# Selected effects-directed assays in water quality monitoring


## A Kruger

orcid.org 0000-0002-3158-0816


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Supervisor: Prof R Pieters

Co-Supervisor: Dr SR Horn

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would also blow over. With that you would remind me that the only constant is the love and presence of God.

## ABSTRACT

Water in South Africa is limited and due to pollution, water of good quality is limited even more. Water is the core of existence, and for this reason it is of utmost importance to monitor and manage the freshwater quality. Chemical analyses alone are not sufficient enough to assess water quality because it will not provide the biological effect which the mixture of compounds would have on organisms exposed to water. It is also not possible to quantify every harmful compound that might be present. The present study used selected bioassays*, in vitro* and *in vivo*, to consider the value of these tools to assess water quality, including selected drinking water and environmental water samples. A small town in the Mpumalanga Province, South Africa, Wakkerstroom was the study area. It offered all types of water at one location because it was important to investigate the water quality of both drinking and environmental water. Thus water and sediment (where applicable) were collected: Potable water from a borehole and a tap in the residential area as well as bottled water sold under the Wakkerstroom brand represented the water fit for human consumption. The aquatic environment was represented by samples from rivers, dams, and various areas in the wetland which is located mostly west of the town.

Samples were collected at the end of the dry season and at the end of the subsequent wet season. Water and sediment were chemically extracted with suitable methods to enrich samples for the in *vitro assays*. The *in vivo* assays were executed on the raw water and sediment and dilutions of thereof.

The biological analyses measured endpoints in the MDA-kb2, H4IIE-*luc*, and HuTu 80 cell lines. These endpoints were: Receptor mediated activity via the aryl-hydrocarbon (AhR), androgen (AR), and glucocorticoid receptors (GR), oxidative stress biomarkers including reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT), lipid peroxidation (LPO) and, non-neuronal acetylcholinesterase (AChE) activity. Viability assays were also run on the same cell lines. Commercially available *in vivo* assays were added when an opportunity presented itself and fairy shrimps (*Thamnocephalus platyurus*) and ostracods (*Heterocypris incongruens*) were used to evaluate water and sediment respectively for one sampling event. The biological endpoints measured were mortality and/or growth in inhibition.

Biological effects mediated via the reporter gene assays were limited: xenobiotic metabolism via the AhR was seen only on two occasions. There was one instance of quantifiable androgen agonism: Wastewater treatment effluent and four events where androgen antagonism were determined. There were many statistically significant responses determined for the various oxidative stress endpoints and most biological endpoints were either increased or decreased in ROS or increased CAT. Both the cell lines used in the oxidative stress assays were equally responsive for the number of responses but not for the same samples. The AChE responses were limited and only the H4IIE-*luc* cells gave quantifiable results. The fairy shrimp *in vivo* assay indicated surprisingly high sensitivity towards the tap water which is likely due to the disinfectant by-products.

The pharmaceuticals, personal care products and pesticides were chemically extracted from the water samples. The extractions were screened for the target compounds using an ultra-high-performance liquid chromatography connected to an UPLC-QTOF-MS. The chemical analyses were used to compliment the biological analyses to provide information on the chemical culprits that possibly added to the measured responses.

Using biological and chemical analyses to compliment each other showed to be of importance as for some of the samples the chemical screening showed little to no results whereas the exact same samples showed responses to the biological analyses.

Biological analyses deemed to be more informative on the effect the water quality would have on the environment and the organisms either living in that water using the water source for household purposes.

**Keywords:** Endocrine disruption; mammalian tissue culture; oxidative stress; sediment, potable, and environmental water.

# LIST OF ABBREVIATIONS

**A**

| | |
|---|---|
| AhR | Aryl-hydrocarbon receptor |
| ARNT | Aryl-hydrocarbon receptor nuclear translator |
| AIP1 | Aryl-hydrocarbon Interacting protein 1 |
| ARA9 | Aryl-hydrocarbon associated protein 9 |
| AHRR | Aryl-hydrocarbon receptor repressor |
| AR | Androgen receptor |
| ARE | Androgen response element |
| ACh | Acetylcholine |
| AChE | Acetylcholinesterase |
| ASE | Accelerated solvent extraction |
| ATCC | American Type Culture Collection |

**B**

| | |
|---|---|
| BEQ | Bioassay equivalents |
| BSA | Bovine serum albumin |

**C**

| | |
|---|---|
| CAT | Catalase |
| $CaSO_4.2H_2O$ | Calcium sulphate dihydrate |
| Cdt | Charcoal dextran treated |
| CYP P450 | Cytochrome P450 |

| | |
|---|---|
| CuZnSOD | Copper and zinc containing superoxide dismutase |
| CV | Coefficient of variance |

**D**

| | |
|---|---|
| DCM | Dichloromethane |
| DWS | Department of Water and Sanitation |
| DWAF | Department of Water Affairs and Forestry |
| DRE | Dioxin response element |
| DHT | Dihydrotestosterone |
| DOC | Dissolved organic carbon |
| Di-PCBs | Di-polychlorinated biphenyls |
| DMEM | Dulbecco’s Modified Eagle’s Medium |
| DPBS | Dulbecco’s phosphate buffer saline |
| DF | Dosing factor |
| DTPA | Diethylenetriaminepenta-acetic acid |

**E**

| | |
|---|---|
| EC | Effect concentration |
| $ECSR_{0.2}$ | Effect concentration suppression ratio at 20% |
| EDCs | Endocrine disrupting compounds |
| EF | Enrichment factor |

**F**

| | |
|---|---|
| FBS | Foetal bovine serum |

| | |
|---|---|
| $Fe^{iv}O$ | covalent oxyferryl species |

**G**

| | |
|---|---|
| GPC | Gel permeation chromatography |
| GR | Glucocorticoid receptor |
| GSH | Glutathione |

**H**

| | |
|---|---|
| $H_2O_2$ | Hydrogen peroxide |
| $H_2SO_4$ | Sulfuric acid |
| $H_2$DCFDA | 2′,7′-Dichlorodihydrofluorescein diacetate |
| HLB | Hydrophilic lipophilic balance |
| HPLC | High-performance liquid chromatography |
| HSP | Heat shock protein |

**I**

| | |
|---|---|
| ISO | International Organization for Standardization |

**K**

| | |
|---|---|
| $K_2MnO_4$ | Potassium permanganate |

**L**

| | |
|---|---|
| L-15 | Leibovitz’s 15 |
| LAR | Luciferase assay reagent |
| LC/MS | Liquid chromatography–mass spectrometry |

**M**

| | |
|---|---|
| MeOH | Methanol |
| MDA | Malondialdehyde |
| $MgSO_4.7H_2O$ | Magnesium sulphate heptahydrate |
| MMTV | Mouse mammary tumour virus |
| mRNA | Messenger ribonucleic acid |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |

**N**

| | |
|---|---|
| $N_2$ | Nitrogen gas |
| NaCl | Sodium chloride |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| $NaHCO_3$ | Sodium bicarbonate |
| NaOH | Sodium hydroxide |
| $NH_4OH$ | Ammonium hydroxide |

**O**

| | |
|---|---|
| OS | Oxidative stress |

**P**

| | |
|---|---|
| PAH | Polycyclic aromatic hydrocarbon |
| PCDD/Fs | Polychlorinated dibenzo-*p*-dioxins and dibenzofurans |
| PPCPs | Pharmaceuticals and personal care products |

**R**

| | |
|---|---|
| REF | Relative extraction factor |

| | |
|---|---|
| REP | Relative effect potency |
| RFUs | Relative fluorescent unit |
| RHP | River Health Programme |
| RLU | Relative light unit |
| ROS | Reactive oxygen species |
| RQIS | Resource quality information sub-directorate |

**S**

| | |
|---|---|
| SANS | South African National Standards |
| SC | Solvent control |
| SPE | Solid phase extraction |
| SPSS | Statistical Package for the Social Sciences |
| SOD | Superoxide dismutase |
| $SR_{0.2}$ | Suppression ratio at 20% |

**T**

| | |
|---|---|
| TCDD | 2,3,7,8-tetrachlorodibenzo-*p*-dioxin |
| TMP | 1,1,3,3-tetramethoxypropane |

**U**

| | |
|---|---|
| UHPLC | Ultra-High-Performance Liquid Chromatography |
| UNEP | United Nations Environment Programme |
| UNDESA | United Nations Department of Economic and Social Affairs |
| UNDP | United Nations Development Programme |

| | |
|---|---|
| UNISA | University of South Africa |
| US EPA | United States Environmental Protection Agency |

**W**

| | |
|---|---|
| WMA | Water management areas |
| WWTP | Wastewater treatment plant |

**X**

| | |
|---|---|
| XAP2 | X-associated protein |

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# CHAPTER 1 INTRODUCTION

No water can be created or destroyed on earth. So, every drop of water that's here has seen the inside of a cloud, and the inside of a volcano, the inside of a maple leaf, and the inside of a dinosaur kidney, probably many times.

– Charles Fishman

## 1.1 General background

Water covers more than 70% of our planet's surface yet it is one of the biggest struggles to get access to clean drinking water (Fishman, 2011). The history of water degradation shows a continuous decrease in the quality of water (Tundisi et al., 2015). Gradually humans have stopped thinking about water, we use more water now than ever but still we do not consider the consequences that our daily activities have on water quality (Fishman, 2011). According to the chief of UNICEF 85 700 children, under the age of 15, die every year due to either water-borne diseases caused by contaminated water or from a lack of water (Shehzad, 2021).

Monitoring water quality is important to enable decisions about water quality management. Emerging contaminants have been known to be present in the environment but their effect on the environment was not fully apprehended (Sauvé & Desrosiers, 2014). These contaminants are ubiquitous in aquatic ecosystems and removal of these contaminants are needed because humans and wildlife often use untreated water from these sources for daily tasks (Valbonesi et al., 2021). Unfortunately, conventional drinking water treatment plants were not designed to remove these types of contaminants. Caffeine and ibuprofen are among the contaminants which the treatment plants can effectively remove but pharmaceuticals such as diclofenac and carbamazepine are not effectively removed (Valbonesi et al., 2021). The presence of disinfection by-products and naturally occurring toxicants pose serious human and animal health threats (Ruiz-lara et al., 2022). Many emerging contaminants act as endocrine disruptors, interfering with the natural hormonal systems of vertebrates, including humans, once they are ingested (Valbonesi et al., 2021).

All environmental, industrial, and metabolic processes are depending on water (Inyinbor Adejumoke et al., 2018). Water plays several roles in living organisms such as acting as a solvent and temperature buffer (Inyinbor Adejumoke et al., 2018). This creates many opportunities for polluted water to cause adverse effects including oxidative damage, metabolic disruption, abnormalities in reproduction and development, endocrine and cardiovascular systems, and may be the cause of several cancer types (Amir et al., 2021; Ruiz-lara et al., 2022; Varticovski et al., 2021).

## 1.2 Problem statement

Monitoring of water quantity and quality is needed to identify the temporal and spatial trends in the ground and surface waters. In South Africa monitoring of water quality is limited due to corruption, lack of expertise, ineffective management of sewage and insufficient finances, which leads to gaps in the monitoring data (Department of Water and Sanitation, DWS, 2019). These gaps allow for incomplete and wrong assessments as well as allowing wrong decision-making (regarding water treatment) to take place. The last proper report available on the Resource Quality Information Systems' website is from 2002, "Report: National Water Resource Quality Status - Inorganic Chemical Water Quality of Surface Water Resources - The Big Picture" (Resource quality information sub-directorate, RQIS, 2018). This report provides information on the chemical water quality of domestic water and irrigated agricultural water by reporting the major inorganic chemical compounds that were present in the surface waters of South Africa. This is now other than the Blue Drop and Green Drop reports from 2022 which are available on the Department of Water and Sanitation's website (DWS, 2022a). The Blue Drop provides information on drinking water quality while the Green Drop reports on the wastewater quality. These reports have not been available for seven years. The South African National Standards 241 (SANS 241) (2015) guidelines are used as regulatory measure and thus the Blue Drop report does not include the status of organic chemicals present in the drinking water (DWS, 2022b). This is because the SANS241 document does not contain guidelines for organic chemicals (SANS 241, 2015) other than phenol and trihalomethanes.

Chemical analyses have limitations because it only targets certain compounds (as determined by the water quality guideline) and overlooks the biological effect and possible toxicity caused by the compounds occurring in mixtures in the water. Water quality testing should move away from relying on chemical analysis only and incorporate biological assays as well (United States Environmental Protection Agency, US EPA, 2018). The chemicals in the environment occur in mixtures and can act antagonistically, additively or synergistically (US EPA, 2018). It is important to learn the cumulative effects on the exposed organisms rather than the identity of each compound within the sample (Brack et al., 2019; US EPA, 2018). *In vitro* bioassays can be used as an alternative to animals

to act as a first tier screen of possible harm to animals prior to comfirming effects with whole body models of selected samples. Tissue culture based *in vitro* assays test for changes in a particular cell type which can predict outcomes such as endocrine disruption, cytotoxicity and oxidative stress (US EPA, 2018) which is the focus of this study. In this study, cell lines were exposed to targeted compound classes in aquatic extracts and *in vivo* assays were conducted of drinking-, surface- and bottled water as well as sediment samples.

It is hypothesised that biological assays provide sufficient information to determine water quality.

### 1.3 Aims and objectives

1. **Evaluate water and sediment samples with biological analysis to determine if these are effective to indicate water quality**

- Select water and sediment to represent a variety of aquatic environments, from potable and bottled water to environmental water and treated waste water.
- Select and test a number of biological analysis to determine their validity to determine water quality.

2. **Investigation of activtion of cellular defence mechanisms by extracting sediment for aryl-hydrocarbon receptor (AhR) ligands.**

- Select appropriate extraction techniques to collect non-polar (dioxin-like) compounds from sediment.
- Determine whether environmental samples elicit xenobiotic metabolism with the H4IIE-*luc* reporter gene assays.

3. **Investigate whether the water and sediment samples contain androgen receptor (AR) or glucocorticoid receptor (GR) ligands able to activate/inhibit the AR, or activate the GR.**

- Extract polar and non-polar compounds from the water through appropriate extraction techniques.

- Determine whether environmental samples elicit (anti-)androgenic and glucocorticoid effects using the MDA-kB2 cells.

4. **Determine whether the collected samples cause oxidative stress and increase or decrease acetylcholinesterase activity.**

- Extracts from aim 3 were given to HuTu 80 and H4IIE-*luc* cells to test for any effect on the reactive oxygen species production, SOD and CAT activity, and MDA.
- The ability of the extracts to increase or decrease the acetylcholinesterase activity was tested.

5. **Investigate the aquatic quality with a limited battery of *in vivo* tests.**

- Directly after sampling, expose *Thamnocephalus platyurus* and *Heterocypris incongruens* to the sampled water and sediment respectively to test for the endpoints: mortality and growth inhibition.

6. **Identify chemicals in the water samples.**

- Extract water samples for pesticides, pharmaceuticals and personal care products.
- Use ultra-high-performance liquid chromatography connected to an UPLC-QTOF-MS to analyse for the presence of the compounds within the mixture.
- Tentatively identify compounds with Compass DataAnalysis 4.3 software, KEGG, and ChEBI.

### 1.4 Structure of dissertation

**Chapter 1: Introduction**

This chapter consists of information sketching a general background on the study including the importance of this study in the form of a problem statement. The study was conducted with focus on several aims, which are also given in this chapter.

**Chapter 2: Literature review**

A literature review was done on the aspects that were involved with this project such as the importance of good water quality, legislation in South Africa regarding the quality of drinking water and the monitoring of the drinking as well as environmental water. The importance of investigating water quality with the use of biological endpoints that can detect xenobiotic metabolism, endocrine disruption, oxidative stress, AChE activity, mortality, and growth inhibition in aquatic invertebrates.

**Chapter 3: Materials and methods**

The description of the study area with information on the ten sampling sites can be found in this chapter. It is important to give thorough descriptions of the methods from the sampling to exposing the cells and analysing the data to explain how the results were obtained. The methods for the chemical analysis with the specification of the instrumentation were also included.

**Chapter 4: Results**

The results from all the assays can be found in this chapter, including the chemical analysis results. The similarities and differences between sites and the different matrixes per site were highlighted. Where the assays failed it was mentioned with the promise of possible explanations in the discussion chapter.

**Chapter 5: Discussion**

This chapter is where there was made sense from all the data obtained. Possible reasons from previous studies were provided to explain the responses seen in this study as well as the absence of responses.

**Chapter 6: Conclusion**

A concluding statement on the outcomes of the study was made including future recommendations that should be considered.

References were done according to the Harvard referencing styling, which was specified by North-West University.

## 1.5 Publication

An article on the importance of effect-based methods to investigate water quality and the monitoring thereof was published in Environmental Science and Pollution Research. The water legislation in South Africa with the situation surrounding the water quality is also discussed.

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# CHAPTER 2 LITERATURE REVIEW

Science is simply the word we use to describe a method of organising our curiosity.

