pellinen R, Beeckman T, Van De Cotte B, Langerbartels C, Kangasjärvi J, Inzé D, Van Breusegem F (2003). Changes in hydrogen peroxide homeostasis trigger an active cell death process in tobacco. Plant J. 33: 621–32.

Dayeh VR, Lynn DH, Bols NC (2005). Cytotoxicity of metals common in mining effluent to rainbow trout cell lines and to the ciliated protozoan, *Tetrahymena thermophila*. Toxicol In Vitro. 19: 399-410.

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

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

Dayeh, VR, Schirmer K, Lee LEJ, Bols NC (2003). The use of fish-derived cell lines for investigation of environmental contaminants. In Hodgson, E. (ed.), Current Protocols in Toxicology. Wiley, New York, pp. 1–17.

De Villiers S, Mkwelo ST (2009). Has monitoring failed the Olifants River, Mpumalanga? Water SA 35**:**671-676.

Dearfield KL, Auletta AE, Cimino MC, Moore MM (1991). Considerations in the U.S. Environmental Protection Agency's testing approach for mutagenicity. Mutat. Res. 258: 259–283.

Dearfield KL, Moore MM (2005). Use of genetic toxicology information for risk assessment.

© University of Pretoria

Environ. Mol. Mutagen. 46:236–245.

Debenest T, Turcotte P, Gagne´F, Gagnon C, Blaise C (2012). Ecotoxicological impacts of effluents generated by oil sands bitumen extraction and oil sands lixiviation on *Pseudokirchneriella subcapitata*. Aquat Toxicol 112– 113: 83–91.

DeNicola D, Stapleton MG (2002). Impact of acid mine drainage on benthic communities in streams: the relative roles of substratum vs. aqueous effects. Environmental Pollution 119: 303-315.

Department of Environment Affairs and Tourism (DEAT) (2005). South African National Cleaner Production and Sustainable Consumption Strategy, Version 2, Draft 3, August 2005; Pretoria, South Africa.

Department of Minerals and Energy (DME) (2004). Operating and Developing coal Mines in the Republic of South Africa. Directory, D2/2004. Department of Minerals and Energy, Pretoria, South Africa.

Department of Minerals and Energy website (2004). Source http://www. dme.gov.za/ Acessesd 23 May 2014.

Department of Water Affairs and Forestry (2002). National Water Resource Strategy. Summary. Proposed first edition, August 2002, Pretoria, 37 pp.

Department of Water Affairs and Forestry (2004). Olifants Water Management Area: Internal Strategic Perspective. Report P WMA 04/0000/00/0304. Department of Water Affairs and Forestry, Pretoria.

Department of Water Affairs and Forestry (2007). Best Practice Guideline H4: Water Treatment.

Department of Water Affairs and Forestry (DWAF) (1998). Government Gazette No. 20526 (8 October 1999), Government notice. no 1191. General Authorizations in terms of Section 39 of the National Water Act, 1998 (Act no 36 of 1998).

Department of Water Affairs, Forestry and Environmental Conservation (1996) South African Water Quality Guidelines, Aquatic ecosystems (1st. Ed.). Department of Water Affairs and Forestry, Pretoria, Vol. 7. pp. 91–99.

Department of Water Affairs, Forestry and Environmental Conservation (1980). Hydrological Research Institute, Technical Report No. TR 108, August 1980. Summarized Water Quality Criteria.

Di Giulio, RT, Benson WH (2002). Interconnections between human health and ecological integrity. Pensacola, Fla., USA, SETAC Press.

Dietz R, Nørgaard J, Hansen JC (1998). Have arctic mammals adapted to high cadmium

© University of Pretoria

levels? Mar. Pollut. Bull. 36: 490-492.

DiGiulio RT, Benson WH, Sanders BM, VanVeld PA (1995). Biochemical mechanisms: Metabolism, adaptation and toxicity. In: Rand GW, editor. Fundamentals of Aquatic Toxicology, 2nd. Washington: Taylor and Francis, 523–561.

Diz HR (1997). Chemical and biological treatment of acid mine drainage for the removal of heavy metals and acidity, PhD Dissertation, Virginia Polytechnic Institute and State University, USA.

Djordjevic VB (2004). Free radicals in cell biology. International Review of Cytology - A Survey of Cell Biology 237: 57–89.

Driescher AC (2008). A water quality study of Loskop Dam and the upper catchment of the Olifants River. MSc Dissertation, University of the Free State, Bloemfontein, South Africa, pp. 1–150.

Druker BJ, Mamon HJ, Roberts TM (1989). Oncogenes, growth factors, and signal transduction. N. Engl. J. Med. 321:1383-1391.

DU Preez HH, Steyn GJ (1992). A preliminary investigation of the concentration of selected metals in the tissues and organs of the tigerfish (Hydrocynus vittatus) from the Olifants River, Kruger National Park, South Africa. Water SA 18: 131-136.

Dumpleton S, Robins NS, Walker JA, Merrin PD (2001). Mine water rebound in South Nottinghamshire: risk evaluation using 3-D visualization and predictive modelling, Quarterly Journal of Engineering Geology and Hydrogeology 34: 307–319.

Durkin TV, Herrmann JG (1994). Focusing on the Problem of Mining Wastes. EPA Seminar Publication/625/R-95/007 on Managing Environmental Problems at Inactive and Abandoned Metals Mine Sites.

Eastern Research Group Inc (ERG) (2006). Site Visit Report Pennsylvania Coal Mine Acid Drainage Treatment Systems. Chantilly, VA. (October). EPA-OW-2004-0032-2311.

Ekwall B (1983). Screening of toxic compounds in mammalian cell cultures. In *Cellular Systems for Toxicity Testing*, Publica- tion No. 209. Williams GM Dunkel VC, Ray VA (eds). New York Academy of Science, 14–17pp.

Ekwall B, Ekwall K (1988). Comments on the use of diverse cell systems in toxicity testing. Atla-alternatives to laboratory animals, 15: 193-201.

Engelbrecht JS (1992). The upper-reaches. Fauna and Flora 48:9-14.

Environment Canada (1990). Biological test method: acute lethality test using rainbow trout. EPS 1/RM/9, Ottawa, Canada, Environment Canada, Environmental Protection Series, p. 1–51.

© University of Pretoria

Environment Canada (1992). Biological Test method: Growth Inhibition Test Using the Freshwater Alga Selenastrum capricornutum. Report EPS 1/RM/25, Environment Canada, Ottawa, ON, Canada.

EPA (1991). Assessment and control of bioconcentrate contaminations in surface water. Office of Health and Environmental Assessment. U.S. Environmental Protection Agency, Cincinnati. Ohio.

Erasmus BJ, Hodgson FDI, Kirstein FE, Roper CB, Smit JS, Steyn PPA, Whittaker RRLG (1981). Geological, hydrological and ecological factors affecting increased underground extraction of coal. SAIMM Vacation School, Johannesburg.

Ercal N, Gurer-Orhan H, Aykin-Burns N (2001). Toxic metals and oxidative stress Part I: Mechanisms involved in metal induced oxidative damage. Current Topics in Medicinal Chemistry 1:529-539.

Escher BI, Hermens JLM (2002). Modes of action in ecotoxicology: their role in body burdens, species sensitivity, QSARs, and mixture effects. Environ. Sci. Technol. 36:4201–4217.

European Environment Agency (EEA) (2001). Late lessons from early warnings: the precautionary principle 1896-2000, Environmental issue report No 22, European Environment Agency.

European Environmental Bureau (EEB) (2000). The environmental performance of the mining industry and the action necessary to strengthen European legislation in the wake of the Tisza-Danube pollution. EEB Document no 2000/016. 32 pp.

Evans DH, Piermarini PM, Choe KP (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev 85: 97-177.

Farkas J, Christian P, Gallego-Urrea JA, Roos N, Hassellöv M, Tollefsen KE, Thomas KV (2011). Uptake and effects of manufactured silver nanoparticles in rainbow trout (*Oncorhynchus mykiss*) gill cells. Aquat. Toxicol. 101: 117-125.

Fatoki S, Ree T, Nakhavhembe J (2010). Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in Surface Waters and Runoff Highway and Urban Environment. In: Rauch, S., Morrison, G. M. and Monzón, A. (Eds.). (Vol. 17). Springer Netherlands, pp: 207-215.

Faust M, Altenburger R, Backhaus T, Blanck H, Boedeker W, Gramatica P, Hamer V, Scholze M, Vighi M, Grimme LH (2001). Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. Aquat.

© University of Pretoria

Toxicol. 56:13.

Feasby G, Jones RK (1994). Report of results of a Workshop on Mine Reclamation, Toronto, Ontario March 10–11, 1994. Workshop hosted by the IGWG-Industry Task Force on Mine Reclamation.

Federal Water Pollution Control Administration (FWPCA). (1968). Stream pollution by coal mine drainage, Upper Ohio River Basin. US Department of the Interior, Ohio Basin Region.

Fent K (2000). Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. Toxicol In Vitro 15:477-488.

Fenton J, Balls M (1993). Replacement of fish in ecotoxicological testing: Use of bacteria, other lower organisms and fish cells *in vitro*. In M. Rchardson (eds) Ecotoxicology monitoring VCH. Weinheim, Germany 71-81 pp.

Ferguson KD, Erickson PM (1988). Pre-Mine Prediction of Acid Mine Drainage. In: Dredged Material and Mine Tailings. Edited by Dr. Willem Salomons and Professor Dr. Ulrich Forstner. Copyright by Springer-Verlag Berlin Heidelberg 1988.

Filman DJ, Brawn RJ, Dankler WB (1975). Intracellular supravital stain delocalization as an assay for antibody-dependent complement-mediated cell damage. J Immunol Methods 6: 189–207.

Finucane DM, Waterhouse NJ, Amarante-Mendes GP, Cotter TG, Green DR (1999). Collapse of the inner mitochondrial transmembrane potential is not required for apoptosis of HL60 cells. Exp. Cell Res. 251**:** 166-174.

Firestone D (1991). Determination of dioxins and furans in foods and biological tissues: reviews and update. Assoc. Official Anal. Chemists 74: 375–384.

Fletcher M, Kelly SP, Part P, O'Donnell MJ, Wood CM (2000). Transport properties of cultured branchial epithelia from freshwater rainbow trout: a novel preparation with mitochondria-rich cells. J. Exp. Biol. 203:1523-1537.

Forstner U, Wittmann GTW (1981). Metal Pollution in the Aquatic Environment, Springer, New York, 473 pp.

Fotakis G, Timbrell J (2006). *In vitro* cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicology letters, 160: 171-177.

Franklin NM, Stauber JL, Markich SJ, Lim RP (2000). pH-dependent toxicity of copper and uranium to a tropical freshwater alga (*Chlorella* sp.). Aquat Toxicol. 48: 275–289.

© University of Pretoria

Freedland JH, Ciriolo MR, Peisach J (1989). The role of glutathione in copper metabolism and toxicity. J Biol Chem 264: 5598-5605.

Freshney I (2001). Application of cell cultures to toxicology. Cell biology and toxicology 17: 213-230.

Freshney RI (2005). Culture of Animal Cells, A Manual of Basic Technique, 5th Ed. Hoboken NJ, John Wiley & Sons.

Fritz KM, Fulton S, Johnson BR, Barton CD, Jack JD, Word DA, Burke RA (2010). Structural and functional characteristics of natural and constructed channels draining a reclaimed mountaintop removal and valley fill coal mine. Journal of the North American Benthological Society 29: 673-689.

Fromm PO (1980). A review of some physiological responses of freshwater fish to acid stress. Env. BioL Fish 5: 79-93.

Fulles W, Howie D, Otto D, Easton J (1996). Policies and legislation applicable to mine water. Literature review: A manual on mine water treatment and management practices in south Africa appendix Volume. WRC Report No 527/1/96. ISBN 1 86845 258 1 ISBN Set No 1 86845 263 8.

Gadagbui BKM, Goksoyr A (1996). Biomarker studies in Tilapia (Oreochromis niloticus) caged in, and in mudfish (Clarias anguillaris) exposed to sediment from a polluted river in Ghana, West Africa. Marine Environmental Research 42: 275.

Galvez F, Tsui T, Wood CM (2008). Cultured trout gill epithelia enriched in pavement cells or in mitochondria-rich cells provides insights into Na+ and Ca2+ transport. Vitro Cell. Dev. Biol .–Animal. 44: 415–425.

Gammeter S, Frutiger A (1990). Short-term Toxicity of Ammonia and Low Oxygen to Benthic Macroinvertebrates of Running Waters and Conclusions for Wet Weather Water Pollution Control Measures. Water Science and Technology, 22: 291-296.

Gardner SC, Fitzgerald SL, Vergas BA, Rodriguez LM (2006). Heavy metal accumulation in four species of sea turtles from the Baja California peninsula, Mexico, BioMetals 19: 91.

Gavin CE, Gunter KK, Gunter TE (1992). Mn^{2+} sequestration by mitochondria and inhibition of oxidative phosphorylation. Toxicol. Appl. Pharmacol. 115: 1–5.

Genter RB, Amyot DJ (1994). Freshwater benthic algal population and community changes due to acidity and aluminum-acid mixtures in artificial streams. Environ. Toxicol. Chem. 13: 369–380.

Gerhardt A (1993). Impact of heavy metals on stream invertebrates with special emphasis on acid conditions. Water Air Soil Pollution 66: 289–314.

© University of Pretoria

Gerhardt A, Janssens de Bisthoven L, Soares AMVM (2004). Macro-invertebrate response to an acid mine drainage: community metrics combined with automated behavioural toxicity bioassay. Environ Pollution 130: 263–274.

Ghosh MC, Ghosh R, Ray AK (2001). Impact of copper on biomonitoring enzyme ethoxyresorufin-O-deethylase in cultured catfish hepatocytes. Environ. Res. Sect, (A) 86: 167–173.

Gibbs PNB, Gore MG, Jordan PM (1985). Investigation of the effect of metal ions on the reactivity of thiol groups in humans: aminolevulinate dehydratase. Biochem. J. 225: 573–580.

Giesy JP, Hilscherova K, Jones PD, Kannan K, M. Machala (2002). Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Mar. Pollut. Bull. 45: 3–16.

Gitari WM, Fatoba, OO, Petrik LF, Vadapalli VRK (2009). Leaching characteristics of selected South African fly ashes: Effect of pH on the release of major and trace species. Journal of Environmental Science and Health, Part A, 44: 206-220.

Godet F, Babut M, Burnel D, Veber A-M, Vasseur P (1996). The genotoxicity of iron and chromium in electroplating effluents. Mutat Res. 370:19–28.

Goudie A (1993). The nature of the environment. 3rd ed., Blackwell Publishers, UK.397pp.

Government Gazette (1984). Requirements for the purification of wastewater or effluent. Gazette No. 9225, Regulation, 991. Geneva, Switzerland.

Gray NF (1996). A substrate classification index for the visual assessment of the impact of acid mine drainage in lotic systems. Water Research 30: 1551-1554.

Gray NF (1997). Environmental impact and remediation of acid mine drainage: a management problem. Enviro Geol. 30: 62-71.

Grim EC, Hill RD (1974). Environmental protection in surface mining of coal. Environment Protection Agency Report, EPA-670:2-74-093.

Grippo RS, Dunson WA (1996). The body ion loss biomarker. 1. Interactions between trace metals and low pH in reconstituted coal mine polluted water. Environ. Toxicol Chem. 15: 1955-1963.

Griscom SB, Fischer NS, Luoma SN (2000). Geochemical influences on assimilation of sediment-bound metals in clams and mussels. Environ. Sci. Technol. 34: 91.

Grobbelaar R, Cruywagen LM, de Necker E, Hodgson FDI (2000). Investigation into geohydrological and hydrochemical aspects relating to mine closure for Transvaal Navigational Colliery. Confidential Report to Ingwe S.A.

© University of Pretoria

Grobbelaar R, Usher B, Cruywagen LM, de Necker E, Hodgson FDI (2002). Long term impact of Intermine flow from Collieries in the Mpumalanga area. WRC Report No 1056/1/04.

Grummt T (2000). Genotoxic substances in the environment. Schriftenr Ver Wasser Boden Lufthyg 106: 63-69

Guerin M.R (1978). Energy sources of polycyclic aromatic hydrocarbons. In Polycyclic hydrocarbons and cancer (Gelboin HV, TS'o,PO eds.) Acad . Press, New York, 1978, 1-42 pp.

Gulden M, Mörchel S, Seibert H (2005). Comparison of mammalian and fish cell line cytotoxicity: impact of endpoint and exposure duration. Aquatic Toxicology 71: 229–236

Gunderson LH, Pritchard L, eds. (2002). Resilience and the Behavior of Large-Scale Ecosystems. Washington, DC: Island Press.

Hahn ME (2002). Aryl hydrocarbon receptors: diversity and evolution. Chem. Biol. Interact, 141: 131–160.

Haider T, Sommer R, Knasmüller S, Eckl P, Pribil W, Cabaj A, Kundi M (2002). Genotoxic response of Austrian groundwater samples treated under standardized UV (254nm)—disinfection conditions in a combination of three different bioassays. Water research 36: 25-32.

Halder M, Worth A (2003). In: Mothersill, C., Austin, B. (Eds.), Validation of Alternative Methods to Animal Testing. Springer- Verlag, Berlin/Heidelberg/New York, 421–433pp.

Halestrap AP, Doran E, Gillespie JP, O'Toole A (2000) Mitochondria and cell death. Biochem. Soc. Trans 28: 170-177.

Halestrap AP, Kerr PM, Javadov S, Woodfield KY (1998). Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. Biochim. Biophys. Acta 1366: 79-94.

Halliwell B, Gutteridge JMC (1999). Free Radicals in Biology and Medicine. Oxford University Press, 1999, Oxford.

Hare L (1992). Aquatic insects and trace metals: Bioavailability, Bioaccumulation, and toxicity. Critical Reviews in Toxicology 22(5/6): 327-369.

Harries HM, Fletcher ST, Duggan CM, Baker VA (2001). The use of genomics technology to investigate gene expression changes in cultured human liver cells. Toxicol In Vitro. 15: 399–405.