– Tim Minchin

## 2.1 Water quality in South Africa

South Africa is classified as a semi-arid country, due to low rainfall that results in frequent drought seasons, and the country has limited water sources (Edokpayi et al., 2018). With an average annual rainfall of 464 mm, South Africa receives about half of the annual amount of rain compared to the rest of the world (857 mm) (United Nations Development Programme, UNDP, 2022). Two factors are driving water scarcity namely economic water scarcity and physical water scarcity (Mnisi, 2020). Economic water scarcity is defined as the lack of investment in water infrastructure and technology which as a result, exacerbates the physical scarcity (Mnisi, 2020). Low rainfall results in freshwater being a physically scarce resource and with the population growing at a rapid rate, there is an increase in water use, which puts this resource under enormous pressure (Du Plessis, 2019; Mnisi, 2020).

In 2005 it was reported that almost all of South Africa's freshwater has been allocated and together with that, water quality was declining. Oberholster and Ashton (2008) reported that by 2030 South African freshwater resources will be depleted and will not meet the needs of the industry or the people. Amongst the southern hemisphere countries, South Africa is unique in the sense that most of the principal metropolitan areas are located on watersheds of river catchments (Oberholster & Ashton, 2008). As the rivers drain away, they provide water supply and transport the waste material to the downstream reservoirs. These downstream reservoirs are becoming progressively enriched. Sewage in South Africa is not treated properly due to incomplete sewer systems or sewage treatment plants that are overloaded (Oberholster & Ashton, 2008).

Contamination of available water sources in South Africa is the biggest threat to sustainable water provision/supply (Edokpayi et al., 2018). The quality of freshwater is decreasing in South Africa due to anthropogenic activities such as mining, deforestation, urbanisation, agriculture, destruction of wetlands and river catchments, and infiltration-percolation from already polluted areas (Pearson et al., 2019; Verlicchi & Grillini, 2020). The already scarce water's quality is degrading rapidly which limits the availability of safe drinking water. Most of the people living in rural communities in South Africa do not have

access to running water, or access to water of decent quality (Pearson et al., 2019). These communities use their nearest water source, often a river or dam, for urination, and defecation and will use water from the same sources for household purposes (cooking, drinking, and washing of clothes) (Pearson et al., 2019). Communities relying on these polluted water sources to support their needs are at risk of various waterborne infections and diseases (Pearson et al., 2019). Proper water quality also ensures high biodiversity, within the aquatic ecosystem as well as the entire landscape, and is necessary to maintain a stable functioning of the aquatic ecosystem (Klimaszyk & Goldyn, 2020).

### 2.2 Water legislation in South Africa

In 1996 the Department of Water Affairs and Forestry (DWAF) (now known as the DWS) set guidelines in place for the quality of water resources in South Africa (DWAF, 1996). DWAF (1996) defined water quality as the aesthetical, chemical, physical and biological properties of the water that determine the water's fitness for several uses and the protection of human and aquatic ecosystems' health and integrity.
It is only two years later that the South African National Water Act (Act No 36 of 1998) was established (Republic of South Africa, 1998). This act states that the water quality should be sustainable for all water users. This act was set in place to protect, conserve, control, manage and use South Africa's water resources. Pollution and degradation of water resources should be reduced and prevented (Republic of South Africa, 1998).

The South African water quality guidelines (1996) document states that the DWAF will modify and update the water quality guidelines regularly, but this has not happened. In 2015 the water quality guidelines for drinking water were updated by South African National Standard (SANS) 241. South African citizens are entitled to drinking water with a quality prescribed by the South African National Standards (SANS) 241 (2015) document. This document specifies the requirements for safe drinking water according to aesthetic, chemical, microbiological, and physical determinants (SANS 241, 2015). The SANS 241 document is now in a draft format after being updated in 2022.

## 2.3 Programs in place for water quality monitoring

### 2.3.1 Sustainable development goal 6

The United Nations Member States, of which South Africa is one, adopted the 2030 Agenda for Sustainable Development in 2015, and 17 sustainable development goals form the core of this agenda (United Nations Department of Economic and Social Affairs, UNDESA, 2022). Sustainable development goal number six works toward ensuring that water and sanitation are available to everyone and that both water and sanitation are sustainably managed by 2030 (Nations Environment Programme, UNEP, 2022). This goal also focuses on the sustainability and quality of environmental water resources because they are important for the survival of overall life on the planet.

Since the United Nation's Sustainable Development Summit in 2015 seven years have passed, halfway to the deadline in 2030, but South Africa has not made much progress (Evans, 2022). South Africa is ranking 108$^{th}$ of 163 countries, with a score of 63.7 out of 100. Sanitation and water related diseases are still the main cause of children under the age of five dying: 297 000 children under five die each year from diarrhoea (Evans, 2022).

### 2.3.2 Blue and Green Drop programmes

The Blue and Green Drop certification programmes were established in 2009. The Department of Water and Sanitation set criteria in place which would be used to assess and measure the municipality's ability to supply drinking water to the community, according to standards in SANS 241, (Water Research Commission, WRC, 2015). Once a year, during an audit, the purification facilities and the raw water pump stations are physically inspected. The Blue Drop certification quickly became the benchmark for excellence in drinking water management and good practices.

The Green Drop certification programme focuses on municipal wastewater quality management (Burges, 2016). This programme compares the results of the performance of the different municipalities and their providers against a standardised scorecard. Critical risk areas are identified within the municipality's wastewater treatment process that requires attention to improve the quality of the wastewater (Burges, 2016).

Since the establishment of these certification programmes in 2009, only five Green Drop and six Blue Drop reports respectively were published. The last report was made available to the public in 2014. The 2016/17 audit was supposably done but was not made available to the public (Bega, 2021). For the next few years, consumers suspected the water quality and services are deteriorating but data were not available to back their suspicions. During the State of the Nation address in 2021 the South African president, Cyril Ramaphosa, stated that the certification programmes will be revived (Bega, 2021; Ratua, 2021). The aim was to do a full Green Drop and a partial Blue Drop audit during 2021 and vice versa during 2022 (Bega, 2021). The South African president's promise was fulfilled in 2022 when Blue Drop and Green Drop reports were released once again. The conclusion was made that the water quality decreased significantly with isolated and rural municipalities' drinking water quality being a concern. The department flagged 11% of the municipalities as high-risk and 23% of municipalities as critical (DWS, 2022a). They found that most rural municipalities struggled to get a score of 50% (DWS, 2022b).

### 2.3.3 Programmes under Resource Quality Information Services' management

The RQIS is part of the Department of Water and Sanitation, and it is their responsibility to oversee several monitoring programmes: i) chemical, ii) microbial, iii) eutrophication, iv) toxicity, v) aquatic ecosystem health as well as vi) radioactivity (which is not discussed in this study) (DWS, 2019a). The RQIS is responsible for the scheduling, consolidation of samples, developing, administering, and visualising the data (DWS, 2019d).

#### 2.3.3.1 National Chemical Monitoring Programme

The South African Department of Water and Sanitation operates this programme since it was implemented in 1970 (Hohls et al., 2003). According to Hohls et al. (2003) samples are to be collected at frequent time intervals across the country at monitoring stations and analysed at the department's laboratories but the last report which I could find on the Department of Water and Sanitation's website was published in 2002 (DWS, 2019b). This programme, when it was still functioning, aimed at providing data and information on the inorganic chemical quality of all water resources in South Africa. Samples were mostly collected at the gauging weirs because monitoring of the surface water quality network

was imposed on the already existing surface water gauging programme (Department of Water Affairs, DWA, 2011).

#### 2.3.3.2 National Microbial Monitoring Programme

The DWS are responsible to administer this programme and to update the microbial water quality national information system – namely the Central Water Quality Database (Luyt et al., 2012). South Africa is divided into 19 water management areas (WMA). In each of these WMAs a regional coordinator was appointed to oversee the running of the programme. These regional coordinators are employees of DWS, and part of their responsibilities are managing the collection and analyses of samples at specific sampling sites every 7–14 days. Every two months the regional coordinators should write a report summarising the trends in the monitoring results (Luyt et al., 2012).

One of the objectives of this programme was to provide the status, trends, and extent of faecal pollution and to provide information that will help with assessing potential human health risk which is associated with faecal polluted water that is used for household purposes (DWA, 2011). The last report with bi-monthly microbial results was published on the department's website dates back to 2012 (DWS, 2019c).

#### 2.3.3.3 National Eutrophication Monitoring Programme

Eutrophication has several negative impacts including aesthetic and recreational impacts, deterioration of water quality, human health impacts, and loss of biodiversity, making eutrophication a concern of water quality. This programme provides information on the water quality status, trends in the concentration of nutrients detected, and also support decision management efforts (DWA, 2011).

The then DWAF was primarily responsible for establishing a national monitoring system as specifically required by the National Water Act, No. 36 of 1998 (DWS, 2002). For a successful monitoring system local monitoring programmes were implemented to monitor single impounds, canals and river reaches (DWS, 2002; DWS, 2019d). The National Eutrophication Monitoring Programme was implemented in 2002 (DWS, 2019d). Throughout South Africa sites are monitored and data on trophic status with related

problems such as cyanobacterial blooms and the excessive growth of water plants are reported. Regional offices, local stakeholders and water control officers assist the RQIS with the samples. The latest report is from 2008 (DWS, 2019d).

#### 2.3.3.4 National Toxicity Monitoring Programme

In the National Water Act (Act 36 of 1998), chapter 14 requires the minister of the then DWAF to create a national monitoring system which can assess water quality as well as the health of ecosystems (Murray et al., 2006). There was a three-year design phase which was wrapped up in three reports: i) a prototype implementation manual, ii) record of decision report and iii) a capacity building plan (Murray et al., 2006). In 2006, at the time of Murray and co-authors' article the programme was in a pilot testing phase over a two-year period during which certain aspects of the design were tested (Murray et al., 2006).

The objective of this programme is to assess, measure and regularly report on the status and trends of the nature and extent of substances that are potentially toxic in all South African water resources. The report should also include selected organisms experiencing potential toxic effects in a manner which will provide support to all strategic management decisions (DWA, 2011). On the Department of Water and Sanitation's website is the last report for this programme published in 2008 (DWS, 2018).

#### 2.3.3.5 National Aquatic Ecosystem Health Monitoring Programme

This programme is managed by the Resource Quality Services and support is provided by the Council for Scientific and Industrial Research, Water Research Commission, and several provincial and regional authorities (DWA, 2011). The River Health Programme (RHP) is one of the most well-known components of this programme. The RHP assess the health and condition of the river systems making primarily use of biological indicators. Biota that live in the river ecosystem provide an integrated and holistic measure of the health or integrity of the river. Indices used by the RHP includes the Fish Response Assessment Index, Index of Habitat Integrity, Geomorphological Driver Assessment Index, Riparian Vegetation Index, and aquatic invertebrates using the South African

Scoring System (DWA, 2011). The last report for this programme was published in 2009 (DWS, 2015).

South Africa has globally been hailed for the good water legislation that is set in place (Takacs, 2016) but in spite of all the monitoring programmes set in place (mentioned above) the implementation thereof has been slipping. Apart from that, the limited water of acceptable quality in South Africa is threatened by corruption, ineffective management of finances and sewage as well as the lack of expertise (DWS, 2019a). The environment and citizens of South Africa need proper water quality testing because their health are depending on available water resources. Globally the quality of the water will never measure up to what it was 50 years ago, but biological analysis can provide early warning signs of a decrease in water quality. This will enable early action taking to prevent further damage to the aquatic ecosystems.

### 2.4 Shift from traditional chemical water quality analysis to biological analysis

The quality of water for regulatory monitoring purposes is traditionally determined using chemical analysis. Water is sampled and sent to laboratories for chemical analysis. The concentrations of preselected chemical compounds within the sample are obtained through these tests (Müller et al., 2018). These analyses are time-consuming, expensive and require certain expertise (Pasika & Gandla, 2020). These analyses also fail to determine the biological effect of complex mixtures of chemicals on water quality and the effect on environmental, animal, and consumers' health (Brack et al., 2019). The chance to overlook the possible harm that chemical mixtures can cause is high. Targeted chemical analysis also does not take into account the presence of unknown chemicals or any transformation products (Escher et al., 2018). To monitor environmental water, it is important to consider multiple chemical contaminants from anthropogenic diffuse emissions and different point sources (Altenburger et al., 2019). On the other hand, biological analysis connects ecological status with chemical contamination, in other words, the results of both biological and chemical analysis will explain which chemicals are the cause of the health status of the ecosystem. Biological analyses take into account additional risks caused by mixtures and unidentified chemicals. It also covers a wide range of toxicity mechanisms (Di Paolo et al., 2016) and provides results of the joint

biological effect of all the active chemicals present in the sample (Escher et al., 2018). Bioassays can be used for the detection of these chemical products (Bonato et al., 2020; Wee et al., 2022). For effective water quality monitoring, Brack and co-authors (2019) recommended to combine chemical analysis with biological analysis.

Pollution in water is neither uniform nor is it constant and ecotoxicological assessments, with the use of sub-lethal bioassays, are viewed as early indicators of adverse effects on the specific test organism (López-López et al., 2006). Biological analysis can firstly be categorised as *in vivo* and *in vitro* assays and secondly whether it tests for acute or chronic toxicity. *In vivo* assays are where a whole organism is exposed to the extract or chemical (Poulsen et al., 2011). *In vitro* assays are considered to be cell-based assays but it also includes enzyme extracts and isolated tissues (eg. liver homogenate which is metabolically active) (Poulsen et al., 2011). Yeast and bacteria are also considered as cell-based assays because it can be grown without sacrificing any test organisms (Poulsen et al., 2011). *In vitro* assays are used to investigate specific mechanism of action including endocrine disruption, oxidative stress, immunotoxicity and mutagenicity (Brack et al., 2019).

### 2.5 Biological effects

Xenobiotic metabolism, endocrine disruption, evidence of oxidative stress and acetylcholinesterase activity are the different biological effects that were investigated in this study. This study will also assess the quality of the water and sediment samples through *in vivo* exposures where mortality and growth inhibition will be used as endpoints.

#### 2.5.1 Effects of xenobiotics on cell metabolism

Chemicals which are not native to the normal biochemistry of an organism are known as xenobiotics. When these chemicals are absorbed by cells they may be metabolised through biotransformation (Escher et al., 2021). The biotransformation process consists of different phases. Phase I is where enzymes, such as cytochrome P450 (CYP P450), change the chemicals through adding functional groups, such as hydroxides, to the molecules (Escher et al., 2021). The CYP P450 enzymes are abundant in the gastrointestinal tract, liver, kidney, and lungs (Xu et al., 2005). During phase II the

functional groups, from phase I, can conjugate with molecular entities and/or enzymes such as glucuronic acid, sulfotransferases, and glutathione S-transferases (Escher et al., 2021; Xu et al., 2005), to result in larger and increased hydrophilic metabolites. The general excretion in the bile and/or urine is enhanced, including an increase in detoxification. However, it should be noted that in certain situations the conjugations with enzymes might produce active metabolites and increase toxicity (Xu et al., 2005).

Specifically liver cells—the liver is the principal site where xenobiotics are metabolised—have a large capacity for biotransformation of xenobiotics (Escher et al., 2021). The aryl-hydrocarbon receptor (AhR), a nuclear receptor, is one of the so-called xenobiotic receptors which responds to compounds such as those that are dioxin-like. Compounds binding to this receptor can cause transcription of genes which encode for metabolic enzymes (Escher et al., 2021). In previous laboratory studies, where vertebrates were exposed to dioxin-like chemicals it resulted in wasting syndrome (organisms experienced progressive weight loss until they had an early death), developmental disorders (malformation) and reproductive disorders (Janošek et al., 2006).

The AhR is a ligand-activated transcription factor which regulates P450 enzymes through gene expression (Tian et al., 2015). The P450 enzymes can mediate toxicological and physiological effects when endogenous and/or exogenous chemicals activate the AhR. The P450 enzymes play a role in host defence, drug metabolism, cell differentiation and detoxification (Tian et al., 2015). This specific receptor also has an important role in the development and differentiation of lymphocytes, and it is involved in steatosis, autoimmune hepatitis, hepatic fibrogenesis and neurotransmission impairment (Tian et al., 2015).

Ligands diffuse into the cell's cytoplasm and bind to the AhR complex (Figure 1). The complex translocates into the nucleus and binds with the AhR nuclear translocator (ARNT) to form a heterodimer (Larigot et al., 2018; Nguyen & Bradfield, 2008). Co-activators (SRC-1, SRC-2, and SRC-3) (not showing in Figure 1) are recruited by the heterodimer and bind to specific DNA sequences which are referred to as dioxin-response-elements (DRE). Binding to the DNA sequences stimulates the transcription of target genes such as AhRR, CYP1A1 and CYP1A2 (Larigot et al., 2018; Tian et al., 2015).