Harris, DL, Lottermoser BG, Duchesne J (2003). Ephemeralacidminedrainage at the Montalbion silver mine, north Queensland, Aust. J. Earth Sci. 50: 797–809.

© University of Pretoria

Harrison PTC, Humfrey CDN, Litchfield M, Peakall D, Shuker, LK (1995). Environmental oestrogens: consequences to human health and wildlife. Leicester: IEH-Med. Res. Council.

Heath R, Coleman T, Engelbrecht J. (2010). Water quality overview and literature review of the ecology of the Olifants River. WRC Report No. TT 452/10. 51 pp.

Heath R, Eksteen MC (2005). Regulating the mining industry, addressing the legacy of abandoned mines and prevent new environmental problems.

Heath RG, Van Zyl HD, Schutte CF, Schoeman JJ (2009). First order assessment of the quantity and quality of non-point sources of pollution associated with industrial, mining and power generation. WRC Report No. 1627/1/09 ISBN 978-1-77005-819-4. November 2009.

Hedin RS, Nairn RW, Kleinmann RLP (1994). Passive Treatment Of Coal Mine Drainage. (1994). US Department of the Interior, Bureau of Mines Information Circular 9389.

Heintz RA, Short JW, Rice SD (1999). Sensitivity of fish embryos to weathered crude oil: Increased mortality of pink salmon (*Oncorhynchus gorbuscha*) embryos incubating downstream from weathered Exxon Valdez crude oil. Environ. Toxicol. Chem. 18:494–503.

Hering D, Johnson RK, Kramm S, Schmutz S, Szoszkiewicz K, Verdonschot PFM (2006). Assessment of European streams with diatoms, macrophytes, macro- invertebrates, and fish: a comparative metric-based analysis of organism response to stress. Freshwater Biology 51: 1757-1785.

Heringa MBM, Screus RHMM, Busser F, Van der Saag PT, Van der Burg B, Hermens JLM (2004). Toward more useful in vitro toxicity data with measured free concentrations. Environ. Sci. Technol 38: 6263–6270.

Hernando MD, Fernández-Alba AR, Tauler R, Barceló D (2005). Toxicity assays applied to wastewater treatment. Talanta 65:358–366.

Herricks EE, Schaeffer DJ (1985). Can we optimize biomonitoring? Environmental management 4:487-492.

Hodgson FDI, Krantz RM (1998). Groundwater quality deterioration in the Olifants River Catchment above the Loskop Dam with specialised investigations in the Witbank Dam Sub-Catchment. WRC Report No 291/1/98.

Hollert H, Dürr, M, Erdinger L, Braunbeck T (2000). Cytotoxicity of settling particulate matter and sediments of the Neckar river (Germany) during a winter flood. Environmental Toxicology and Chemistry 19: 528–534.

© University of Pretoria

Holling CS (1973**)**. Resilience and stability of ecological systems. Annual Review of Ecology and Systematics, 4: 1-23

Howells G D, Brown D J. Sadler AK (1983). "Effects of acidity, calcium, and aluminum on fish survival and productivity - a review." Journal of the Science of Food and Agriculture 34: 559-570.

Hutchinson TH, Barrett S, Buzby M, Constable D, Hartmann A, Hayes E, Huggett D, Laenge R. Lillicrap AD, Straub JO, Thompson RS (2003). A strategy to reduce the numbers of fish used in acute ecotoxicity testing of pharmaceuticals. Environmental Toxicology and Chemistry 22: 3031–3036.

Huuskonen SE, Ristola, TE, Tuvikene A, Hahn M, Kukkone JVK, Lindstrom- Seppa (1988). Comparison of two bioassays, a fish liver cell line (PLHC-1) and a midge (*Chironomous riparius*) in monitoring fresh water sediments. AquaTox 44: 47-67.

Hwang, P.P. and Yang, C.H. (1997). Modulation of cadmium pretreated tilapia *(Oreochromis mossambicus)* larvae. *Fish Physiol. Biochem.* 16: 403 – 410.

Iger Y, Balm PHM, Jenner HA, Wendelaar Bonga SE (1995). Cortisol induces stress-related changes in the skin of rainbow trout (*Oncorhynchus mykiss*). General Comparative Endocrinology 97:188–198.

Iji OT, Serem JC, Bester MJ, Venter EA, Jan G. Myburgh JG, McGaw LJ (2016)**.** Generation of reactive oxygen species in species relevant cell lines as a bio-indicator of the safety of treated acid mine water**.** Manuscript submitted to Water SA.

Jarvis AP, Younger PL (2000). Broadening the scope of mine water environmental impact assessment: a UK perspective. Environmental Impact Assessment Review 20:85-96.

Jarvis AP, Younger PL (2001). Passive treatment of ferruginous mine waters using high surface area media. Water Research 35: 3643-3648.

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

Johnson CA, Thornton I (1987). Hydrological and chemical factors controlling the concentrations of Fe, Cu, Zn, and As in a river system contaminated by acid mine drainage. Water Research 21: 359-365.

Johnson DB, Hallberg KB (2005). Acid mine drainage remediation options: a review. Science of the Total Environment 338:3–14

Johnson DB, Rolfe S, Hallberg KB, Iversen E (2001). Isolation and phyloge- netic

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characterization of acidophilic microorganisms indigenous to acidic drainage waters at an abandoned Norwegian copper mine, Environ. Microbiol. 3: 630–637.

Johnson RK, Hering D, Furse MT, Clarke RT (2006). Detection of ecological change using multiple organism groups: metrics and uncertainty. Hydrobiologia 566: 115-137.

Jones KC, Voogt PD (1999). Persistant Organic Pollutants (POPs): state of the science. Environmental Pollution 100: 209-221.

Jönsson EM, Brunström B, Ingebrigtsen K, Brandt I (2004). Cell-specific CYP1A expression and benzo[*a*]pyrene adduct formation in gills of rainbow trout (*Oncorhynchus mykiss*) following CYP1A induction in the laboratory and in the field. Environ Toxicol Chem 23: 874- 82.

Jonsson, ME, Carlsson C, Smith RW, Pärt, P (2006). Effects of copper on CYP1A activity and epithelial barrier properties in the rainbow trout gill. Aquat. Toxicol. 79: 78–86.

Jos A, Repetto G, Rios JC, Hazen MJ, Molero ML, del Peso A, Salguero M, Fernandez-Freire P, Perez-Martın JM, Camean A (2003). Ecotoxicological evaluation of carbamazepine using six different model systems with eighteen endpoints. Toxicology *in Vitro* 17: 525–532.

Jose S, Mohandas, A, Philip R, Bright Singh I.S (2010). Primary hemocyte culture of Penaeus monodon as an in vitro model for white spot syndrome virus titration, viral and immune related gene expression and cytotoxicity assays. Journal of Invertebrate Pathology 105: 312–321.

Kamer I, Rinkevich B (2002). *In vitro* application of the comet assay for aquatic genotoxicity: considering a primary culture versus a cell line. Toxicol. *In Vitro* 16: 177–184.

Karr JR, Chu EW (1999). Restoring Life in Running Waters: Better Biological Monitoring. Island Press, Washington DC, 1206 pp.

Karr JR1991. Biological integrity: A long-neglected aspect of water resource management. Ecological Applications 1: 66-84.

Katsuo D, Kim D, Yamaguchi K, Matsuyama Y, Oda T (2007). A new simple screening method for the detection of cytotoxic substances produced by harmful red tide phytoplankton. Harmful Algae 6: 790-798.

Kavlock J (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors. Environmental Health Perspectives 104: 715-740.

Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, Wilson EM (1995). Persistent DDT metabolite p,p-DDE is a potent androgen receptor antagonist. Nature 375: 581-585.

© University of Pretoria

Kelly EN, Schindler DW, Hodson PV, Short JW, Radmanovich R, Nielsen CC (2010). Oil sands development contributes elements toxic at low concentrations to the Athabasca River and its tributaries PNAS 107: 16178-16183

Kelly M (1988). Mining in the freshwater environment. Elsevier Applied Science, London, U.K.

Kelly RJ, Gruner TM, Furlong JM, Sykes AR (2006). Analysis of corrinoids in ovine tissues. Biomed. Chromatogr 20: 806–814

Kelly SA, Havrilla CM, Brady TC, Abramo KH, Levin, ED (1998). Oxidative stress: established mammalian and emerging piscine model systems. Environ Health Perspect. 106: 375-384.

Kelly SP, Wood CM (2001). Effect of cortisol on the physiology of cultured pavement cell epithelia from freshwater trout gills. Am. J. Physiol. 281:R811-R820.

Kelly SP, Wood CM (2002). Cultured gill epithelia from freshwater tilapia (Oreochromis niloticus): Effect of cortisol and homologous serum supplements from stressed and unstressed fish. J. Memb. Biol 190: 29–42.

Kennedy CJ, Walsh PJ (1994). The effects on the uptake and metabolism of benzo[a]pyrene in isolated gill cells of the gulf toadfish, *Opsanus beta*. Fish Physiol. Biochem 13: 93 – 103.

Kfir R, Prozesky OW (1981). Detection of toxic substances in water by means of a mammalian cell culture technique. Water Res 15: 553–559.

Kienzler A, Tronchère X, Devaux A, Bony S (2012). Assessment of RTG-W1, RTL-W1, and PLHC-1 fish cell lines for genotoxicity testing of environmental pollutants by means of a Fpg-modified comet assay. Toxicology *in Vitro* 26: 500–510.

Kiffney PM, Clements WH (1994). Effects of heavy metals on a macroinvertebrate assemblage from a Rocky Mountain stream in experimental microcosms. J N Am Benthol Soc 13: 511-523.

Kilemade M, Lyons-Alcantara M, Rose T, Fitzgerald R, Mothersill C (2002). Rainbow trout primary epidermal cell proliferation as an indicator of aquatic toxicity: an in vitro/in vivo exposure comparison. Aquatic Toxicology 60: 43–59.

Kilemade M, Quinn B (2003). In: Mothersill, C., Austin, B. (Eds.), *In vitro*/*In vivo* Bridging Approaches—Validating the Relevance of *In vitro* Techniques with Reference to the Whole Organism in the Natural Environment. Springer-Verlag, Berlin/Heidelberg/New York, 377–398 pp.

© University of Pretoria

Kimmel WG (1983). "The impact of acid mine drainage on the stream ecosystem." Pennsylvania Coal: Resources, Technology and Utilization". Pennsylvania Academic Science Publications 424-437 pp.

Kinani S, Bouchonne S, Creusot, N, Bourcier S, Balaguer P, Porcher JM, Aït-Aïssa S (2010). Bioanalytical characterisation of multiple endocrine and dioxin-like activities in sediments from reference and impacted small rivers. Environ. Pollut 158:74-83.

Klobucar, GIV, Pavlica M, Erben R, Papes D (2003). Application of the micronucleus and comet assays to mussel *Dreissena polymorpha* haemocytes for genotoxicity monitoring of freshwater environments. Aquatic Toxicology 64:15-23.

Kocan RM, Sabo KM, Landolt ML (1985). Cytotoxicity/ genotoxicity: the application of cell culture techniques to the measurement of marine sediment pollution, Aquat. Toxicol. 6:165–177.

Koizumi T, Shirakura H, Kumagai H, Tatsumoto H, Suzuki KT (1996). Mechanism of cadmium-induced cytotoxicity in rat hepatocytes: cadmium-induced active oxygen-related permeability changes of the plasma membrane. Toxicology 114:125–134.

Koldas S (2000). Notes of environmental inspections in South African Mines. Department of Minerals and Energy of South Africa p. 125.

Kolosov D, Kelly SP (2013). A role for tricellulin in the regulation of gill epithelium permeability. Am. J. Physiol. 304: R1139-R1148.

Komulainen, H (2004). Experimental cancer studies of chlorinated byproducts, Toxicology198: 239–248.

Koryak M, Shapiro MA, JL Sykora (1972). Riffle zoobenthos in streams receiving acid mine drainage. Water Research 6: 1239-1247.

Koutsogiannaki S, Evangelinos N, Koliakos G, Kaloyianni M (2006). Cytotoxic mechanisms of Zn2+ and Cd2+ involve Na+ /H+ exchanger (NHE) activation by ROS. Aquat Toxicol. 78: 315–324.

Kramer NI, Hermens JLM, Schirmer K (2009). The influence of modes of action and physicochemical properties of chemicals on the correlation between in vitro and acute fish toxicity data. Toxicol In Vitro 23: 1372−1379.

Kriel JP (1975). Planning and Progress in the Development of the Water Resources of South Africa with Special Reference to South Western Cape, Congress of the South African Association for the Advancement of Science, Stellenbosch.

Kurelec B (1993). The genotoxic disease syndrome. Mar. Environ. Research. 35: 341-348.

Lah B, Žinko B, Tišler T, Romana Marinšek-Logar (2005). Genotoxicity Detection in

© University of Pretoria

drinking water by Ames test, Zimmermann test and comet assay. Acta Chim. Slov. 52: 341–348.

Larsson P (1985). Contaminated sediments of lakes and oceans act as sources of chlorinated hydrocarbons for release to water and atmosphere. Nature 317: 347−349.

Lawrence J, Williams B (1991). Water quality- how best to monitor, Wand wt, May, 1991.

LeBel CP, Ischiropoulos H, Bondy SC (1992). Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol. 5: 227-231.

LeCluyse EL (2001). Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. Eur J Pharm. Sci. 13:343-368.

Lee HS, Hwang PP, Lin HC. (1996). Morphological changes of integument chloride cells to ambient cadmium during the early development of the teleost, *Oreochromis mossambicus.* Environ. Biol. Fishes 45: 95 – 102.

Lee LEE, Clemons JH, Bechtel DG, Caldwell SJ, Han KB, Pasitschniak-Arts M, Mosser DD, Bols NC (1993). Development and characteriza- tion of a rainbow trout liver cell line expressing cytochrome P450- dependent monooxygenase activity. Cell Biol Toxicol. 9: 279-294.

Leguen I, Cauty C, Odjo N, Corlu A, Prunet P (2007). Trout gill cells in primary culture on solid and permeable supports. Comp.Biochem. Physiol A. 148: 903-912.

Leguen I, Peron S, Prunet P (2011). Effects of iron on rainbow trout gill cells in primary culture. Cell Biol. Toxicol. 27: 311-319.

Leonards PEG (1997). PCB's in Mustelids, PhD thesis, Free University Amsterdam.

Lesser MP (2006). Oxidative stress in marine environments: biochemistry and physiological ecology. Ann Rev Physiol. 68: 253–278.

Levine SL, Oris JT (1999). CYP1A expression in liver and gill of rainbow trout following waterborne exposure: implications for biomarker determination. Aquat. Toxicol 46: 279–287.

Li J, Eygensteyn J, Lock RAC, Wendelaar Bonga SE, Flik G (1997). Na^{+} and Ca^{2+} homeostatic mechanisms in isolated chloride cells of the teleost *Oreochromis mossambicus* analyzed by confocal laser scanning microscopy. J. Exp. Biol, 200: 1499-1508.

Lilius H, Sandbacka M, Isomaa B (1995). The use of freshly isolated gill epithelial-cells in toxicity testing. Toxicol. *In Vitro* 9: 299-305.

Lindegaard C (1995). Classification of water-bodies and pollution. The Chironomidae:

© University of Pretoria

Biology and ecology of non-biting midges. Edited by P.O. Armitage. P.S. Cranston and L.C.V. Pinder by Chapman & Hall. ISBN 041245260 X.

Liu T, Biddle D, Hanks AN, Brouha B, Yan H, Lee RM, Leachman SA, Grossman D (2006). Activation of dual apoptotic pathways in human melanocytes and protection by surviving. J Invest Dermatol 126: 2247–2256.

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

Livingstone DR (2001). Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull. 42: 656-666.

Lloyd DR, Phillips DH, Carmichael PL (1997). Generation of putative intra-strand cross-links and strand breaks in DNA by transition metal ion-mediated oxygen radical attack. Chem Res Toxicol 10: 393–400.

Lopez DL, Overly B, Robbins EI, Carroll KC. (1999). The role of flow regime on the chemical evolution of acidic waters discharged from an abandoned underground coal mine. In: Sudbury 99 Mining and the Environment II, Conference Proceedings Sudbury, Ontario, Canada, September 12-16 1999, Vol. I, 89-98 pp.

Lu WQ, Chen XN, Yue F, Jenter C, Gminsk R, LI XY, Xie H, Mersch-Sundermann V (2002). Studies on the *in vivo* and *in vitro* mutagenicity and the lipid peroxidation of chlorinated surface (drinking) water in rats and metabolically competent human cells. Mutat Res 513: 151–157.

Lund BO, Miller DM, Woods JS (1993). Studies on Hg(II)-induced H2O2 formation and oxidative stress in vivo and in vitro in rat kidney mitochondria. Biochem Pharmacol 45: 2017–2024.

Luoma SN, Rainbow PS (2005). Why is metal bioaccumulation so variable? Biodynamics as a Unifying factor Concept. Environmental Science and Technology 39: 1921–1931.

Lushchak OV, Kubrak OI, Nykorak MZ, STorey KB and Lushchak VI (2008). The effect of potassium dichromate on free radical processes in goldfish: possible protective role of glutathione. Aquat. Toxicol 87: 108–114.

Madeira VS, Echert AB, Schneider CH, Schneider IAH (2005). Sulfate reduction in acid mine drainage through anaerobic treatment with sulphate reducing bacteria. In: Proceedings of the XXI Ores National Treatment Dating Extractive agents Metallurgy and interference processes, degradation and environmental damage November, Natal RN.

Magwood S, George S (1996). *In vitro* alternatives to whole animal testing. Comparative

© University of Pretoria

cytotoxicity studies of divalent metals in established cell lines derived from tropical and temperate water fish species in a neutral red assay. Marine Environmental Research 42: 37–40.

Mallatt J (1985). Fish gill structural changes induced by toxicants and other irritants: A statistical review. Can J Fish Aquat Sci 42: 630– 648.

Mallatt J, Bailey JF, Lampa SJ, Evans MA, Brumbaugh S (1995). A fish gill system for quantifying the ultrastructural effects of envi- ronmental stressors: Methylmercury, Kepone, and heat shock. Can J Fish Aquat Sci 52: 1165–1182.