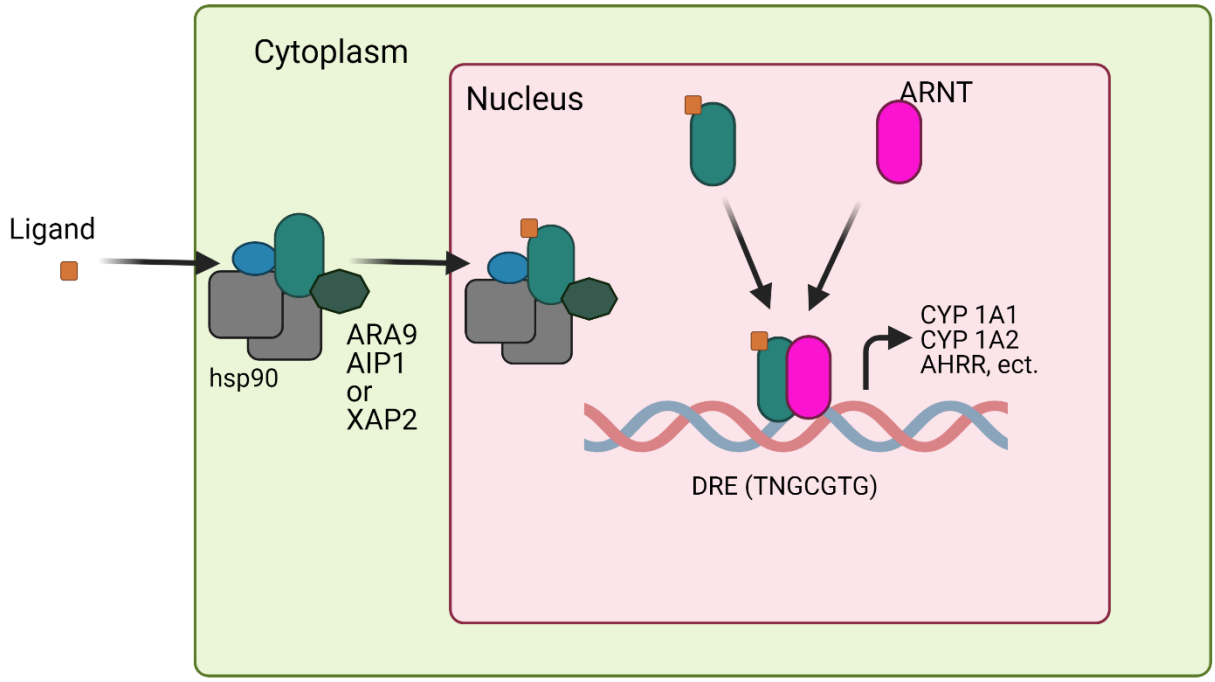


**Figure 1:** Mechanism of action of the aryl-hydrocarbon receptor (adapted from Nguyen & Bradfield, 2008) (created in Biorender). (HSP- heat shock protein, ARA9- Aryl hydrocarbon receptor-associated protein 9, AIP1- Aryl hydrocarbon receptor-interacting protein 1, XAP2- X-associated protein, AhR- aryl hydrocarbon receptor, DRE- dioxin response element, ARNT- AhR nuclear translocator, AHRR- aryl hydrocarbon receptor repressor).

The formation of dioxins might be local, but the environmental distribution is global (WHO, 2016). These compounds are by-products from manufacturing processes of some pesticides, herbicides, smelting, incomplete burning of hospital waste, and the choline bleaching of paper pulp to name a few (World Health Organization, WHO, 2016). Dioxins are hydrophobic which allows them to be strongly adsorbed to soil components and organic materials (Kirkok et al., 2020). The dioxins in the sediment and soil will seep into ecosystems and water sources creating the risk to poison future generations of shrimp, fish, crops and vegetables (Nhung et al., 2022).

The toxic effects of dioxins are fundamentally caused by AhR mediated dysregulation of the gene expression (Lindén et al., 2010). When a compound has a high affinity for the AhR the effect will have a high potency (Lindén et al., 2010). According to structure studies for high affinity binding to the AhR that the ligand should be hydrophobic, planar,

rectangular, and with maximum dimensions of 14 x 12 x 5 $x10^{-10}$ Å (Omiecinski et al., 2011). The greater the proportion of the receptor that is occupied by the ligand, the greater the possibility for the transformed receptor-ligand complex to interact with the dioxin response element found on the DNA (Lee et al., 2015).

#### 2.5.1.1 Recombinant H4IIE-*luc* rat hepatoma cell line

A genetically modified version of the rat hepatoma cell line (H4IIE) was used in this study to investigate AhR-ligand activation.

Rat hepatoma cells (H4IIE) were used to derive the recombinant H4IIE-*luc* cell line (Tillit et al., 1991) by transfecting the H4IIE cells with a luciferase reporter gene plasmid known as pGudLuc1.1 (Aarts, 1995). The pGudLuc1.1 plasmid was prepared through subcloning the 1810 bp fragment which contains the mouse mammary tumour virus promotor (MMTV) (Aarts, 1993). The promotor is under AhR mediated control and this cell line also contains a firefly (*Photinus pyralis*) luciferase gene and as a result can measure the induction of cytochrome P450 (Aarts, 1993). Light is produced once the AhR is activated, and the amount of light produced is equivalent to the number of receptors activated (Aarts, 1995).

### 2.5.2 Endocrine disruption

Pollutants such as pharmaceuticals, preservatives and insecticides end up in the water resources. Water consumers are exposed to these substances when they use the water for household and recreational purposes. These substances mimic or inhibit endogenous hormone action and cause disruption of normal endocrine signalling (Klopčič et al., 2015). Aquatic biota are also exposed to these compounds. Water utility systems are designed to remove selective pollutants with the result that persistent and hydrophilic EDCs are not removed from the water at these utility systems (Wee et al., 2022). These compounds are released back into the environment and even advanced treatment technology is not able to remove these compounds due to various transport pathways and the various physiochemical properties of EDCs (Wee et al., 2022). In the end, humans are exposed to EDCs when drinking water (Wee et al., 2022).

Compounds which are interfering with the hormone systems often indirectly affect the reproduction and health of an organism; these compounds are known as endocrine disrupting compounds (EDCs) (WHO, 2021). Endocrine disrupting compounds interfere with homeostasis of the body when they interact with hormone receptors and disturb numerous processes (Burkhardt-Holm, 2010). These compounds alter the biosynthesis of hormones, hormone release and/or storage, hormone recognition, and binding of receptors to name a few. The alterations potentially cause detrimental effects on plants, animals, humans and finally the entire ecosystem (Burkhardt-Holm, 2010). In aquatic birds, the concentration EDCs tend to be 100 times smaller in the surrounding water than in the body tissue. In humans and land-living vertebrates such as reptiles and birds, the abnormalities for exposure to EDCs include a decrease in the quality of the sperm, lower sperm count, neurological and immunological effects (Olujimi et al., 2010, WHO, 2021).

Endocrine disruptive effects had been blamed for declining alligator, fish and bird populations, the feminisation of amphibian and fish species and the eggshell thinning found in certain bird species (Kasonga et al., 2021). After the oral intake of the compound or extensive exposure of aquatic organisms, the endocrine disrupting effects can occur anywhere in the body and are not limited to a specific area or process (Brand et al., 2013).

The endocrine system responds to small changes in hormone levels with the result that even low concentration EDCs detected in the environment cannot be ignored (Varticovski et al., 2021). These compounds have effects on male and female reproduction, neuronal function, metabolism, obesity, cardiovascular and endocrine systems, and several cancer types (Varticovski et al., 2021). Studies found that in human foetal development there is a very sensitive window for effects induced by sex EDCs (EDCs that mimics sex hormones), it can have long-lasting consequences (Grindler et al., 2018, Ohtani et al., 2018, Varticovski et al., 2021). *In utero* exposure to drugs with androgenic activity cause female pseudohermaphrodism in rats, hamster, hedgehog, rabbit, cattle, and monkey (Grumbach & Ducharme, 1960). Studies have shown that dysregulation of androgens can result in polycystic ovaries in human females which are associated with obesity, infertility and insulin resistance to name a few consequences (Varticovski et al., 2021). It has also

been proven that most prostate cancer patients respond well to androgen deprivation because of the link between androgen and the development as well as the progression of prostate cancer (Dehm & Tindall, 2007; Varticovski et al., 2021).

#### 2.5.2.1 MDA human breast carcinoma cell line

The parent cell line, MDA-MB-453 (human breast cancer), was stably transfected with a luciferase reporter plasmid construct. This construct is also driven by the MMTV promotor (Wilson, 2002). In addition to the MMVT promotor being androgen responsive, glucocorticoids can also activate the promotor (Wilson, 2002). As a result, this cell line can stably express glucocorticoid- and androgen responsive receptors which can be used for the detection of hormone receptor antagonists and agonists.

The androgen receptor (AR) is a nuclear receptor that is activated through bindindg of testosterone, or dihydrotestosterone (DHT) or synthetic androgenic steroids (Hotchkiss et al., 2008). Androgen receptor is bound to an androgen regulates gene expression which acts as a DNA binding transcription factor (Hotchkiss et al., 2008). Testosterone is the hormone activating the AR in the Wolffian duct during the mammalian sexual differentiation while in the hair follicles, urogenital tubercle, and urogenital sinus DHT is the main hormone activating the AR (Hotchkiss et al., 2008). There are specific time windows during puberty and sexual differentiation when the actions of the androgen regulate the gene expression for normal development. During puberty and adulthood, after the early organisation, androgens activate the male-typical, sexually dimorphic behaviours and during adult life it is important for maintaining a normal reproduction function (Hotchkiss et al., 2008). Androgens also play important roles in the development and maintenance of the brain, muscle, bone, skin and hair (Hotchkiss et al., 2008).

Glucocorticoid is a steroid hormone which is synthesised in the adrenal cortex (Vilasco et al., 2011). This hormone regulates biological functions such as cell growth, apoptosis, and inflammatory responses. Glucocorticoids are also important for maintaining basal, stress-related homeostasis and to regulate a wide spectrum of physiological functions which are essential for life (intermediary metabolism, reproduction, and activities of the cardiovascular and central nervous systems) (Klopčič et al., 2015; Timmermans et al., 2019).

The process of androgen and glucocorticoid binding to their receptors up until the luciferase production happens in more or less the same way as the ligand binding to the AhR. When an androgen binds to the AR it leads to a change in receptor conformation. This results in the heat-shock proteins being dissociated, dimerization and the androgen-AR complex being transported from the cytoplasm into the nucleus (Figure 2) (Hotchkiss et al., 2008). In the nucleus, the AR dimer binds to the androgen response element (ARE) which is a specific sequence of the DNA. The process of transcription will start which will increase the synthesis of messenger RNA (mRNA). Ribosomes will translate the mRNA into specific proteins (Hotchkiss et al., 2008).

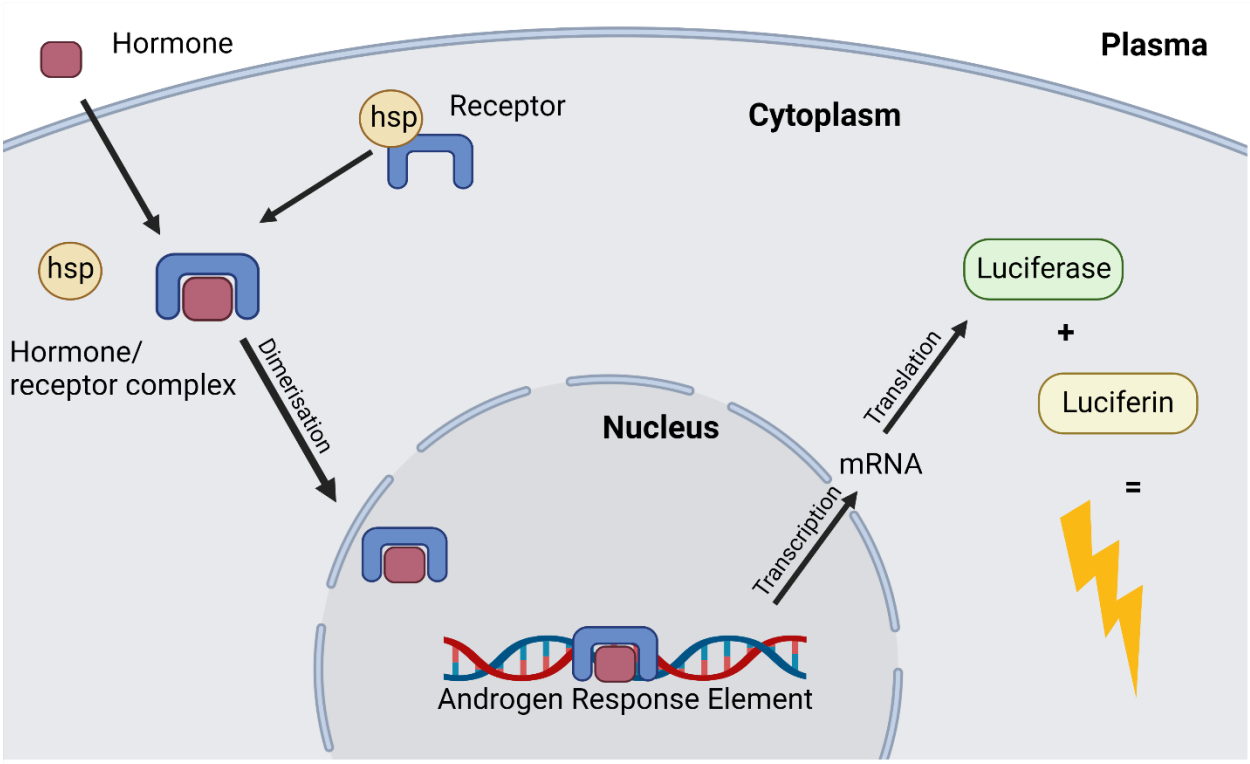


**Figure 2:** Mechanism of action of the androgen receptor (adapted from Hotchkiss et al., 2008) (created in BioRender). (hsp: heat shock protein, mRNA: messenger ribonucleic acid).

The glucocorticoid hormone easily diffuses across cell membranes into the cytoplasm, where it can interact with the glucocorticoid receptor (GR). Heat shock proteins dissociate from the receptor complex because of a change in the receptor conformation after the ligand interacted with the receptor (Scheschowitsch et al., 2017). Dimerization takes place before the glucocorticoid-GR complex translocates into the nucleus. Inside the

nucleus the GR dimers bind to the glucocorticoid response element to exert their effects (Vilasco et al., 2011).

### 2.5.3 Oxidative stress

Oxidative stress (OS) is caused when there is an imbalance between the production of reactive oxygen species (ROS) and the antioxidant systems' ability to detoxify these reactive intermediates (Katerji et al., 2019). ROS are free radicals which are chemically reactive, unstable and contain oxygen which is produced as natural by-products of aerobic cellular metabolism. Free radicals have one or more unpaired electrons in their molecular orbital (Nandi et al., 2019). These radicals take electrons from other molecules to stabilize themselves. Free radicals have the potential to oxidize cellular biomolecules such as lipids, proteins, and nucleic acids (Nandi et al., 2019). These radicals are formed during normal cellular metabolism as β-oxidation of fatty acids, mitochondrial electron transport chain, and cytochrome P450 mediated reactions (Nandi et al., 2019). ROS are needed at low to moderate concentrations for physiological processes including cell death, homeostasis, immune defence against pathogens, induction of mitogenic response, and intracellular cell signalling (Katerji et al., 2019). However, exogenous sources including biotransformation of xenobiotic compounds, diet, ionizing radiation, lifestyle, stress, smoking, and UV light can induce ROS (Bonato et al., 2020, Katerji et al., 2019). When excessive amounts of ROS are present in the body they can cause damage to DNA, lipids, and proteins (Katerji et al., 2019).

Living organisms possess antioxidant defence systems which are responsible for regulating the levels of these ROS (Katerji et al., 2019). The first line of defence against ROS is enzymatic antioxidants including catalase (CAT), glutathione peroxidase, methionine sulfoxide reductases, peroxiredoxins, and superoxide dismutase (SOD) (Bonato et al., 2020). This first line of defence cannot guarantee to cover the entire risk of OS due to certain compounds produced by detoxification reactions. Some of these products are reactive and have the potential to be cytotoxic for example, hydrogen peroxide produced by SOD (Bonato et al., 2020). As a result, it is important to have a joint coordinated action of several enzymatic compounds to prevent any further damage, such

as glutathione (GSH) and metallothioneins which are biosynthesised by the cell (Bonato et al., 2020).

Superoxide dismutase converts superoxide radicals into molecular oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$) (Weydert & Cullen, 2009). This enzyme can be found in three different areas in the body: i) manganese containing SOD which is found in the mitochondria, ii) copper and zinc containing SOD (CuZnSOD) that is found in the nucleus and the cytoplasm; and iii) the SOD which is expressed extracellularly in some tissues. The CuZnSOD covers 90% of the overall SOD activity in a eukaryotic cell (Weydert & Cullen, 2009).

Catalase is an important antioxidant enzyme and is found in all aerobic organisms. CAT converts $H_2O_2$ into oxygen and water in a two-step reaction (Nandi et al., 2019). The reaction starts with the formation of a spectroscopically distinct intermediate compound I, covalent oxyferryl species [$Fe^{IV}O$].

[$Fe^{IV}O$] has a porphyrin π-cation radical because of one hydrogen peroxide molecule being reduced. During the second step redox reactions reduce compound I through a two-electron transfer: from the electron donor which is the second $H_2O_2$ molecule and produces oxygen, water, and the free enzyme

Enzyme [porphyrin Fe(IV)•O] + $H_2O$ → Enzyme [porphyrin Fe(III)] + $H_2O$ + $O_2$
(free enzyme)

(Nandi et al., 2019). CAT malfunctioning or deficiency is associated with several diseases including Wilson disease, anaemia, schizophrenia, vitiligo, diabetes mellitus, and cardiovascular disease (Nandi et al., 2019).