Mance G (1987). Pollution Threat of Heavy Metals in Aquatic Environments. London: Elsevier Applied Science.

Mangena SJ, Brent AC (2006). Application of a Life Cycle Impact Assessment framework to evaluate and compare environmental performances with economic values of supplied coal products. J Cleaner Prod 14:1071-1084.

Maree JP, Greben HA, de Beer M (2004). Treatment of acid and sulphate –rich effluents in an integrated biological/chemical process. Water SA 30: 2.

Markich SJ, Camilleri C (1997). Investigation of metal toxicity to tropical biota: Recommendations for revision of the Australian water quality guidelines. Supervising Scien- tist Report 127, Supervising Scientist, Canberra.

Maron, DM, Ames BN (1983). Revised methods for the *Salmonella* mutagenicity test, Mutation Res.113: 173-215.

Martineau D, Lemberger K, Dallaire A, Labelle P, Lipscomb TP, Michel P, Mikaelian I (2002). Cancer in wildlife, a case study: Beluga from the St. Lawrence estuary, Québec, Canada. Environmental Health Perspectives 110: 285–292.

Maruoka S (1978). Estimation of toxicity using cultured mammalian cells of organic pollutants recovered from Lake Biwa. Water Res. 12: 371–375.

Mazzeo DEC, Matsumoto ST, Levy CE, Angelis DF, Marin-Morales MA (2013). Application of micronucleus test and comet assay to evaluate BTEX biodegradation. Chemosphere 90: 1030–1036.

McCulloch WL, Goodfellow WL, Black JA (1993). Characterization, identification and confirmation of total dissolved solids as effluent toxicants. Environ Toxicol and Risk Assess. 2: 213–227.

McKim J, Schmieder P, Veith G (1985). Absorption dynamics of organic chemical transport across trout gills as related to octanol–water partition coefficient. Toxicol Appl Pharm 77: 1–10.

© University of Pretoria

McKim J, Schmieder P, Veith G (1985). Absorption dynamics of organic chemical transport across trout gills as related to octanol–water partition coefficient. Toxicol Appl Pharm 77: 1–10.

McKnight DM, Feder GL (1984). The ecological effect of acid conditions and precipitation of hydrous metal oxides in a Rocky Mountain stream. Hydrobiologia 119: 129–138.

Mdegela RH, Braathen M, Mosha RD, Skaare JU, Sandvik M (2010). Assessment of pollution in sewage ponds using biomarker responses in wild African sharptooth Catfish (*Clarias gariepinus*) in Tanzania. Ecotoxicology 19: 722–734.

Means B, Tiff H (2004). Comparison of three methods to Measure acidity of coalmine Drainage. 2004 National Meeting of the American Society of Mining and Reclamation. Lexington, KY. Unknown. EPA-HQ-OW-2006-0771-0142.

Metcalfe-Smith JL (1991). Biological water quality assessment of rivers based on macroinvertebrate communities. Rivers Handbook. Monitoring Programmes, National Water Research Institute, Environment Canada. Contribution no. 91-71.

Midgley DC, Pitman WV, Middleton BJ (1994). Surface Water Resources of SA 1990. WRC Report No 298/1.4/94 Second Edition.

Milavec PJ (2000). Abandoned mine drainage abatement projects: successes, problems and lessons learned. Proceedings of the National Association of Abandoned Mine Land Programs. National Abandoned Mine Land Conference, Steamboat Springs, CO. Available from: http://www.dep.state.pa.us/dep/deputate/min-res/bamr/amd/amd_abatement_projects.htm.

Millennium Ecosystem Assessment (2005). Ecosystems and Human Well-being: Synthesis. Island Press, Washington, DC.

Miller MR, Hinton DE, Stegeman JJ (1989). Cytochrome P-450E induction and localization in gill pillar (endothelial) cells of scup and rainbow trout. Aquat. Toxicol. 14: 307 – 322.

Mine Environment Neutral Drainage (MEND) (2001). "List of Potential Information Requirements in Metal Leaching/ Acid Rock Drainage Assessment and Mitigation Work". Mining Environment Neutral Drainage Program. W. A. Price, CANMET, Canada Centre for Mineral and Energy Technology.

Mitchelmore CL, Chipman JK (1998). DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutat. Res. 399: 135-147.

Mittler R (2002). Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7: 405–10.

© University of Pretoria

Mochida K (1986). Aquatic toxicity evaluated using human and monkey cell culture assays. Bull Environ Contam Toxicol. 36: 523–526.

Moore MN (1992). Molecular cell pathology of pollutant-induced liver injury in flatfish: use of fluorescent probes. Mar Ecol Prog Ser. 91: 127-133.

Mortelmans K, Zeiger E (2000). The Ames *Salmonella*/microsome mutagenicity assay. Mut Res -Fund Mol Mech 455: 26-60.

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

Mota Marques DML, Suminsky M, Brum CP (2000) Created ecosystems (wetlands): Importance of macrophytes on pH control of acid mine drainage, coal and the environment. Ecology Center of the Federal University of Rio Grande do Sul (Org). Porto Alegre, Publisher of UFRGS.

Mothersill C,Austin B (2003). *In vitro* methods in aquatic toxicology. Chichester, UK: Praxis Publishing Ltd, 17-29 pp.

Naicker D, Myburgh JG, Botha CJ (2007). Establishment and validation of primary hepatocytes of the African sharptooth catfish (*Clarias gariepinus*) Chemosphere 68: 69–77.

National Service Center for Environmental Publications (2006). US EPA Engineering Issue Management and Treatment of Water from Hard Rock Mines.

National Water Quality Management Strategy (2000). Australian and New Zealand Guidelines for Fresh and Marine Water Quality. Volume 1, PAPER No. 4, Australian and New Zealand Environment and Conservation Council.

Nehls S, Segner H (2001). Detection of DNA damage in two cell lines from rainbow trout, RTG-2 and RTL-W1 using the comet assay. Environ Toxicol Chem 16: 321-329.

Nemes Z, Dietz R, Luth JB (1979). The pharmacological relevance of vital staining with neutral red. Experientia 35: 1475–1476.

Nishimoto M, Yanagida GK, Stein JE, Baird WM, Varanasi U (1992). The metabolism of benzo (a) pyrene by English sole (*Parophrys vetulus*): comparison between isolated hepatocytes in vitro and liver in vivo. Xenobiotica 22: 949–961.

Nobergh- King TJ, Ausley LW, Burton DT Goodfellow WI, Miller JL, Waller WT (2005). Toxicity Reduction and Toxicity Identification Evaluations for effluents, ambient waters and other aqueous Media. Pansacola, Fl, USA: Society of environmetal toxicology and

© University of Pretoria

chemistry (SETAC). 496p Volume 1 edited by Teresa Norberg-King. Toxicity reduction evaluation case study 152-163 pp.

O'Brien J, Wilson I, Orton T, Pognan F (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European Journal of Biochemistry, 267: 5421-5426.

OECD (Organisation for Economic Co-operation and Development). OECD Guideline for Testing of Chemicals: 202 Fish, Acute Toxicity Test; 1992.

Ohe T, Watanabe T, Wakabayashi K (2004). Mutagens in surface waters: a review. Mutat Res. 567: 109–149.

Olajire AA, Altenburger R, Kuster E, Brack W (2005). Chemical and ecotoxicological assessment of polycyclic aromatic hydrocarbon – contaminated sediments of the Niger Delta, Southern Nigeria. Sci. Tot. Environ 340: 123–36.

Open Source Epidemiologic Statistics for Public Health Version 3.03a updated

Organisation for Economic Co-operation and Development (OECD) (1992). OECD 203. OECD Guideline for Testing of Chemicals. Test No. 203: Acute Fish Test.

Organisation for Economic Co-operation and Development) (OECD) (1995). OECD Guideline for Testing of Chemicals: 202 Fish, Acute Toxicity Test reformatted.

Overly BM (1997). Variations in Chemical and Bacterial Species of Acid Mine Drainage Affecting the Snow Fork Basin, Ohio: The Esco #40 Underground Mine. M.S. Thesis, Ohio University.

Owens A, Burke J (2009): A Consolidated Approach to Implementing Best Practice Guidelines, Water Use Licence Conditions and Integrated Water and Waste Management Plans in the Mining Environment – Opportunities and Challenges. – In: Water Institute of Southern Africa & International Mine Water Association: Proceedings, International Mine Water Conference. – p79-88, Pretoria (Document Transformation Technologies).

Palmer R and O'Keffe J (1990). Transported material ina small river with multiple impoundments. Freshwater Biol 24: 181-191.

Pandeya S, Parvezb S, Ansaria RA, Alia M, Kaura M, Hayata F, Ahmada F, Raisuddin, S (2008). Effects of exposure to multiple trace metals on biochemical, histological and ultrastructural features of gills of a freshwater fish, *Channa punctata* Bloch. Chemico-Biological Interactions 174: 183–192.

Park SY, Choi J (2007). Cytotoxicity, genotoxicity and ecotoxicity assay using human cell and environmental species for the screening of the risk from pollutant exposure. Environ Int. 33: 817–822.

© University of Pretoria

Parsons JD (1977). Effects of acid mine wastes on aquatic ecosystems. Water Air and Soil Pollution 7: 333-354.

Part P, Norrgren L, Bergstrom E, Sjoberg P (1993). Primary cultures of epithelial cells from rainbow trout gills. Journal of Experimental Biology 175: 219–232.

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

Pegram GC, Quibeli G, Gorgens AHM (1997). The development of a guide for the assessment of non-point sources in South Africa, *in Proceedings of 8th South African National Hydrology Symposium*, 17-19th November, University of Pretoria. Taken from http://www.wrc.org.za/sanciahs.

Pereira Ede J, Panek AD, Eleutherio EC (2003). Protection against oxidation during dehydration of yeast. Cell Stress Chaperones. 8: 120–124.

Petersen Jr, RC, Maden BL, Wilbach MA, Magadza, CHD, Paarlberg A, Kullberg, A, Cummins, KW (1987). Stream management. Emerging Global similarities. Ambio. 16: 166-179.

Petit PX, Goubern M, Diolez P, Susin SA, Zamzami M, Kroemer G (1998). Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition. FEBS Lett 426: 111-116.

Planas D (1996). Acidification effects In R.J Stevenson et al (eds) Algal ecology: Freshwater benthic ecosystems. Academic. 497-530 pp.

Playle RC, Wood C M (1989). Water chemistry changes in the gill micro- environment of rainbow trout--experimental observations and theory. Journal of Comparative Physiology B Biochemical Systemic and Environmental Physiology, 159, 527-537.

Poland A, Knutson JC (1982). 2,3,7,8-Tetrachlorodibenzo-*p*- dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annu. Rev. Pharmacol. Toxicol. 22, 517–554.

Poljsak B, Gazdag Z, Jenko-Brinovec S, Fujs S, Pesti M, Bélagyi J, Plesnicar S, Raspor P (2005). Pro-oxidative vs antioxidative properties of ascorbic acid in chromium (VI)-induced damage: an in vivo and in vitro approach. J Appl Toxicol. 25: 535–548.

Pourahmad J, O'Brien PJ (2000). Acomparison of hepatocyte cytotoxic mechanisms for Cu2q and Cd2q. Toxicology 143:263–273.

Pulido MD, Parrish AR (2003). Metal-induced apoptosis: mechanisms Mutat 533: 227–241.

Pulles W, Banister S, Van Biljon M (2005). The development of appropriate procedures towards and after closure of underground gold mines from a water management

© University of Pretoria

perspective. WRC report no 1215/1/05.

Rachlin JW, Perlmutter A (1968). Fish cells in culture for study of aquatic toxicants. Water research 2:409-414.

Rahn PH, Davis AD, Webb CJ, Nichols AD (1996). Water quality impacts from mining in the Black Hills, South Dakota, USA. Environ Geol 27: 38–53.

Ramos K, Cox LR (1987). Primary cultures of rat aortic endothelial and smooth muscle cells: an in vitro model to study xenobiotic-induced vascular cytotoxicity. *in vitro* cellular and developmental biology 23: 288-296.

Rees KR (1980). Cells in culture in toxicity testing: a review. J. R. Soc. Med. 73:261–264.

Reifferscheid G, Grummt T (2000). Genotoxicity in German surface waters - Results of a collaborative study. Water, Air and Soil Pollution 123: 67-79.

Repetto G, Sanz P (1993). Neutral red uptake, cellular growth and lysosomal function: in vitro effects of 24 metals. Altern. Lab. Anim. 21:501–507.

Resh VH, Grodhaus G (1983). Aquatic insects in urban environments. *In* Urban Entomology: Interdisciplinary Perspectives G.W. Frankie and C.S. Koehler (ed). Praeger Publishers, New York. 247-276 pp.

Richardson, D, Dorris, TC, Burks, S, Browne, RH, Higgins, ML, Leach, FR (1977). Evaluation of a cell culture assay for determination of water quality of oil-refinery effluents. Bull Environ Contam Toxicol 18: 683-690.

Rogero SO, Lugão AB, Ikeda TI, Cruz AS (2003). Test *in vitro* cytotoxicity: a comparative study between two methods Mater Res 6: 317–320.

Rosemond AD, Reice SR, Elwood JW, Mulholland PJ (1992). The effects of stream acidity on benthic invertebrate communities in the southeastern United States. Freshwater Biology 27: 193-209.

Rosenberg, DM, Resh VH (1993). Freshwater Biomonitoring and Benthic Macroinvertebrates. Chapman and Hall, New York.

Rosenkranz AR, Schmaldient S, Stuhlmeier KM, Chen W, Knapp W, Zlabinger GJ (1992). A microplate assay for the detection of oxidative products using 2′,7′-dichlorofluorescin-diacetate. J Immunol Methods. 156: 39–45

Rossman W, Wytovich E, Seif JM (1997). Abandoned Mines— Pennsylvania's Single Biggest Water Pollution Problem. Pennsylvania Department of Environmental Protection, Rachel Carson State Office Building, 400 Market Street, Harrisburg, Pennsylvania 17105. Available from: http://www.dep.state.pa.us/dep/deputate/minres/

© University of Pretoria

bamr/mining_012397.htm.

Round FE (1993). The identity of Synedra closterioides Grun. and its transference to a new genus Psammosynedra. Diatom Research 8: 209-213

Rusche B, Kohlpoth M (1993). The R1-cytotoxicity test as a replacement for the fish test stipulated in the German Waste Water Act. In Braunbeck, T., Hanke, W. and Segner, H. (eds.). Fish ecotoxicology and ecophysiology. VCH, Weinheim. 81-92 pp.

Safe SH (1994). Polychlorinated biphenyls (PCBs)-environmental impact, biochemical and toxic responses, and implications for risk assessment. Critical Review Toxicology 24: 87-149.

Saito H, Iwami S, Shigeoka T (1991). *In vitro* cytotoxicity of 45 pesticides to goldfish scale (gfs) cells. Chemosphere 23: 525–537.

Sandback M, Pärt P, Isomaa B (1999). Gill epithelial cells as tools for toxicity screening-comparison between primary cultures, cells in suspension and epithelia on filters. Aquat. Toxicol. 46: 23–32.

Sandin L, Johnson RK (2004). Local, landscape and regional factors structuring benthic macroinvertebrate assemblages in Swedish streams. Landscape Ecology 19: 501-514.

Schaeffer WI (1990). Terminology associated with cell, tissue and organ culture, molecular genetics. Tissue Culture Association Terminology Committee. In Vitro Cellular and Developmental Biology 26: 91-101.

Schirmer K (2006). Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. Toxicology 224: 163–183.

Schirmer K, Chan AGJ, Greenberg BM, Dixon DG, Bols NC (1997). Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicol In Vitro. 11:107–119.

Schirmer K, Dayeh VR, Bopp S, Russold S, Bols NC (2004). Applying whole water samples to cell bioassays for detecting dioxin-like compounds at contaminated sites. Toxicology 205: 211–221.

Schirmer K, Tom DJ, Bols NC, Sherry JP (2001). Ability of fractionated petroleum refinery effluent to elicit cyto and photocytotoxic responses and to induce 7-ethoxyresorufin-O-deethylase activity in fish cell lines. Sci.Total Environ. 271: 61-78.

Schlezinger JJ, Keller J, Verbrugge LA, Stegemann JJ (2000). 3, 3', 4, 4'-tetrachlorobiphenyl oxidation in fish, bird and reptile species: relationship to cytochrome P450 1A inactivation and reactive oxygen production. Comp Biochem Physiol. 125C: 273-286.

© University of Pretoria

Schnurstein A, Braunbeck KT (2001). Tail moment versus tail length application of an *in vitro* version of the comet assay in biomonitoring for genotoxicity in native surface water using primary hepatocytes and gill cells from Zebra fish (*Danio rerio*) Ecotox Enviro Safety 49: 187-196.

Schwab R (2002). Government experiences with mine closure: meeting objectives rather than actions. WISA Mine Water Division. Mine Closure Conference. Randfontein, South Africa. 23–24 October 2002.

Schwarzenbach RP, Escher BI, Fenner K, Hofstetter TB, Johnson CA, Sorensen MB (2006). Metal poisoning in fish. CRC Press, Boca Raton, Florida.

Scrimgeour GJ, Andd W (1996). Aquatic ecosystem health and integrity: problems and potential solutions. Journal of the North American Benthological Society 15:254-261.

Scullion J, Edwards RW (1980). The effects of coal industry pollu- tants on the macroinvertebrate fauna of a small river in the South Wales coalfield. Freshwater Biology 10: 141–162

Segner H (1998a). Fish cell lines as a tool in aquatic toxicology. EXS. 86: 1- 38.

Segner H (1998b). Isolation and primary culture of teleost hepatocytes. Comp Biochem Physiol 120: 71–81.

Segner H (2004). Cytotoxicity assay with fish cells as an alternative to the acute lethality assay with fish. ATLA. Alternatives to Laboratory Animals 32, 375–382.

Segner H, Lenz D (1993). Cytotoxicity assays with the rainbow trout R1 cell line. Toxicology in Vitro 7: 537–540.