Lipid peroxidation is the process during which free radicals attack lipids that contain double carbon-carbon bonds, especially the polyunsaturated fatty acids which involve the abstraction of hydrogen from the carbon and the insertion of oxygen (Ayala et al., 2014). This results in hydroperoxides and lipid peroxyl radicals. Well-known targets of lethal peroxidative modification and damage are cholesterols, phospholipids, and glycolipids (Ayala et al., 2014). The process of lipid peroxidation consists of three phases: initiation,

propagation, and termination. During the initiation phase the pro-oxidants such as the hydroxyl radical abstract an allylic hydrogen forming the carbon-centred lipid radical ($L^{\bullet}$). The $L^{\bullet}$ quickly reacts with oxygen in the propagation phase to form a lipid peroxy radical ($LOO^{\bullet}$) (Ayala et al., 2014). This radical abstracts hydrogen from another lipid to form a new $L^{\bullet}$ and lipid hydroperoxide (LOOH). Nonradical (stable) products are formed during the termination phase when antioxidants such as vitamin E donates a hydrogen atom to the $LOO^{\bullet}$ species. The primary product of lipid peroxidation is LOOH. Propanol, hexanal, and malondialdehyde are secondary products of lipid peroxidation. Malondialdehyde is used as a biomarker for lipid peroxidation because of its facile reaction with thiobarbituric acid (Ayala et al., 2014).

The potential of the extracts to cause oxidative stress was investigated on the HuTu 80 (duodenum) and H4IIE-*luc* (liver) cells, because in these organs absorption and biotransformation of compounds are taking place. The liver cells were discussed in section 2.5.1.

#### 2.5.3.1 Small intestine cell line

One of the cell lines with which oxidative stress responses were determined in this study is the Hutu 80 cell line.

The Hutu 80 cell line is human small intestine, duodenum adenocarcinoma cells that was harvested from a Caucasian male (ATCC, 2022). The mammalian small intestine is the organ that mainly serves as a site for absorption of water, nutrients, and xenobiotics (Kaminsky & Zhang, 2003). The gastrointestinal tract acts as a defence line because it is the third most exposed to chemicals and drugs after the skin and lungs (Ryu et al., 2021). The intestinal tract metabolises the ingested xenobiotics and absorbs the parent xenobiotics and metabolites. The parent xenobiotics and metabolites might cause damage to the gastrointestinal tract during the absorption processes. If this is the case the toxic reactions will lead to the loss of function of the intestinal barrier and ultimately cause a degradation in the system's defence (Ryu et al., 2021).

### 2.5.4 Acetylcholinesterase activity

Acetylcholine (ACh) is a molecule that is a neurotransmitter (Beckmann & Lips, 2013; Forsgen et al., 2009). This molecule is also released by non-neuronal cells such as intestinal epithelium, in keratinocytes of the skin, urinary bladder, in various cell types present in the airway, and in cells located in the walls of blood vessels and activity of acetylcholinesterase enzeme has also been found in organs such as the pancreas, placenta, liver, intestine, heart, and kidney (Beckmann & Lips, 2013; Forsgen et al., 2009; Pérez-Aguilar et al., 2015). The system composed of ACh-synthesising enzymes, receptors, transporters, and degrading enzymes outside of the nervous system is known as the non-neuronal cholinergic system (Beckmann & Lips, 2013). The enzyme AChE recycles ACh to produce an acetate group and choline (Lionetto et al., 2011). The inhibition of AChE activity is an endpoint commonly used in water quality assessment for specific neurotoxicity (Escher et al., 2021). This assay has been used for measuring several types of environmental water extracts (Al-Ghais 2013; Gaitonde et al., 2006). Although Neale and Escher (2013) recommended that this assay should not be used to measure water quality of samples rich with dissolved organic carbon (DOC) as concentrations as low as 2 mg/L suppressed the AChE activity with the result that it is not clear whether the inhibition of AChE activity is due to chemicals present in the sample or DOC, which is often found in surface and wastewater (Neale & Escher, 2013).

### 2.5.5 Acute toxicity using *in vivo* assays

Acute toxicity refers to short-term exposure to a relatively high concentration of the chemicals with a lethal endpoint whereas chronic toxicity is exposure to a lower concentration over a longer period with sublethal (growth inhibition, reproduction, and swim behaviour) and lethal endpoints (US EPA, 1994). These toxicity tests, both acute and chronic and give an indication of whether the contaminant is bioavailable. It considers the effect of parameters such as pH, hardness, and total dissolved solids of the testing medium have on the organisms, and these toxicity tests determine the total nature of the toxic effect (sublethal or lethal) (US EPA, 1994). Organisms such as algae, duckweed, *Daphnia magna*, zebrafish and frogs are typically used for these toxicity testing.

Although this was not one of the main aims of this study in which the focus was on *in vitro* assays, two *in vivo* test kits were gifted to us during the second year of the study, and they were put to use. *In vivo* tests are useful tests to evaluate whole water toxicity because the organisms are exposed to the raw water or sediment under controlled conditions (ISO 14380:2011; ISO 14371:2012).

From an ecotoxicological perspective, crustaceans are of interest because they are primary consumers and a major part of the zooplankton in aquatic ecosystems (ISO 14380:2011). Toxicants do not only occur in the water column, but are also present in the sediment and tend to accumulate in the sediment (ISO 14371:2012). The benthic ostracods were exposed to the sediment collected. Beaver fairy shrimps (*Thamnocephalus platyurus*), standardised test according to the International Standard Organization, were exposed to the water collected.

#### 2.5.5.1 *Thamnocephalus platyurus* (Crustaceans)

Crustaceans are primary consumers and play a key role in the aquatic food web with the result that they are model species used to study chronic and acute aquatic toxicity (Lavado et al., 2021). If chemicals are affecting the health of this beaver-tail fairy shrimp freshwater crustacean, the possibility exists that the adverse effect might extend to the predators and the ecosystem itself (Lavado et al., 2021). The fairy shrimps were more sensitive than the other standard crustacean test animal, *Daphnia magna* in a study by Blinova (2000). Various river water samples and wastewater samples were tested with a battery of tests, which apart from the crustacean tests also included the green algae, *Selenastrum capricornutum,* duckweed *Lemna minor,* and the ciliate protozoan *Terahymena thermophila*.

#### 2.5.5.2 *Heterocypris incongruens* (Ostracod)

This ostracod species is truly representative of an ecological benthic species because a small part of its life this species is swimming in the water while for the greater part of its life this species is in direct contact with the sediment (Khanal et al., 2014). Studies done by Chial and authors have revealed that *H. incongruens* is indeed sensitive to river sediment which are contaminated (Chial et al., 2003a; Chial et al., 2003b). The sensitivity

of this species are similar to the midge larva, *Chironomus riparius*, and the amphipod, *Hyalella azteca*, that are usually used in toxicity tests investigating whole-sediment samples (Chial et al., 2003a; Chial et al., 2003b).

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# CHAPTER 3 MATERIALS AND METHODS

The more I study nature, the more I stand amazed at the work of the Creator. Science brings men nearer to God.

– Louis Pasteur

In this study, the aim was to assess the quality of different types of water (surface-, drinking, and wastewater) by determining the biological effects posed by chemicals in these sources. Water and sediment samples were collected in and around Wakkerstroom and transported back to the laboratory at the North-West University, Potchefstroom Campus (Figure 3).

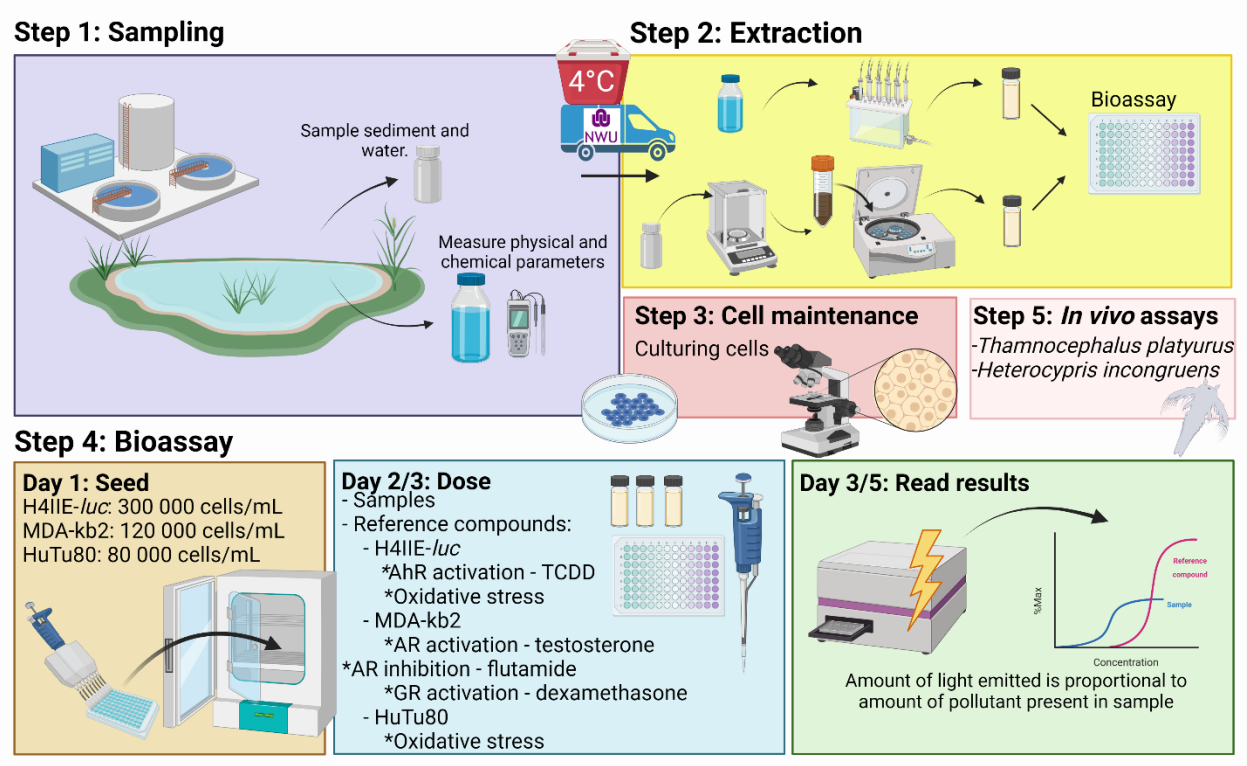


**Figure 3:** Steps followed in this study from sample collection to obtaining the results (created in BioRender).

### 3.1 Sampling sites

Wakkerstroom is a small town located in the Highveld (inland plateau of South Africa with an altitude of less than 2 100 m but more than 1 500 m (Language et al., 2020)), on the border between the Mpumalanga and KwaZulu-Natal Provinces, where the annual rainfall is between 600 and 800 mm per year (Cadman, 2007), which is almost double the annual rainfall of South Africa at 440 mm (Tempelhoff, 2019). Small steep tributary streams rise in the hills to form the Wakkerstroom River which flows into Martin's Dam, upstream of a wetland (Joubert & Ellery, 2013). Most of the town is situated to the east of the wetland (Figure 4). The lower reaches of the wetland re-forms into the Thaka River which enters the KwaZulu-Natal Province into the Zaaihoek Dam, a short distance from the wetland. The wetland stretches over more or less 1 000 ha (Joubert & Ellery, 2013), which is

northwest of the small town. The Wakkerstroom River forms part of the Tugela River upper catchment (Stockdale et al., 2021). Wakkerstroom had a population of 6 852 during the 2011 census (Frith, 2011). An informal settlement forms the east side of the town. Cattle farms surround the town and wetland.

After discussions with the Wakkerstroom community and experts from the local branch of BirdLife South Africa sanctuary, ten specific sites that represent different types of water in Wakkerstroom were selected. Water and sediment (where available) were collected upstream and downstream from the wetland and in the wetland itself (Figure 4) (Table 1).

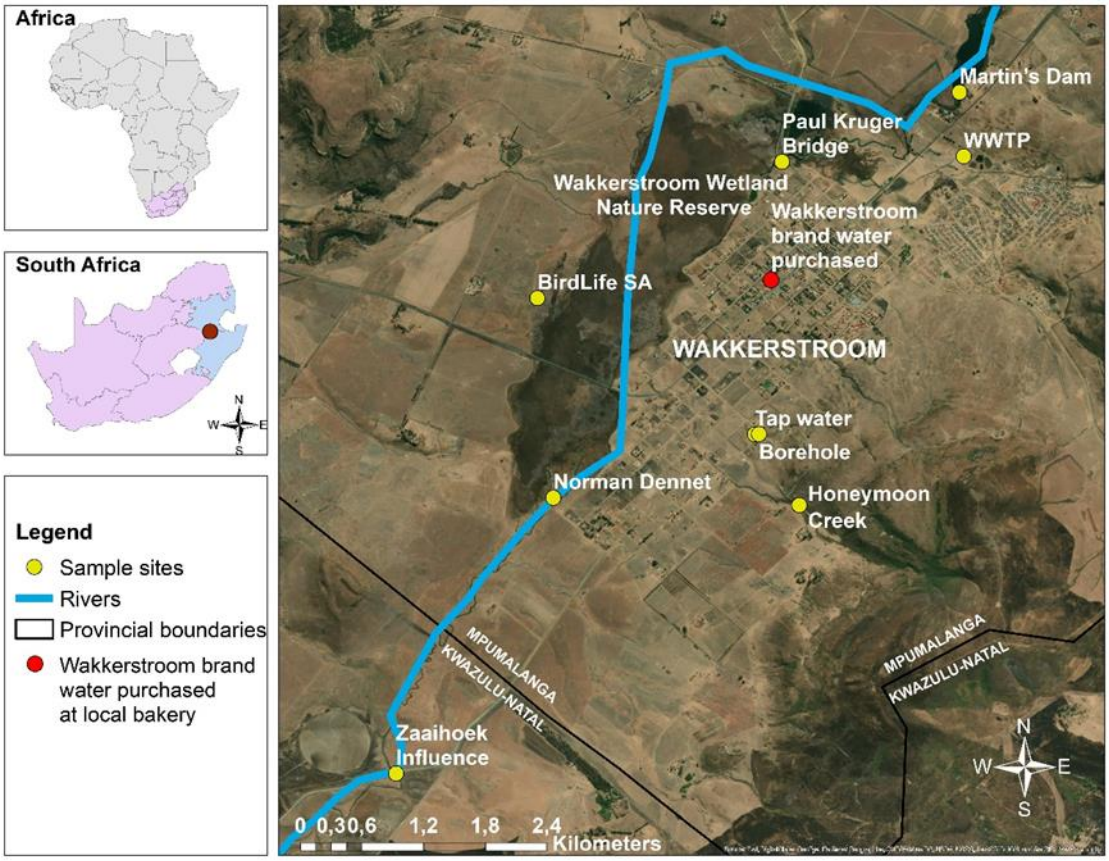


**Figure 4:** Map of Wakkerstroom, showing the locations of the ten specific sampling sites (WWTP-Wastewater Treatment Plant).

### 3.2 Sample collection

Samples from the dry and wet season were collected to investigate whether the concentration of chemicals has a greater effect during the dry season because during the dry season the

chemicals are anticipated to be more concentrated. In the wet season, it is expected that a rise in water level after the seasonal rainfall will dilute the concentration of chemicals but the concentration of chemicals might also increase due to chemicals from the surrounding area might be transported into the river. Dry season samples were collected during September 2021 and the wet season samples were collected in February 2022. Water was collected at each of the ten sites (Table 1) in 1 L glass Schott bottles which had been pre-cleaned (acid washed). The Wakkerstroom branded water was purchased in town at a local supermarket. This water is collected at a natural fountain upstream from Wakkerstroom in the direction of Paul Pietersburg. Composite sediment samples were collected at five sites (Table 1) into pre-cleaned stainless-steel containers, mixed thoroughly and dispensed into wide-mouth high-density polyethylene bottles (250 g) (Thermo Scientific, 2104-0008). All the samples were kept at 4°C while transported to the laboratory. Chemicals of interest might be light sensitive thus the bottles were covered to protect against UV degradation.