Seibert H, Balls M, Fentem JH, Bianchi V, Clothier RH, Dierickx PJ, Ekwall B, Garle MJ, Go ́mez-Lechon MJ, Gribaldo L, Gulden M, Liebsch M, Rasmussen E, Roguet R, Shivrastava R, Walum E (1996). Acute toxicity testing in vitro and the classification and labelling of chemicals. The report and recommendations of ECVAM workshop 16. Alter. Lab. Anim. 24: 499 – 510.

Sesso A, Marques MM, Monteiro MMT, Schumacher RI, Colquhoun A, Belizário J, Konno SN, Felix TB, Botelho LAA, Santos VZC, Silva GR, Higuchi ML, Kawakami J (2004). Morphology of mitochondrial permeability transition: morphometric volumetry in apoptotic cells. Anat. Rec. A Discov. Mol. Cell. Evol. Biol 281: 1337-1351.

Shailaja MS, D'Silva C (2003). Evaluation of impact of PAH on a tropical fish, *Oreochromis mossambicus* using multiple biomarkers. Chemosphere 53: 835–841.

Shimada T, Guengerich FP (2006). Inhibition of human cytochrome P450 1A1, 1A2, and 1B1-mediated activation of procarcinogens to genotoxic metabolites by polycyclic

© University of Pretoria

aromatic hydrocarbons. Chem Res Toxicol 19: 288-94.

Sies M (1999). Glutathione and its role in cellular functions. Free Radicals Biol Med. 27: 916–921.

Simpi B, Hiremath SM, Murthy KNS, Chandrashekarappa KN, Patel AN, Puttiah ET (2011). Analysis of water quality using physico-chemical parameters hosahalli tank in Shimoga. Global J Science Frontier Res. 11: 31-34.

Singh NP, McCoy MT, Tice RR, Schneider EL (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175: 184-191.

Siroka Z, Drastichova D (2004). Biochemical markers of aquatic environment contamination CYP1A in fish. A review. Acta Vet. Brno 73: 123–132.

Skelton P (1993). Freshwater Fishes of South Africa. Southern Boekuitgewers (Edms) Bpk, Halfweghuis, Cape Town, South Africa, pp 89–380.

Skousen J, Lilly R, Hilton T. (1993). Special chemicals for treating acid mine drainage. GreenLands 23: 34-41.

Smeets JMW, Voormolen A, Tillitt DE, Everaarts JM, Seinen W, Van den Berg M, (1999). CYP1A induction, benzo[a]pyrene metabolism, and nucleotide adduct formation in fish hepatoma cells: Effect of pre-exposure to 3,3' 4,4', 5-pentachlorobiphenyl. Environ. Toxicol. Chem 18: 474–480.

Smith RW, Jönsson M, Houlihan DF, Pärt P (2001). Minimising aerobic respiratory demands could from the basis to sub-lethal copper tolerance by rainbow trout gill epithelial cells in vitro. Fish Physiol. Biochem. 24: 157–169.

Smolowitz RM, Schultz ME, Stegeman JJ (1992). Cytochrome P4501A induction in tissues, including olfactory epithelium, of topminnows (*Poeciliopsis* spp.) by waterborne benzo[*a*]pyrene. Carcinogenesis 12: 2395–2402.

Smolowitz RM, Schultz ME, Stegeman JJ (1992). Cytochrome P4501A induction in tissues, including olfactory epithelium, of topminnows (*Poeciliopsis* spp.) by waterborne benzo[*a*]pyrene. Carcinogenesis 12: 2395–2402.

Sola F, Masoni A, Isaia J (1994). Effects of lead loads on branchial osmoregulatory mechanisms in the rainbow trout (*Oncorhynchus mykiss)* J Appl Toxicol 14:343–349.

Soucek DJ, Cherry DS, Currie RJ, Latimer HA, Trent GC (2000). Laboratory to field validation in an integrative assessment of an acid mine drainage-impacted watershed. Environ Toxicol Chem 19:1036-1043.

South Africa Weather Service (2007). SA Weather Bureau http://weathersa.co.za.

Spielmann H, Liebsch M (1992). BALB/C 3T3 cytotoxicity test protocol INVITTOX no. 46.

© University of Pretoria

DB-ALM, ECVAM database service on Alternative Methods to Animal Experimentation. http://ecvam-dbalm.jrc.cec.eu.int/. Springer Verlag, Berlin, Heidelberg et New York, 2nd revised edition, 486 pp.

Spry DJ, Wiener JG (1991). Metal Bioavailability and Toxicity to Fish in Low- Alkalinity Lakes: A Critical Review. Environmental Pollution 71: 243-304.

Sracek O, Choquette M. Gélinas P, Lefebvre R, Nicholson RV (2004). Geochemical characterization of acid mine drainage from a waste rock pile, Mine Doyon, Québec, Canada, J. Contam. Hydrol. 69: 45–71

Stahl RG (1991). The genetic toxicology of organic compounds in natural waters and wastewaters. Ecotox Environ Safety 22: 94-125.

Stegeman JJ, Brouwer M, Di Giulio RT, Förlin L, Fowler BA, Sanders BM, Van Veld PA. (1992). Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Huggett RJ, Kimerle RA, Mehrle PM, Bergman HL, editors. Biomarkers: Biochemical, Physiological and Histological markers of Anthropogenic stress., Boca Raton, FL: Lewis; 235-335 pp.

Stegeman JJ, Lech JJ (1991). Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. Environ Health Perspect 90: 101–109.

Stevenson RJ, White KD (1995). A comparison of natural and human determinants of phytoplankton communities in the Kentucky River Basin, USA. Hydrobiologia 297: 201-216.

Steward AR, Zaleski J, Sikka HC (1990). Metabolism of benzo(a)- pyrene and transbenzo(a)pyrene-7,8-dihydrodiol by freshly isolated hepatocytes of brown bullheads. Chem. Biol. Interact 74: 119– 138.

Stohs SJ, Bagghi D (1995). Oxidative mechanisms in the toxicity of metal ions. Free Radicals Biol. Med. 18: 321–336.

Strmac M, Braunbeck T (2000). Isolated hepatocytes of rainbow trout Oncorhynchus mykiss as a tool to discriminate between differently contaminated small river systems. Toxicol *In Vitro* 14: 361–377.

Stumm W, Morgan JJ, (1996). Aquatic Chemistry. Wiley, Inc., New York, NY.

Tanneberger K, Knöbel M, Busser FJ, Sinnige TL, Hermens JL, Schirmer K (2013). Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. Environ. Sci. Technol 47: 1110-1119.

Taylor EJ, Maund SJ, Pascoe D (1991). Evaluation of a chronic toxicity test using growth of

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the insect Chironomus riparius Meigen. In: Jeffrey DW, Madden B, editors. Bioindicators and Environmental Management. London, UK: Academic Press; 1991. 343–52 pp.

Tenbrink BJE, Woudstra JH (1991). Towards an effective and rational water management: The aquatic outlook project-intergraing water management, monitoring and research. Eur.Water Pollut. Control 1: 20-27.

Thompson JG (1980). Acid mine water in South Africa and their amelioration. Water SA 6: 130-134.

Tice RR, Agurell E, Anderson D *et al.* (2000). Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ. Mol. Mutagen. 35: 206–221.

Townsend CR, Riley RH (1999). Assessment of river health: accounting for perturbation pathways in physical and ecological space. Freshwater Biology. 141: 393–405.

Tshwete l, Gunther P, Mey W, Niekerk AV (2006). eMalahleni (Witbank) mine water reclamation project. Paper presented at the *WISA Biennial Conference and Exhibition*, Durban.

Tsuboy MS, Angeli JPF, Mantovani MS, Knasmüller S, Umbuzeiro GA, Ribeiro LR (2007). Genotoxic, mutagenic and cytotoxic effects of the commercial dye CI disperse blue 291 in the human hepatic cell line HepG2. Toxicol *in vitro* 21:1650–1655.

Ueng YF, Ueng TH (1995). Induction and purification of cytochrome P4501A from 3-methylcholanthrene treated tilapia (*Oreochromis niloticus x Oreochromis aureus)* Arch. Biochem. Biophys. 322: 347 – 356.

United States Environmental Protection Agency (1983). Neutralization of Acid Mine Drainage, Design Manual. USEPA- 600/2-83-001, Cincinnati, OH.

United States Environmental Protection Agency (1994). Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, EPA 600/7-91-002, Washington DC.

United States Environmental Protection Agency (2005). Mountaintop mining/valley fills in Appalachia. Final programmatic environmental impact statement. Region 3, US Environmental Protection Agency, Philadelphia, Pennsylvania. (Available from: http://www.epa.gov/ region3/mtntop/index.htm).

United States Environmental Protection Agency (2006). Database of Environmental Toxicity Data from Data Submitted under the Toxic Substance Control Act (TSCA), Public Law 94-469, 90 Stat. 2003, October 11, 1976. Washington DC: OPPT, USEPA, 1400 Pennsylvania Ave., N.W.

© University of Pretoria

United States Environmental Protection Agency (USEPA) (1982). Development Document for Effluent Limitations Guidelines and Standards for the Coal Mining Point Source Category. EPA 440/1-82/057. Washington, DC. (October).