**Table 1:** Location and description of sampling sites. Water was sampled from each of the sites. If sediment was sampled, it was indicated in the third column.

| Site name | GPS co-ordinates | Collected sediment | Site description |
|---|---|---|---|
| Tap Water | 27°22'07.9"S 30°08'22.8"E | ✘ | Water was sampled at a resident's house. The water was prepared by the local purification plant. |
| Borehole | 27°22'07.8"S 30°08'24.1"E | ✘ | Water was sampled at a resident's house. |
| Wakkerstroom Brand Water | 27°21'19.4"S 30°08'28.0"E | ✘ | A 5 L Wakkerstroom brand water was purchased at the local supermarket. The water is from a local natural fountain. |
| Zaaihoek Influence | 27°23'54.5"S 30°06'29.8"E | ✓ | The Wakkerstroom River (Thaka River) flows from the Wakkerstroom wetland and enters the Zaaihoek Dam. The samples were collected up-stream from the Zaaihoek Dam. Cattle were grazing near the sampling site. |
| Norman Dennet | 27°22'27.8"S 30°07'19.3"E | ✓ | Close to the last house in town towards the Zaaihoek Dam. It was evident from tracks and cow dung that cattle grazed this area. A mesh-like material covered the banks of the stream. |
| Honeymoon Creek | 27°22'30.18"S 30°08'36.8"E | ✓ | Samples were collected in a 3 m deep, and 4 m wide ditch carved by the creek. Cattle were grazing on the edge of the creek. This stream originates from the Ossewakop mountain. |
| BirdLife SA | 27°21'25.1"S 30°07'14.3"E | ✓ | The site is approximately 500 m east of the BirdLife SA buildings in the wetland itself. Samples were collected between the reeds. |
| Paul Kruger Bridge | 27°20'42.2"S 30°08'31.4"E | ✓ | Sampled from a walkway over the water which is underneath a road bridge. The road leads to Amersfoort, a nearby town 56 km northwest from Wakkerstroom. |

| Site name | GPS co-ordinates | Collected sediment | Site description |
|---|---|---|---|
| Wastewater treatment plant | 27°20'40.5"S 30°09'28.6"E | ✘ | Treated water (effluent) was collected as it exited the treatment plant just before it disappears into a pipe. The pipe was surrounded by dense reeds. |
| Martin's Dam | 27°20'20.3"S 30°09'27.3"E | ✘ | Samples were collected near the dam wall, closest to the wetland. The surface of the water was covered with duckweed. Wakkerstroom's water purification plant is next to the dam wall, only a fence between the dam and the water treatment works. Glass bottles, plastic and paper polluted the grass area next to the dam and were also found in the water. |

## 3.3 Extraction methods

### 3.3.1 Solid-phase extraction of water samples for biological analysis

Target compounds were extracted from the sampled water using solid-phase extraction (SPE) to investigate AR and GR activity of the extracts and also if these extracts caused oxidative stress. Whether these extracts caused an increase or decrease in acetylcholinesterase activity in the cells were determined. This extraction method was performed at the University of Pretoria at the Environmental Chemical Pollution and Health Research Unit under the supervision of Dr. Natalie Aneck-Hahn.

Once the samples were back at the laboratory, the pH of the sampled water was adjusted to 3 before the filtering. Two filter papers were stacked: 47 mm filter paper (Macherey-Nagel, MN GF-1, 0.7 µm particle retention) was the top filter and 0.2 µm S-Pack white gridded filter (47 mm) (Pall Corporation, 66234) was the second layer. Both filters wer.e used to remove small soil particles from the sample. The water samples were filtered 250–300 mL at a time (De Jager et al., 2011). The extraction was done using a SPE manifold connected to a vacuum pump and Oasis® hydrophilic lipophilic balanced 5cc 200 mg LP glass cartridges (Oasis®, 186000683). The cartridges were preconditioned with 5 mL deionised water (18.2 MΩ.cm) (from in-house Milli-Q® EQ 7000 Ultrapure Water Purification System), 5 mL analytical grade methanol (MeOH) (Honeywell, 67-56-1) and again 5 mL deionised water. Five millilitres of the sample were added at a time to the reservoir of the cartridge and the valve of the cartridge was opened to allow the sample to pass through at a flow rate of 10 mL/min. Once the whole sample (1 L) passed through the cartridge was left to dry before the target compounds were

eluted with 5 mL MeOH. This was done without vacuum and the methanol dripped through the cartridge by gravitational pull only. Eluates were evaporated to dryness using a gentle stream of nitrogen gas ($N_2$). The extracts were reconstituted with 1 mL MeOH and stored in amber glass screw top vials in the -80˚C until bioassays commenced.

### 3.3.2 Solid-phase extraction of water samples for chemical analysis

To extract pesticides from the sampled water a hydrophilic and lipophilic balance (HLB) cartridge was used. The Oasis® 3 cc HLB (30 µm, 60 mg) cartridges (Waters®, WAT058883) were conditioned twice with 1 mL MeOH and twice with 1 mL water. The 1 L water samples were passed through under vacuum at 10 mL/min (Riches et al., 2011). Cartridges were washed twice with 5% MeOH made up in water before they were eluted with 1 mL MeOH (2x).

A second extraction was then employed to extract pharmaceutical and personal care products (PPCPs) for chemical analysis is different from the SPE method described in section 3.3.1. A tandem configuration allowed for a three-tiered extraction mechanism (3 different elution solvents) which used anion exchange, cation exchange and reverse-phase (Mallet et al., 2017). This configuration was used to ensure retention of neutral, basic, and acidic PPCPs. A Waters® 6-cc Oasis® MCX cartridge (Thermo Fisher Scientific, 50-818-609) was connected below the Waters® 6-cc Oasis® MAX cartridge (Thermo Fisher Scientific, 50-780-194) (Figure 5). Both the cartridges were conditioned with 5 mL MeOH, and 5 mL $H_2O$ before the 1 L water samples were passed through under vacuum at 10 mL/min using a bottle to SPE adapter (Mallet et al., 2017). After the samples passed through, the cartridges were disconnected to be washed and eluted separately with specific steps.

The Waters® 6-cc Oasis® MAX cartridges were washed with 5 mL 5% ammonium hydroxide ($NH_4OH$) solution (Sigma-Aldrich, 1336-21-6). The cartridges were eluted with 5 mL MeOH to elute the neutral PPCPs and secondly with 5 mL MeOH that contained 5% formic acid (Sigma-Aldrich, 64-18-6) to elute the acidic PPCPs. Both the elutes were collected in a 20 mL glass bottle. The Waters® 6-cc Oasis® MCX cartridges were washed with 5 mL 5% formic acid and eluted with 5 mL MeOH that contained 5% $NH_4OH$ to elute the basic PPCPs (Figure 5). The elutes (n=3) from both (MAX and MCX) cartridges and

the HLB cartridges were pooled and evaporated to dryness using a gentle stream of $N_2$ (Figure 5). The elutes were reconstituted using 900 µL ammonium formate diluted in water (10 mM) (2 x 450 µL) (Sigma-Aldrich, 540-69-2) and stored in screw top amber vials.

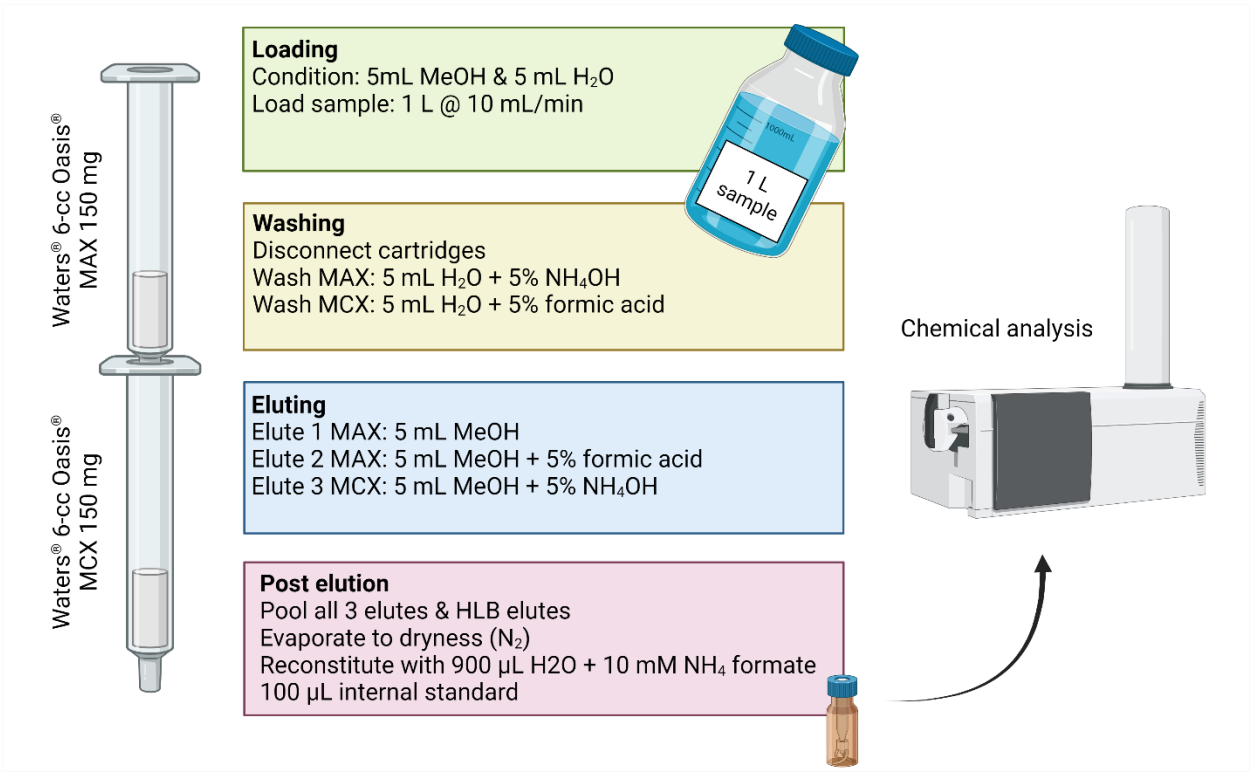


**Figure 5:** Processing of samples and preparation of extracts containing pharmaceutical and personal care products (created in BioRender).

### 3.3.3 Extraction of sediment for biological analysis

In this study two different extraction methods were used to extract the targeted compounds from the sediment. The extracts for the MDA-kb2 cell line were extracted based on a method found in literature (Kinani et al., 2010). A combination of polar and non-polar solvents was used to extract both polar and non-polar compounds from the sediment with the process of sonication. The accelerated solvent extraction method was used to extract non-polar compounds from the sediment using only hexane (non-polar

solvent) because well-known AhR ligands are traditionally non-polar (dioxin-like compounds) and these were targeted to activate the AhR in the H4IIE-*luc* cells.

#### 3.3.3.1 Ultrasonication extraction of sediment

Compounds from air-dried ground sediment (5 g) were extracted with 10 mL of hexane (Honeywell, 110-54-3) and acetone (2:1). The sediment mixture was sonicated three times for 10 minute intervals (Figure 6) (Kinani et al., 2010) and after each sonication step the supernatants were combined. Anhydrous sodium sulphate (BDH laboratory reagents, 7757-82-6) was added to the supernatant before centrifugation. The supernatant was mixed with activated copper to remove cytotoxic elemental sulphur. The mixtures were shaken for an hour before it was filtered through 0.45 µm polytetrafluoroethylene syringe filters (Membrane Solutions, SFPTFE030045NB) (Kinani et al., 2010). The solvent was evaporated to near dryness before reconstituted with 1 mL MeOH and stored at -20˚C until bio-assays commenced.

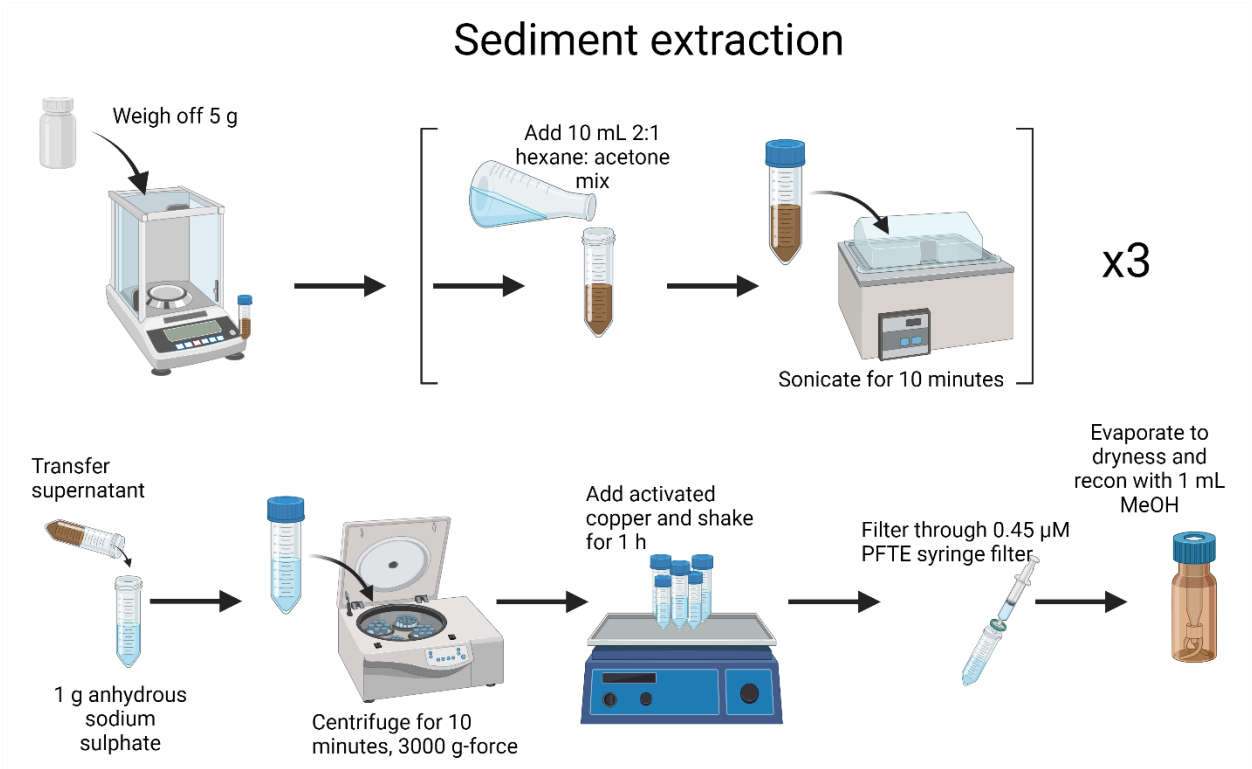


**Figure 6:** The steps followed to extract target compounds from sediment (created in BioRender).

#### 3.3.3.1.1 Preparation of activated copper

Copper powder (Aldrich Chemistry, 266086-2KG) (25 g) was added to a 500 mL Schott bottle and covered with HCl (Rochelle Chemicals, 7647-01-0). This procedure was done in a fume hood. The mixture was swirled several times until the copper changed to a reddish colour. This was the indication that the oxide was removed from the copper's surface. The acid was discarded. The process was repeated until the copper was uniformly reddish brown. The copper was rinsed with tap water, followed by five times (20 mL) deionised water (18.2 MΩ.cm) (from in-house purification system, ELGA PURELAB Ultra Mk 2 version 3),, analytical grade acetone (Honeywell, 67-64-1), followed by MeOH. Dried activated copper was stored in a closed Schott bottle in a desiccator.

### 3.3.3.2 Accelerated solvent extraction of sediment

Sediment was air-dried and ground into a fine powder by the use of a mortar and pestle. Sediment (20 g) was mixed with an equal amount of anhydrous diatomaceous earth (Thermo Scientific, 062819) to remove any trace water molecules from the sediment (Figure 7). The extraction cell of the DIONEX150 accelerated solvent extractor (ASE) was pre-washed with a phosphate-free soap, rinsed with deionised water (18.2 MΩ.cm) followed with three rinses of acetone and then three hexane rinses. The extraction cell was allowed to dry before a cellulose filter (Thermo Fisher Scientific, 056780) was placed at the outlet end of the cell. The sediment and anhydrous diatomaceous earth mixture was added to the cell and another cellulose filter was placed on top. The lid of the cell was closed tightly. A 3:1 analytical grade dichloromethane (DCM) (Honeywell, 75-09-2) and hexane mixture was used as the extraction solvent. The Dionex150 parameters were set to 100˚C, 10 342,14 kPa, (10 minutes heat with 5 minutes static time), 60% flash volume, and nitrogen purge time of 100 seconds, and two cycles (McCant et al., 1999). The extract was collected in a pre-cleaned collection bottle and transferred to a TurboVap® flask. A gentle stream of nitrogen gas was used to evaporate the extract until dryness in the TurboVap® II (Caliper LifeSciences). Subsequently, the extract was reconstituted with 10 mL hexane and transferred to a separation funnel for the acid treatment.

To remove all the non-persistent AhR ligands such as PAHs, 15 mL of 98% concentrated $H_2SO_4$ (Merck, 1041578) was added, and the separation funnel closed, before carefully mixing the acid with the organic phase. Vertebrate bodies are known to metabolise the more transient AhR ligands, and these are less toxic or not toxic at all (Lamoree et al., 2004). The organic and acid phases were allowed to separate before the acid phase was discarded. This acid treatment was repeated until the acid phase was clear, but not more than six times because it would break down the target compounds. The samples were further washed with an aqueous solution of 5% NaCl and the different phases were allowed to separate. Aqueous NaOH (20%, 15 mL) was used to wash the sample for a maximum of 15 minutes before a final 5% sodium chloride wash followed to remove traces of the potassium hydroxide. Each time the phases were allowed to separate before the bottom phase was collected and discarded.

The remaining organic solvent was evaporated from the sample to near dryness with a gentle nitrogen stream in the TurboVap® II. Dichloromethane (2 mL) was used to reconstitute the samples before they were transferred to clear glass 15x45 mm screw neck total recovery vial (Waters®, 186002520) (Furlong et al., 1996). The sample DCM mixtures were subjected to gel permeation chromatography (GPC) which is size-exclusion chromatography that separates analytes based on size. The recovery vials were weighed before and after it was filled with the sample and after it was injected into the GPC, to keep track of potential losses during this clean-up process. This is an established clean-up step which was used to remove elemental sulphur because it is toxic to cells (Furlong et al., 1996). Previously (See 3.3.3.1), the elemental sulphur was removed with activated copper, but the GPC was operational again when this extraction process was in progress.