United States Environmental Protection Agency (USEPA) (1986). Ambient Aquatic Life Water Quality Criteria for Lead. Washington, DC: Office of Water, Regulations and Standards, Criteria and Standards Division.

USDA (1993). "Acid Mine Drainage form Impact of Hard Rock Mining on the National Forests: A Management Challenge." USDA Forest Service, Program Aid 1505

USDA-Natural Resources Conservation Service; EPA - Environmental Protection Agency (2002). Handbook of constructed wetlands: a guide to creating wetlands for: agricultural wastewater, domestic wastewater, coalmine drainage, storm water in the Mid-Atlantic Region. v. 1. Washington, D.C. USDA/NRCS/EPA. 53 p.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007). Free radicals and antioxidants in normal physiological functions and human disease. Int. J Biochem Cell Biol. 39: 44–84.

Valko M, Morris H, Cronin MT (2005). Metals, toxicity and oxidative stress. Curr Med Chem. 12: 1161–1208.

Van der Merwe W, Grobler DC (1990). Water quality management in the RSA: Preparing for the future. Water SA 16: 49-53.

Van der Oost R, Beyer J, Vermeulen PE (2003). Fish Bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149.

Van der Schalie WH, Gardner HS, Bantle JA, De Rosa CT, Reif JS, Reuter RH, Backer LC, Burger J, Folmar LC, Stokes WS (1999). Animals as sentinels of human health hazards of environmental chemicals. Environ. Health Perspect 107:309–315.

Van Doren SR, Hall MS, Frazier LB, Leach FR (1984). A rapid cell culture assay of water quality. Bull Environ Contam Toxicol. 32: 220–226.

Van Staden CM (1979). How ERPM uses lime to solve unique underground water problem. Coal, Gold and Base Minerals of Southern Africa 27: 100-109.

Verschaeve L, Van Staden J (2008). Mutagenic and antimutagenic properties of extracts from South African traditional medicinal plants. Journal of Ethnopharmacology. 119: 575-587.

Vevers WF, Jha, AN (2008). Genotoxic and cytotoxic potential of titanium dioxide (TiO2) nanoparticles on fish cells *in vitro*. Ecotoxicology 17: 410–420.

Viarengo A, Nott JA (1993). Mechanisms of heavy metal cation homeostasis in marine invertebrates. Comp Biochem Physiol. 104: 355–372.

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Visoottiviseth P, Chanwanna N (2001). A newly established cell culture from hybrid catfish (Clarias gariepinus x Clarias macrocephalus) for screening toxicity of triphenyltin hydroxide. Appl. Organometal. Chem 15: 463–472.

Vureen L, (2009). World mine-water experts to gather in Pretoria. http://www.wrc.org.za/News/Pages/Worldmine. Accessed 15th May 2012.

Walker PA, Bury NR, Hogstrand C (2007). Influence of culture conditions on metal- induced responses in a cultured rainbow trout gill epithelium. Environ. Sci. Technol. 41: 6505–651.

Walker PA, Kille P, Hurley A, Bury NR Hogstrand C (2008). An in vitro method to assess toxicity of waterborne metals to fish. Toxicol. Appl. Pharmacol. 230: 67-77.

Walling C (1975). Fenton's reagent revisited. Accounts Chem Res. 8:139-142.

Walmsley RD, Butty M (Eds.) (1980). The Limnology of some selected South African Impoundments. A collaborative report by the Water Research Commission and the National Institute for Water Research, Pretoria, South Africa.

Wang W, Qin Y, Song D, Wang K (2008). Column leaching of coal and its combustion residues. Coal Geology 75: 81-87.

Ward JV (1998). Riverine landscapes: Biodiversity patterns, disturbance regimes and aquatic conservation. Biological Conservation 83: 269-278.

Warhurst A. Noronha L. (2000). Environmental Policy in Mining: Corporate Strategy and Planning for Closure. London: Lewis Publishers.

Water research commission (1998). Quality of domestic water supplies. Volume 1: Assessment Guide. Second Addition. 59pp.

Water Research Commission (WRC) (2009). First order assessment of the quantity and quality of non-point sources of pollution associated with industrial, mining and power generation. Report to the WRC Report No. 1627/1/09. ISBN 978-1-77005-819-4

Weeks JM, Sorokin N, Johnson IJ, Whitehouse P, Ashton D, Spurgeon D, et al (2004). Biological Test Methods for Assessing Contaminated Land: Stage 2-A Demonstration of the Use of a Framework for the Ecological Risk Assessment of Land Contamination. UK: Environmental Agency.

Wendelaar Bonga SE (1997). The stress response in fish. Physiology Reviews 77: 591–625

Weyermann J, Lochmann D, Zimmer A (2005) A practical note on the use of cytotoxicity assays. Int. J. Pharmaceut. 288: 369–376.

Wildeman T, Dietz J, Gusek J, Morea S (1993). Handbook for Constructed Wetlands Receiving Acid Mine Drainage. Risk Reduction and Engineering Laboratory, Office of

© University of Pretoria

Research and Development, US Environmental Protection Agency (USEPA), Cincinnati, Ohio.

Williams WD (1987). Salinization of rivers and streams: an important environmental hazard. Ambio 16:180–185.

Winckler J (1974). Vital staining of lysosomes and other cell organelles of the rat with neutral red. Prog Histochem Cytochem 6:1–89.

Winston DW, Di Giulio RT (1991). Prooxidant and antioxidant mech- anisms in aquatic organisms. Aquat Toxicol 19:137 – 161.

Wolf K, Quimby MC (1969). Fish cell and tissue culture. In *Fish Physiology* WS Hoar, Randall DJ (eds). Academic Press: New York.

Wolf W, De Comber M, Douben P, Gimeno S, Holt M, Léonard M, Lillicrap A, Sijm D, van Egmond R, Weisbrod A, Whale G (2007). Animal use replacement, reduction, and refinement: development of an integrated testing strategy for bioconcentration of chemicals in fish. Integr. Environ. Assess. Manag. 3:3–17.

Wood CM (2001). Toxic responses of the gill In: Schlenk, D, Benson, W.H (Eds.), Target Organ Toxicity in Marine and Freshwater teleost. Taylor & Francis, London, 1–89 pp.

Wood CM (2001). Toxic responses of the gill. In: Schlenk, D., Benson, W.H (Eds.), Target Organ Toxicity in Marine and Freshwater teleosts. Taylor & Francis, London, 1–89 pp.

Wood CM, Kelly SP, Zhou B, Fletcher M, O'Donnell M, Eletti B, Pärt P (2002). Cultured gill epithelia as models for the fresh water fish gill. Biochim. Biophys. ACTA. 1556: 72-83.

Wright JF (1995). Development and use of a system for predicting the macroinvertebrate fauna in flowing waters. Aust.J. Ecol 20: 181-197.

Xue W, Warshawsky D (2005). Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. Toxicol Appl Pharmacol 206: 73-93.

Younger PL (1997). The longevity of mine water pollution: A basis for decision-making. The Science of the total environment 194/195: 457–466.

Younger PL (2001). Passive treatment of ferruginous mine water using high surface area media. Elsevier science Ltd. Great Britain.

Younger PL, Robins NS (2002). Challenges in the characterization and prediction of the hydrogeology and geochemistry of mined ground. In: Younger, P.L., Robins, N.S. (Eds.), Mine Water Hydrogeology and Geochemistry. Geol. Soc. Spec. Pub, vol. 198, 1–16 pp.

ZahnT, Hauck C, Holzschuh J, Braunbeck T (1995). Acute and sublethal toxicity of seepage waters from garbage dumps to permanent cell lines and primary cul- tures of hepatocytes from rainbow trout (*Oncorhynchus mykiss*): a novel approach to environmental risk

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assessment for chemicals and chemical mixtures. Zbl. Hyg. 196: 455 – 479.

Zakaria, MP, Takada H, Tsutsumi S, Ohno K, Yamada, J, Kouno E (2002). Distribution of Polycyclic Aromatic Hydrocarbons (PAHs) in rivers and Estuaries in Malaysia: A Widespread Input of Petrogenic PAHs. Environmental Science and Technology 36: 1907-1918.

Zalasiewicz J, Williams M, Steffen W, Crutzen P (2010). The new world of the Anthropocene. Environ. Sci. Technol. 44: 2228-2231.

Žegura B, Sedmak B, Filipic M (2003). Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2. Toxicon 41:41–48.

Zhou B, Liu W, Wu RSS (2005). Cultured gill epithelial cells from tilapia (*Oreochromis niloticus*): a new in vitro assay for toxicants. Aquatic Toxicology 71: 61–72.

Zhuang J, Dinsdale D, Cohen GM (1998). Apoptosis, in human monocytic THP.1 cells, results in the release of cytochrome c from mitochondria prior to their ultra condensation, formation of outer membrane discontinuities and reduction in inner membrane potential. Cell Death Differ 5: 953-962.

Zurbuch P (1996). Early results from calcium carbonate neutralization of two West Virginia Rivers acidified by mine drainage. In: Proceedings, 17th West Virginia Surface Mine Drainage Task Force Symposium, West Virginia University, Morgantown, WV. http://www.wvmdtaskforce.com/ accessed 20 September 2015.

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Appendix 1: Protocol and Animal Ethics approval

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