The GPC system comprises of a Waters 2487 dual λ absorbance detector, Waters $717_{plus}$ autosampler, Waters 1515 isocratic High-performance liquid chromatography pump and Waters fraction collector III and two Envirogel GPC clean-up columns (SERNO: 0006474896-4, 0006474896-3) connected in series. A reference solution containing 63 mg/mL corn oil (Sigma-Aldrich, 8001-30-7), 0.05 mg/mL perylene (Sigma-Aldrich, 198-55-0), 0.2 mg/mL sulphur (Sigma-Aldrich, 7704-34-9), 2.5 mg/mL bis(2-ethylhexyl) phthalate (Sigma-Aldrich, 117-81-7), 0.5 g/mL methoxychlor (Sigma-Aldrich, 72-43-5)

was used to calibrate the system. The retention times of the calibration standards were used to calculate at which time the fraction without sulphur should be collected. Dichloromethane was the mobile phase used and the flow was set at 5 mL/min for 30 minutes. The fraction containing the sample was collected in a TurboVap flask and evaporated to near dryness before it was reconstituted with 1 mL hexane in 2 mL amber screw top vials and stored at 4˚C until it was used in a bio-assay.

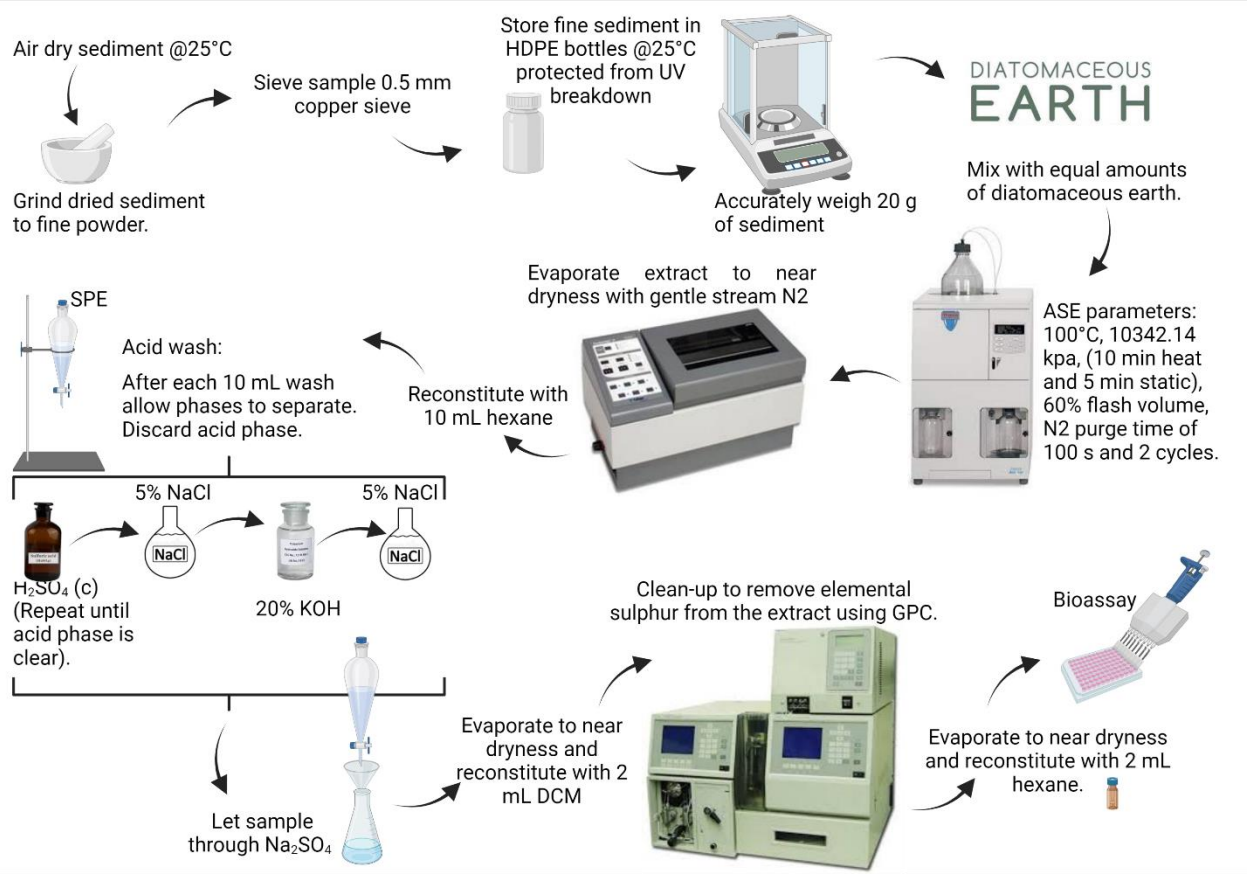


**Figure 7:** The steps followed to extract dioxin-like compounds from sediment (created in BioRender).

### 3.4 *In vitro* biological assays

In this study three different cell lines were used to investigate the water quality using different biological endpoints. The H4IIE-*luc* cell line, (rat hepatoma cells), measures aryl-hydrocarbon receptor activation whereas the MDA-kb2, human breast cancer, cell line is used to investigate AR and GR activity. The HuTu 80, duodenum adenocarcinoma, cell line is used together with the H4IIE-*luc* cell line to test for oxidative stress because the

duodenum and liver are where absorption and biotransformation of xenobiotics take place (Escher et al., 2021; Kaminsky & Zhang, 2003).

### 3.4.1 Principle of H4IIE-*luc* reporter gene assay

The H4IIE-*luc* cell line was derived from rat hepatoma cells and contains the aryl hydrocarbon receptor (AhR) (Tillit et al., 1991). The cells are stably transfected with the luciferase reporter gene under transcriptional control of various dioxin-responsive enhancers (Tillit et al., 1991). When the receptor is activated, luciferase is expressed and in the presence of the substrate, luciferin, light is emitted. The amount of light emitted is directly proportional to the number of receptors activated (Aarts, 1993). The reporter gene assay using H4IIE-*luc* cells was originally established to detect dioxin-like compounds including dioxin-like polychlorinated biphenyls (dl-PCBs), polychlorinated dibenzofurans (PCDD/Fs) and a few polycyclic aromatic hydrocarbons (PAHs) (Aarts, 1993). All these compounds elicit a response through the AhR and mediate detrimental effects such as impairments in nervous, immune and reproduction systems (Tian et al., 2015).

### 3.4.2 Principle of MDA reporter gene assay

The MDA-MB-453, human breast cancer, parent cell line was stably transfected with an androgen-responsive luciferase reporter plasmid. This plasmid is driven by the mouse mammary tumour virus promoter (Wilson et al., 2002). MDA-kb2 cell line is used to test for androgenic and glucocorticoid receptor activity through agonists and antagonists (Wilson et al., 2002). Once either of the receptors are activated the enzyme luciferase is expressed and in the presence of luciferin, light is produced. The amount of light produced is directly proportional to the number of receptors activated or inhibited in the sample (Denison et al., 2004).

### 3.4.3 Cell maintenance

The H4IIE-*luc* and MDA-Kb2 cell lines were provided by the University of Saskatchewan (Canada). The HuTu 80 cell line (HTB-40) was obtained from the American Type Culture Collection (ATCC) in the United Kingdom.

**Table 2:** Overview of the growth conditions of the different cell lines used in this study.

| Cell line | Growth medium (contain 10% foetal bovine serum (FBS) | Seeding density cells/mL | | Received 1% antibiotic-antimycotic |
|---|---|---|---|---|
| | | Reporter gene assay | Oxidative stress | |
| H4IIE-*luc* | Low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 3.7 g $NaHCO_3$ | 300 000 | 80 000 | ✗ |
| MDA-kb2 | Leibovitz's (L-15) (13.8 g/L)) | 120 000 | Not applicable | ✓ |
| HuTu 80 | Low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 3.7 g $NaHCO_3$ | Not applicable | 80 000 | ✓ |

Catalogue numbers and suppliers of consumables: antibiotic-antimycotic (Celtic, L0010-100, amphotericin B, penicillin G and streptomycin), DMEM (Sigma-Aldrich, D2902-1L), NaHCO3 (Sigma-Aldrich, S5761-1KG), L-15 (Sigma-Aldrich, L4386-50L), FBS (Biowest, S181G-500)

Frozen cells (1 mL) were collected from cryopreservation, thawed and added to 11 mL of their respective growth medium (Table 2). The cells were centrifuged at 125 *g* for 5 minutes and the supernatant was discarded. Using 12 mL medium, the pellet of cells was resuspended and placed in a tissue culture dish (TPP, 93100). The dish was placed in the Thermo Electron Corporation Forma Series II incubator with humidified air, supplemented with 5% $CO_2$ and at 37˚C, except for the MDA-kb2 cells which grow in the absence of the 5% $CO_2$.

All the cells were inspected under an inverted microscope after two days to investigate whether they have attached and to make sure there is no bacterial contamination. When the plates were confluent, the cells were passaged to ensure the cells do not overgrow, deplete the nutrients in the medium and die as a result. After more than two passages, the cells in confluent tissue culture dishes were used for assays.

Working inside the bio-safety cabinet class II, the medium was removed, and the cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (Sigma-Aldrich, D5652-10L) three times before adding 1.5 mL trypsin (Biowest, S181G-500) (trypsin and DPBS 1:9 mixture) and incubated for three minutes before the trypsin was removed (Whyte et

al., 2004). The cells were washed from the culture dish using 12 mL supplemented medium and aliquots were transferred to new culture dishes at desired ratios (1:3) (cell suspension:medium) for continuous cell growth.

All experiments were conducted in a sterile environment to prevent bacterial and fungal contamination. The laboratory surfaces and bio-safety hood were frequently sprayed with 70% ethanol especially before and after working with the cells and between working with different cell lines. Cell handling took place within the bio-safety hood (Davis, 2002). Schott bottles and lids were autoclaved and sprayed with 70% ethanol before placing it in the bio-safety hood. The medium of the respective cell lines was filtered using 0.22 µm, 250 mL bottle top filters (AEC Amersham SOC, 596-4520) after it was made and before the media were given to the cells. This will remove any bacteria that might be present in the powder and water used to prepare the medium.

### 3.4.4 Reporter gene assays

The assays were performed with growth medium (Table 2) that contained FBS stripped from its hormones. The hormones were removed from the FBS by treating the FBS with dextran treated charcoal (dtc). Heated FBS (56ºC for 30 min) was gradually poured onto dtc (Sigma-Aldrich, C6241-20G) in a ratio of 33 mL:0.2 g, and shaken for 45 min at 45ºC (Dang & Lowik, 2005; Mesalam et al., 2017). The FBS and charcoal mixture was centrifuged for 20 min at 1000 g and the supernatant was decanted into 50 mL centrifuge tubes containing 0.2 g dtc, shaken for 45 min at 45ºC, followed by the centrifugation step.

The hormone removal step was necessary because hormones can bind to the receptors and thus they were removed to prevent false positive results. For the reporter gene assays, white-walled 96-well plates with transparent bottoms (TPP, 92196) were used. The outer wells received 250 µL DPBS to create the same micro-environment across the cell-bearing wells because only the inner 60 wells were seeded with cells (300 000 cells/mL H4IIE-*luc* and 120 000 cells/mL MDA-kb2) (Table 2). The density of the cells was determined with the use of an automated cell counter, the Luna-II (Logos Biosystems, Brightfield cell counter).

After 24 h for the H4IIE-*luc* cell line and 48 h for the MDA-kb2 cell line, the attachment of the cells was confirmed by visual inspection (Larsson et al., 2014) and the cells were

exposed to the reference compounds, sediment-, and water-extracts in triplicate. After the exposure period was completed (24 h for H4IIE-*luc* and 48 h for MDA-kb2) the assay plates were inspected under the inverted microscope and confluency of the cells in each of the wells was noted. The plates were washed three times with DPBS containing additional $CaCl_2$ (0.11 g/L) (Sigma-Aldrich, C-2661) and $MgSO_4 \cdot 7H_2O$ (0.25 g/L) (Sigma-Aldrich, 23,039-1). The $Ca^{2+}$ and $Mg^{2+}$ were added because these ions are required in the light-producing reaction when the luciferase reacts with the luciferin. The plates were tapped dry over paper towels before 25 µL lysis buffer (Promega, ADE3971) was added to the cell containing wells. Afterwards, plates were placed in the -80°C freezer (10 minutes) for flash freezing to ensure lysis of the cells.

Frozen plates were placed on the flat bed shaker to allow the plates to thaw. Luminescence was read in the dark because the luciferase assay reagent (LAR) (20 mM tricine (Sigma-Aldrich, T5816-25G), 2.67 mM $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich, 23,039-1), 33.3 mM DTT (Melford, D11000-5.0), 470 µM beetle luciferin (Promega, E160C), 270 µM CoA (Melford, MEL-C70800-0.1), 530 µM ATP (Melford, B3003), 0.1 mM EDTA (Sigma-Aldrich, 318884-500ML), and 1.07 mM $(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$ (Villeneuve et al, 1999)) is light sensitive. A multimode microplate reader (Berthold multimode microplate reader model Tristar LB941) was used to determine the luminescence expressed as relative light units (RLUs). The microplate reader automatically injected 100 µL LAR into each well. The luciferase enzyme produced by the cells cleaves the luciferin substrate in the LAR and light is emitted. The amount of light produced is directly proportional to the number of receptors activated or inhibited.

### 3.4.5 H4IIE-*luc* reporter gene assay details

The cells were exposed in triplicate to each concentration of the reference compound, water, and sediment extracts. On day two the cells received the reference compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Industrial Analytical, DRE-GA09011171TO) (0.0004, 0.0015, 0.0059, 0.0178, 0.053, and 0.16 ng/mL), sediment (0.08, 0.25, 0.74, 2.22, 6.67, and 20 g/mL) and water (0.01, 0.031, 0.093, 0.278, 0.833, 2.5 µL/mL) extracts. The rest of the assay was commenced as explained in section 3.4.4.

### 3.4.6 MDA-kb2 reporter gene assay details

The MDA-kb2 assay was commenced and only if light was emitted the assay was repeated with the AR blocked. The AR was blocked to determine whether light was emitted due to GR or AR activation. If light was still emitted with the AR blocked only then would it be possible to know that it was GR activation and if no light was emitted, the light produced in the previous assay was due to AR activation.

As a precautionary measure, 1% antibiotic-antimycotic was added to the stripped L-15 medium, because during the maintenance of the cells bacterial growth was noted in one of the plates. Even though that plate was discarded, it did share the same incubator, the decision was made to grow the cells on antibiotics. It would have placed too much stress on the cells to seed them without antibiotics because the hormones were already removed from the stripped medium. The cells were seeded and placed in the incubator. After 48 h the cells were exposed to 2.5 µL of the water (0.003, 0.01, 0.09, 0.28, 0.83, 2.5 µL/mL) and sediment (0.02, 0.06, 0.19, 0.56, 1.67, 5 g/mL) extracts in triplicate. A separate set of cells also received the AR receptor agonist (reference compound) testosterone (0.008, 0.047, 0.142, 0.283, 0.567, 0.85 ng/mL) (Sigma-Aldrich, 86500-1G) in triplicate.

For the assay to investigate GR activation on day one at the start of the assay, the cells were seeded using medium which contained a background of 555.6 ng/mL flutamide (Sigma-Aldrich, F9397-1G). The concentration flutamide was chosen based on the flutamide dose-response curve, where 80% of the AR would be blocked (Creusot et al., 2014). The rest of the seeding occurred the same as mentioned above (3.4.4.). After 48 h the cells were exposed to samples that had shown activation in the first activation assay, and the reference compound dexamethasone (0.9, 4.5, 22.5, 112.5, 562.5, 843.75 ng/mL) (Sigma-Aldrich, D4902-100MG).

The medium used for seeding the cells for the AR inhibition assays was commenced with a background of 0.283 ng/mL testosterone, resulting in 50% of the ARs being activated, and the rest of the seeding commenced as mentioned above (3.4.4.). The plates were incubated for 48 h before the cells were exposed to the samples and the AR antagonist (reference compound) flutamide (0.0206, 0.0617, 0.185, 0.556, 1.667, 5 µg/mL) and the

cells were exposed to the same water and sediment extract concentrations. In the presence of an inhibitor, the amount of light emitted by the cells will diminish.

### 3.4.7 MTT viability assay

The principle of this assay is based on the ability of viable cells to reduce the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution through mitochondrial dehydrogenase to form violet-blue formazan crystals (Mossmann, 1983).

This assay was performed concurrently with the reporter gene assays (3.4.5 & 3.4.6). The only difference was that the cells were seeded in transparent 96-well microplates with a flat transparent bottom (TPP, 92196) and the inner 60 wells including column 12 was seeded with cells on day one of the assay. This assay was performed to ensure that the cells were viable at the end of the exposure period and that the unknown environmental samples were not cytotoxic. In the case of no responses seen in the reporter gene assays, this assay will confirm that the absence of a response was not due to dead cells but because the extracts did not contain agonists or antagonists. The luminescence assay's results should be reconsidered when cytotoxicity was observed in the viability assay because the absence of light might be due to dying or dead cells, and not the lack of ligands present in the samples.

For the MTT viability assay, the cells were exposed to the samples on the same day as the reporter gene assays. At the end of the exposure time, the cells were inspected, and the confluency of each well was noted. The medium in column 12 was replaced with 200 µL MeOH to kill the cells and to create a negative control. After five minutes the medium in the rest of the plate was discarded and the plates were washed with DPBS three times. The plates were patted dry upside down on paper towels before 100 µL MTT solution (0.5 mg/mL tetrazolium salt thiazolyl blue (Sigma-Aldrich, M5655-1G) (prepared in the dark) was added to each well. The plates were incubated for two hours. The MTT solution was discarded, and each well received 200 µL dimethyl sulphoxide (Sigma-Aldrich, 34869-1L) to dissolve the violet-blue formazan crystals. After 30 minutes the absorbance was read at 560 nm with the microplate reader.

### 3.5 Oxidative stress

Two cell lines (HuTu 80 and H4IIE-*luc*) were used to test whether the water and sediment samples cause oxidative stress. The number of reactive oxygen species was investigated as well as testing for the first line of defence enzymes catalase, superoxide dismutase. Along with these the protein content was determined to express the results in terms of the amount of protein present per well as it is not possible to know the number of cells present in each well. Lastly, lipid peroxidation was also to be measured. The lipid peroxidation assays did not result in any useful data, but the method is still explained here because the assays were done.

#### 3.5.1 Reactive oxygen species

This assay was done using the method described in Wang and Joseph (1999) based on 2',7'-dichlorofluorescin being oxidized in the presence of reactive oxygen species to dichlorofluorescein which is highly fluorescent (Wang & Joseph, 1999).

HuTu 80 and H4IIE-*luc* cells were seeded on day one with 80 000 cells/mL in a 24 well plate (1 mL/well). Upon seeding the cells received an antibiotic antimycotic mixture (1%). The plate was placed in the incubator (5% $CO_2$, 37˚C) to allow for cell attachment to the bottom of each well. On day two of this assay, the plate was dosed with 10 µL of the water (0.28 µL/mL) and sediment (0.56 g/mL) extracts (n=3) before the plate was returned to the incubator. Six wells on each plate were not dosed and served as the untreated control wells.

After 24 h three of the control wells (on each plate) were dosed with 10 µL $H_2O_2$ (3.5 ng/mL) (Sigma-Aldrich, 7722-84-1), to act as a positive control because for the assay to be valid the mean relative fluorescent units (RFUs) of the cells that received $H_2O_2$ should be higher than the RFUs of the untreated cells. The plate was incubated for 45 min. The plates were washed with DPBS three times. The lights were switched off. In each well 200 µL 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) (Sigma-Aldrich, D6883-250MG) was added before the plates were incubated for 30 min. $H_2$DCFDA was removed and the plates were washed again with DPBS (three times).

The cells were trypsinised (150 µL trypsin/well) for 3 min and the cells were collected with 500 µL DPBS. The cell suspension of each well was transferred to a labelled 2 mL microcentrifuge tube (Lasec, P2TUB003C) and was centrifuged (room temperature, 1000 g, 4 min) before the supernatant was discarded. The pellet of cells was resuspended with 800 µL DPBS and 200 µL of this cell suspension was added in triplicate to a 96-well black flat bottom cell culture Corning®microplate (Sigma-Aldrich, 3924). Excitation of the cell contents was done at 480 nm and subsequent fluorescence was quantified at 535 nm (Wang & Joseph, 1999).

The raw data was analysed in Excel® where the mean, standard deviation and the % CV were calculated. The RFUs of each sample (treated) and the control (untreated) cells were compared and SPSS software was used to determine statistically and practically significant differences (Mann-Whitney U ($p<0.05$) and Cohen's d-value ($d\geq0.8$)). See section 3.9.

### 3.5.2 Superoxide dismutase content, catalase activity and protein determination

The method for SOD is based on inhibiting the pyrogallol autoxidation that is catalysed through superoxide radicals (Kim et al., 1995). The CAT activity assay measures the decomposition of hydrogen peroxide ($H_2O_2$) catalysed by the catalase enzyme (Cohen et al., 1970). The protein content was determined because it represents the number of cells present in each well. The determination was done based on the protein's ability to bind to the Coomassie blue dye which leads to a colour change from brown to blue. The binding to the dye caused an increase in absorption at 595 nm, which is measured (Bradford, 1976).

The plates were seeded and exposed in the same manner as described in section 3.5.1. Three control wells per assay plate only received cells with supplemented medium. Exposure duration was 24 h after which the medium was removed from all the wells and the cells were trypsinised (150 µL trypsin/well) for 3 minutes. The trypsin's activity was stopped by adding 500 µL DPBS. The cell suspension was transferred to 2 mL microcentrifuge tubes before centrifugation (room temperature, 1 000 g, 4 min). The supernatant was discarded carefully to avoid losing the cell pellet. The cell pellet was resuspended with 270 µL of ice-cold potassium phosphate buffer (ice-cold) (potassium

dihydrogen phosphate (Sigma-Aldrich, 7778-77-0) was used to adjust 0.09 M dipotassium hydrogen phosphate's (Sigma-Aldrich, 7758-11-4) pH to 7.4). Microcentrifuge tubes containing the cell suspension were sonicated (Scientech, Labotec, 704) (30 seconds, medium intensity) and centrifuged for 10 min at 4°C and 10 000 g. The supernatant was used for the following assays.

Superoxide dismutase: In a 96-well white-walled plate with a transparent bottom, 4 µL of the supernatant (3.5.2) was added in triplicate. Tris buffer (4 µL) (Sigma-Aldrich, 77-86-1) blanks were included before 245 µL diethylenetriaminepenta-acetic acid (DTPA) (Sigma-Aldrich, 67-43-6)/Tris buffer (1:49 mix, 1 nM DTPA mixed with 50 mM Tris buffer (pH 7.5). The mix was aerated for 20 minutes, and pH was adjusted to 8.2) was added to all the wells. In the dark, 4 µL pyrogallol (24 nM mixed with 10 mM hydrogen chloride) (Sigma-Aldrich, 87-66-1) was added to initiate the reaction (Del Maestro & McDonald 1987). The absorbance of the kinetic reaction was measured at 560 nm, every 30 seconds for 4.5 min on the microplate reader. The raw data for each well across the 4.5 minutes were analysed in Excel® where a single unit of SOD was expressed as the number of enzymes which inhibited the pyrogallol's autoxidation rate by 50%. The percentage of pyrogallol was determined by converting the rate in each well to a percentage. This value was divided by 50 because 50% pyrogallol inhibition is equal to one SOD unit. To express the data in terms of SOD activity in units the 50% pyrogallol values were multiplied by 125 (1 SOD unit = 125 ng/mL). The dilution factor was taken into consideration by multiplying by 4 (SOD activity in ng/mL) and the fraction pyrogallol present in each well was taken into account by multiplying by 100. Finally, the ng/mL SOD values were expressed in terms of the protein content (ngSOD/mgprotein) (Del Maestro & McDonald, 1987). The statistical significance was determined for the samples where the ngSOD/mgprotein of each sample was compared to the ngSOD/mgprotein of the untreated cells (the control). The IBM SPSS program was used to run the Mann Whitney U ($p<0.05$) and Kolmogorov-Smirnov Z statistical tests. The Kolmogorov-Smirnov Z test was used to determine whether the data was normally distributed, but since was not not distributed normally the non-parametric Mann-Whitney U test was used for the statistical analysis. Practical significance was determined with the use of the Cohen d test ($d\geq0.8$).

Catalase: Sample supernatants (prepared in section 3.5.2) were added 10 µL in triplicate to a 96-well white plate with a transparent bottom. The enzymatic reaction was initiated once 93 µL $H_2O_2$ (6 mM: 62 µL 30% $H_2O_2$ was added to 10 mL phosphate buffer) was added. The plates were incubated and 19 µL $H_2SO_4$ (6N: 1.68 mL $H_2SO_4$ stock solution were mixed with 8.32 mL deionised water) were added immediately after the 3 min to each well to stop the reaction. The lights were switched off. After 130 µL potassium permanganate (($K_2MnO_4$) (1.9 mM: 0.0316 g $K_2MnO_4$ was mixed with 100 mL deionised water) (Sigma-Aldrich, 7722-64-7) (Cohen et al., 1970) was added to each well the absorbance was measured immediately at 490 nm with a SpectraMax® iD3 (Molecular Devices®). The decomposition reaction between the remaining $H_2O_2$ and $K_2MnO_4$ causes a colour change. To calculate the decomposition of $H_2O_2$ through catalase the following equation was used:

$$K = \log\left(\frac{S0}{S3}\right) x \frac{2.3}{t}$$

where= K is the constant for the first kinetic reaction,
S0= initial substrate concentration and
S3 = substrate concentration at 3 minutes,
2.3 = a constant and used as the first-order kinetic factor and it is divided by the 3 minutes (t) time interval.

The K-value was then expressed in terms of the protein content, µmol$H_2O_2$/min/mg protein (Cohen et al., 1970). The CAT activity of the samples was compared to the CAT activity of the untreated cells to determine statistical significance with the Kolmogorov-Smirnov Z and Mann-Whitney U ($p<0.05$) test, using the SPSS program. As explained in section 3.5.2. the data was tested for normal distribution using the Kolmogorov-Smirnov Z, but the data was not distributed normally the Mann-Whitney U test was used instead for the statistical analysis. The Cohen d test was performed to determine the practical significance ($d\geq0.8$).

Protein content determination: The supernatant (5 µL) was added to the wells of a 96-well white-walled plate with a clear bottom and three 5 µL deionised water blanks were included. Bovine serum albumin (BSA) (Sigma-Aldrich, P0914-1AMP) (5 µL) (0, 62.5,

125, 250, 500, 1 000, 2 000 µg/mL) was used to prepare a protein calibration curve. The lights were switched off before 245 µL of Bradford's reagent (Sigma-Aldrich, B6916-500ML) was added and the absorbance (optical density) was read at 590 nm (Bradford, 1976). A calibration curve was plotted with the mean absorbance on the y-axis and the µg/mL BSA on the x-axis. The protein calibration curve is used to determine the slope and intercept. Protein concentration of all the cells was determined through using the equation: y=mx+c where the individual absorbance reading is y, m is the slope and c is the intercept. The equation is used to determine x in µg/mL and divided by 1000 to provide the x-value in mg/mL. The final x-value of each reading was used to normalise the SOD and CAT biomarkers as well as the lipid peroxidation and the acetylcholinesterase activity.

### 3.5.3 Lipid peroxidation (Malondialdehyde (MDA)) assay

The principle of the lipid peroxidation assay is based on MDA's (final product of lipid peroxidation) reactivity with thiobarbituric acid to produce a red product (Janero, 1990). This assay was seeded and exposed as described above (3.5.1). Untreated control wells were also included. Collecting the cells from each well was done in the same manner as mentioned in section 3.5.2 until after discarding the supernatant from the microcentrifuge tubes that were centrifuged. The pellets in the microcentrifuge tubes were resuspended with 200 µL Tris-HCl/sucrose buffer (25 mM Tris-HCl (Sigma-Aldrich, 1185-53-1) was mixed 1:1 with 250 nM sucrose (Sigma-Aldrich, 57-50-1)) and vortexed. Microcentrifuge tubes containing the cell suspensions were sonicated for 30 seconds at medium intensity. The tubes were centrifuged for 10 min (10 000 g at 4°C).

In 15 mL centrifuge tubes the following was added: 12.5 µL sample supernatant, 187.5 µL 20% glacial acetic acid ($CH_3COOH$, with pH of 3.5) (Merck Chemicals, 64-19-7), 187.5 µL 0.8% thiobarbituric acid (Sigma-Aldrich, 504-17-6), 25 µL sodium dodecyl sulphate (SDS, 8.1%) (Sigma-Aldrich, 151-21-3), and 87.5 µL deionised water (18.2 MΩ.cm) (Ohkawa et al., 1979). Malondialdehyde standard, (1,1,3,3-tetramethoxypropane (Sigma-Aldrich, 102-52-3)) (2, 3, 4, 5, 6, 7, and 8 nM) were included to create a calibration curve which was also added (12.5 µL) to their own 15 mL centrifuge tubes. The samples were placed in a water bath (95°C) for 30 minutes and afterwards, it was placed on ice for 10 minutes.

Deionised water (125 µL) and 625 µL n-butanol (Honeywell, 71 36 3) and pyridine (Sigma-Aldrich, 110 86 1) solution (15:1) were added (Ohkawa et al., 1979). The samples were vortexed before it was centrifuged for 10 minutes (2 700 g at room temperature). In a white 96-well transparent bottom plate, 150 µL of the lipid layer (top layer) of each sample was loaded in triplicate including the MDA standards and three wells of deionised blanks. The optical density was measured at 532 nm with a SpectraMax® iD3 (Jentzsch et al., 1995; Ohkawa et al., 1979).

A calibration curve was plotted with nM MDA standard on the x-axis and the mean absorbance on the individual MDA concentrations on the y-axis. The calibration curve was used to calculate the slope and intercept. Using the equation y=mx+c, where the individual absorbance values were y and with using the slope and intercept the x-values were calculated. The x-values were divided by the corresponding protein content x-values. Finally, the lipid peroxidation was expressed as mmol/mg protein. The Kolmogorov-Smirnov Z and Mann-Whitney U ($p<0.05$) tests were used for determining statistical significance on the SPSS program. The use of these two tests are explained in section 3.5.2 and due to the data from this test also not normally distributed the Mann-Whitney U test was used for the statistical analysis. The practical analysis was measured with the Cohen d test ($d\geq0.8$).

### 3.6 Acetylcholinesterase activity

For this AChE activity assay, the rate at which the yellow colour is produced, is measured. This is accomplished through measuring the colour response when thiocholine is continuously reacting with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) to produce 5-thio-2-nitro-benzoic acid (TNB) which is a yellow anion (Ellman et al., 1961). The colour response is measured with a spectrophotometer and the absorbance of TNB measured is directly proportional to the AChE activity (Zimmerman et al., 2008). This assay was seeded as described in 3.5.1. The rest of the assay commenced as mentioned in section 3.5.3.

After the microcentrifuge tubes were centrifuged for 10 min (10 000 g at 4°C) the lights were switched off because the Ellman's reagent and acetylthiocholine iodide are light sensitive. In the dark, ice-cold potassium phosphate buffer (210 µL), 10 µL 10 mM DTNB

(Sigma-Aldrich, D218200-1G) (Ellman's reagent), and 10 µL 30 mM acetylthiocholine iodide (Sigma-Aldrich, 01480-5G) were added to the wells of white 96-well transparent bottom plates (Ellman et al., 1961). The plates were tapped gently to mix the solutions in the wells before incubating the plates for five minutes at 37°C. The samples (10 µL) were added in triplicate in the dark. Absorbance was measured immediately at 412 nm every minute for six minutes, with the first reading starting at zero minutes (Ellman et al., 1961). This kinetic reaction's data was analysed in Excel® where the mean of every time interval for all the wells. The slope was calculated for each well across the 6 min time interval. Reaction rate was determined by dividing the slope by six. Finally, the AChE activity was expressed as absorbance/min/mg protein by dividing the reaction time by the corresponding protein content. Statistical significance was calculated using the tests Mann-Whitney U ($p<0.05$) and Kolmogorov-Smirnov Z in the SPSS program. As mentioned in section 3.5.2. the data was tested for normal distribution but since the data was not distributed normally the Mann-Whitney U test was used to test for statistical significance. The Cohen d test ($d\geq0.8$) was used to test for practical significance.

### 3.7 Acute toxicity - *In vivo* biological assays

These *in vivo* test kits were provided by MicroBioTests Inc, (Belgium). These tests were used to screen the samples for toxicity. Unfortunately, these assay test kits were only received in time for the second sampling session, thus the organisms were not exposed to the dry season samples.

#### 3.7.1 Thamnotoxkit F

The principle of this acute toxicity test is to measure the lethal effects caused by a water sample by exposing *Thamnocephalus platyurus* (beaver fairy shrimp) for 24 h to a water sample. This test is applicable for aqueous extracts, sewage or industrial effluents, freshwater, stable chemical substances, and the toxins of cyanobacteria (ISO 14380:2011).

Moderately hard synthetic water (standard freshwater) was used to hatch the cysts and to prepare the toxicant dilution. A volumetric flask (1 L) was filled with 800 mL 18.2 MΩ.cm deionised water before adding 96 mg/L $NaHCO_3$, 120 mg/L $CaSO_4.2H_2O$,

123 mg/L $MgSO_4.7H_2$ 4 mg/L KCl. The flask was filled with deionised water to make up 1 L. This standard was stored in the dark at 4°C. Prior to hatching the cysts, this standard freshwater was aerated for 15 minutes. The cysts were pre-hydrated by adding 1 mL diluted standard freshwater (2.5 mL standard solution mixed with 17.5 mL deionised water) to the vial containing the cysts. The vial was closed and shaken for 30 minutes. The mixture containing the hydrated cysts was transferred to a polystyrene Petri dish (5 cm in diameter) before the vial was rinsed with diluted standard freshwater to make sure all the cysts were in the Petri dish. The Petri dish was placed in a low temperature IncoCool Labotec incubator (model 356) (25°C for 22 h, continuous illumination (3 000–4 000 lux)).

Half an hour before exposure started, the water sample dilutions were added to the wells (1 mL from column 2–6) (Figure 8) from the lowest to the highest concentration (6.25, 12.5, 25, 50 and 100%). Column 1 was filled with 1 mL standard freshwater as well as row D. After the cysts were hatched the petri dish was collected and under a dissection microscope, the cysts were transferred to a 24 well plate. The larvae were transferred from the Petri dish to column 1 (row A–D) (± 50 larvae/well) of each 24 well plate (Figure 8). These wells were used to rinse the larvae before they were transferred to column 2–6 (10 larvae/well). The transfer took place in the sequence from $A_1$ to $A_2$, $A_3$ until $A_6$. The same sequence was followed for all the remaining rows (B-D). The plates were incubated for 24 h at 25°C in the dark.

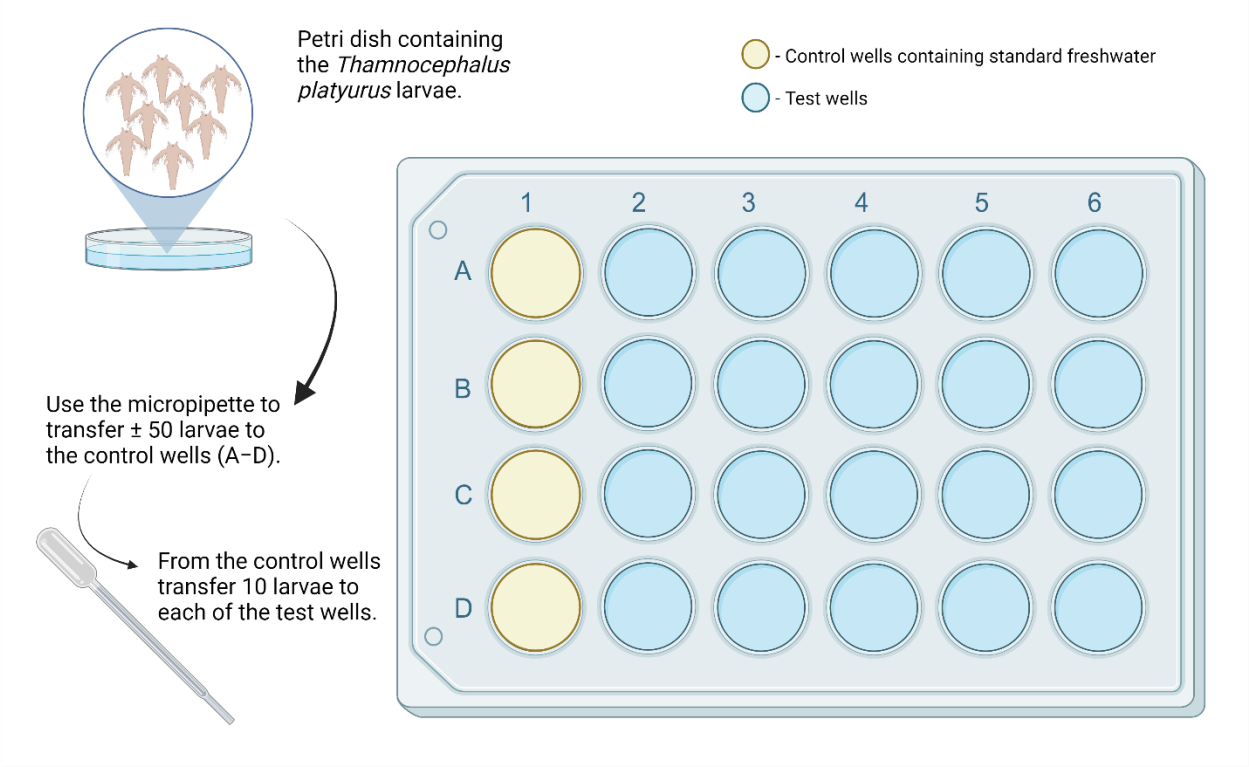


**Figure 8**: Illustration of Thamnotoxkit F plate layout and Thamnocephalus platyurus larvae distribution (created in BioRender).

The plates were collected from the incubator after exposure and the amount of dead *T. platyurus* per well was noted. The results were expressed in terms of the %mortality per concentration of each sample. For the validity of this test, it was important that the mortality in the control wells ($A_1$, $B_1$, $C_1$, $D_1$) should not exceed 10%.

These tests were gifted but there were limited supplies so there were not three replicates of a test but two.

### 3.7.2 Ostracodtoxkit F

An ostracodtoxkit F test kit was used to perform this *in vivo* direct contact toxicity test for freshwater sediment. The kit was provided by MicroBioTests Inc, the main laboratory is located in Belgium but there are suppliers worldwide.

This test is used to measure sublethal and lethal effects of contaminated sediment on ostracod crustacean *Heterocypris incongruens* to through exposure for six days

(ISO14371:2012). At the end of exposure period, the mortality and growth of the crustaceans are obtained and compared to the results from the crustaceans exposed to the reference sediment (ISO14371:2012).

Standard freshwater is also required in this test, and it was prepared the same as described in section 3.6.1. The freshwater standard was aerated for 15 minutes before 8 mL of the standard was placed in a Petri dish. A vial containing the *H. incongruens*' cysts was opened, and 1 mL standard freshwater was added to the vial. The vial was shaken to ensure a better hatching result. The vial's contents were emptied into the Petri dish and the vial was rinsed twice with 1 mL standard freshwater to secure a complete transfer of the cysts to the Petri dish. For the next 52 h the Petri dish was placed in a low temperature IncoCool Labotec incubator (model 356) (25°C, continuous illumination (3000–4000 lux)).

The hatching *H. incongruens* eggs were provided with food 48 h after hatching was initiated. The ostracodtoxkit F provided tubes filled with Spirulina powder, one of these tubes were filled with standard freshwater. A vortex mixer was used to obtain a homogenous suspension. This suspension was added to the hatching *H. incongruens* in the Petri dish for the remainder of the incubation time.

After 52 h of hatching has passed the length of ten ostracods was measured. A glass micropipette was used to transfer the ostracods into one of the thin-bottom multiwells. Lugol fixative solution (one drop) was added to the well to immobilise the ostracods. A micrometer slip was placed on a microscope slide and set to the stage of a dissection stereomicroscope (Nikon, SMZ445). The ostracods were placed one at a time on the slip to measure the length. The micrometer slip has intervals of 50 µm and the average length of a freshly hatched ostracod range between 150–250 µm. To ensure the ostracods have sufficient food supply for the duration of the exposure, an algal food suspension was prepared. The storage medium from one of the vials containing algal beads was removed before adding 7 mL matrix dissolving medium. A vortex mixer was used to shake the contents until the matrix surrounding the algae has dissolved and released the microalgae free. The vial was centrifuged (10 min, 1400 *g*) before discarding the supernatant. Deionised water (18.2 MΩ.cm) (10 mL) was added to the vial and the algae were re-suspended using a vortex mixer. Centrifugation followed (10 min, 1400 *g*) and the

supernatant was discarded before 10 mL of the standard freshwater was added to re-suspend the algae. The algal suspension was poured into a 25 mL volumetric flask and topped to the 25 mL mark with standard freshwater.

Standard freshwater (2 mL) (Figure 9) was added to each well of the six-well test plates before adding 1 000 µL of sediment to each well. One 6-well plate received reference sediment while the remaining five 6-well plates received the sampled sediment. The test plates were gently shaken to ensure the even distribution of the sediment in every well. The volumetric flask containing the algal suspension was shaken to ensure a homogenous algal suspension before adding 2 mL of the suspension to each well. Standard freshwater (5 mL) was added to the lid of the hatching Petri dish and under the dissection microscope, the ostracod neonates were transferred to the lid using a glass micropipette. Ten ostracods were transferred to each well of the six test plates (Figure 9). The plates were incubated for six days at 25°C in total darkness.

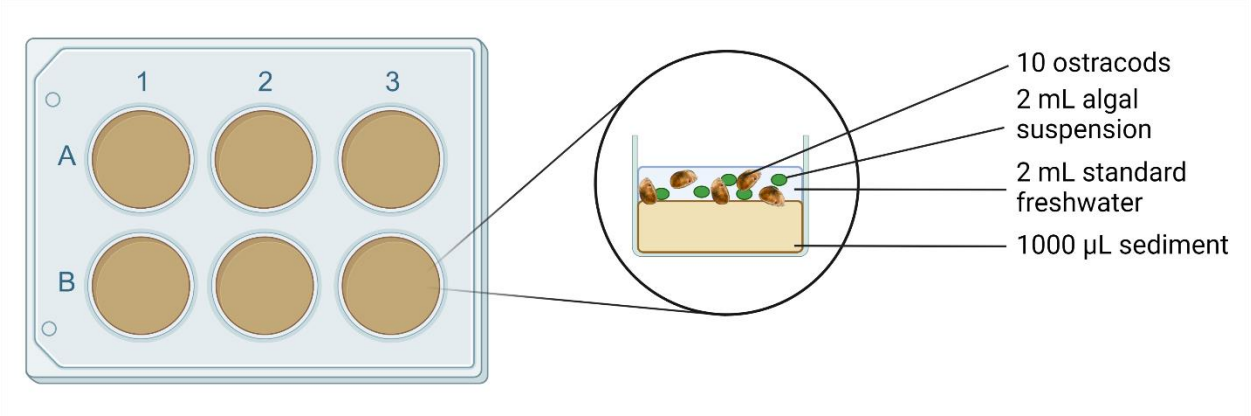


**Figure 9:** Illustration of the contents to be added to each well of the test plates (created in BioRender).

The surviving ostracods were recovered, and their lengths were measured after six days of exposure. A glass micropipette was used to recover the ostracods directly from each well containing the reference sediment. The ostracods were transferred to a well of the length measurement multiwell plate, which came with the kit. Ostracods recovered from the same exposure were kept together and placed in the same well of the length measurement multiwell plate. A drop of Lugol fixative was added to every well containing recovered ostracods. The number of living ostracods in each well was noted.

Growth inhibition was determined by comparing the length of the surviving ostracods exposed to the sampled sediment to the surviving ostracods exposed to the reference sediment. This sub-lethal effect was only determined for sampled sediment that caused mortality of less than 30%. As a precaution, the length of all the surviving ostracods was measured and only the data of those with a mortality of less than 30% was used. The procedure used to measure the length of the surviving ostracods was the same as described above.

### 3.8 Instrumental chemical analysis

The chemical analysis was performed at the University of South Africa at their Institute of Nanotechnology and Water Sustainability under the supervision of professor Hlengilizwe Nyoni. The analysis was performed as follows:

Water samples were extracted as described in section 3.3.2. The Dionex Ultimate 3000 Ultra-High-Performance Liquid Chromatography (UHPLC) system was used to separate the analytes. The system was equipped with an ACQUITY UPLC BEH C18 column (100 mm x 2.1 mm, 1.7 µm) (Waters®, 186002352). The column's temperature was maintained at 35°C and the volume of samples injected was 5 µL. Water and methanol both with 0.1% formic acid was respectively mobile phase A and B. The optimised chromatographic method (reverse phase) was as follows: initial mobile phase composition (98% A, 2% B) was kept constant for one minute which was followed by a linear gradient increase from 2% B to 100% B for nine minutes. The mobile phase was kept at 100% B for two minutes before dropping back to 2% B in 12.1 minutes and kept constant at 2% B for two minutes. The flow rate was 0.3 mL/min and the total run time was 14 minutes. The UHPLC system was connected to an Ultra-High resolution quadrupole time-of-flight mass spectrometer Impact II Bruker (Bruker Daltonics) which operated in positive ion mode and equipped with electrospray ionisation. The LC/MS accurate mass spectra were recorded ranging from 50–1600 m/z while the accurate mass measurements were obtained by means of a sodium formate calibrant solution (Sigma-Aldrich, 141–53–7) which was delivered by a KD Scientific external syringe pump. Bruker Compass DataAnalysis 4.3 software was used to process the recorded data.

### 3.9 Data and statistical analysis

The reporter gene assays: The raw data was processed in Microsoft Excel®, where the mean, standard deviation, and coefficient of variation (CV) were calculated. For quality assurance, it was important that the CV had to be less than 20%. The samples' RLUs were expressed in terms of the maximum light emitted by the reference compound. This was referred to as the %TCDDmax, %testosteronemax, %flutamidemax or %dexamethasonemax for the different reporter gene assays. %TCDDmax, %testosteronemax and %dexamethasonemax refer to the maximum amount of light emitted by the reference compound, whereas %flutamidemax refer toe the maximum inhibition/decrease of light. The dose-response curves were created for the reference compound as well as the samples by plotting the %TCDDmax, %testosteronemax, %flutamidemax or %dexamethasonemax on the y-axis and the logarithm of the reference compound and samples' concentrations, on the x-axis. The effective concentration (EC) was calculated for both the reference compounds and the samples. Relative potency (REP) of each sample was determined by using the EC values: the ratio of $EC_{20\text{-}80}$ of each sample was divided by the ratio of $EC_{20\text{-}80}$ of the reference compound (Villeneuve et al, 1999).

Data from the inhibition assay was reported as suppression ratio at 20% ($SR_{0.2}$) because most of the samples did not cause a full dose response curve (Escher et al., 2021). Also, at 20% the variability is often larger than when compared to the agonist mode while 10% is frequently not above the variability of the reference compound (Escher et al., 2021). Because the water and sediment samples in this study were extracted to concentrate the target compounds (enrichment) in the case of the water samples, or to get the target compounds into solution, in the case of the sediment, the relative extraction factor (REF) was calculated for plotting on the x-axis and represent the 'concentration' of the exposure.

$$REF = \frac{mass\ or\ volume\ extracted}{final\ volume\ in\ bioassay}$$

$$= EFxDF$$

$$EF = \frac{mass\ or\ volume\ extracted\ (kg\ or\ L)}{final\ volume\ of\ extract\ (L)}$$

$$DF = \frac{volume\ of\ extract\ dosed\ (L)}{final\ volume\ in\ bioassay\ (L)}$$

Where:

REF = relative extraction factor

EF = extraction factor

DF = dosing factor

The suppression ratio which belongs on the y-axis was calculated as follows:

$$SR = 1 - \frac{activation\ of\ sample}{activation\ of\ agonist}$$

Where:

SR = suppression ratio

Activation of sample = RLU elicited by sample

Activation of agonist = RLU elicited by solvent control wells in plate that received agonist to elicit a background signal that can be reduced in the presence of an antagonist (Escher et al., 2021)

The SR was used to determine whether the signal of the sample suppressed the signal of the agonist. To calculate the linear regression of $SR_{0.2}$ through the zero point the following equation was used $EC_{SR0.2}$=0.2/Slope-intercept.

The software, IBM Statistical Package for Social Sciences (SPSS) (version 27) was used to investigate statistically significant differences. Due to the unequal variance, sample size, and the not normal distribution of the data a non-parametric Mann Whitney U test was performed. Statistical significance was set at $p<0.05$ for the Mann Whitney U test, where the samples were compared to the control cells to determine the statistical significance between the samples and the reference compounds used. Cohen's d test was performed to determine the practical significance using the following equation:

$$d = \frac{|\bar{x}_E - \bar{x}_K|}{S_K}$$

where: $\bar{x}_E$ = experimental mean

$\bar{x}_K$ = control mean

$S_K$ = standard deviation of the control group.

The sign of the difference between the experimental and control mean was not taken into consideration. Practical significance is when the difference is large enough to cause an effect in practice (Ellis & Steyn, 2003). With practical significance the size of the effect is independent of the sample size. A value of d≥0.8 is considered to be practically significant.

The MTT viability assay: The mean, standard deviation, and coefficient variance (%CV) for the absorbance of each sample, solvent control (SC), and the MeOH killed cells were calculated in Excel®. The mean of the MeOH killed cells was subtracted from the individual readings of the samples' responses. The percentage viability was calculated by expressing the samples' responses in terms of the solvent control which represented 100% viable cells. The mean values and standard deviation were plotted on a graph. The statistical significance of each sample compared to the SC was determined with the non-parametric Mann-Whitney U test with the SPSS software.

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# CHAPTER 4 RESULTS

Science is a gift from God.

He gave it to us so we can know Him more.

– Joe Francis

This study investigated whether biological analyses could be used to determine the quality of environmental and drinking water by using biological assays based on modes of action or endpoints. The *in vitro* biological assays to investigate endpoints for endocrine disruption used in this study were activation and inhibition of AR, GR, and AhR, while the oxidative stress biomarkers such as ROS, SOD, CAT, and MDA were investigated. The ability of the water and sediment extracts to inhibit the activity of non-neuronal acetylcholinesterase in liver and duodenum cells was determined too. The *in vitro* assessment of the water quality was lastly supplemented using *in vivo* tests with the endpoints of mortality and growth inhibition.

## 4.1 Effects of xenobiotics on cell metabolism

### 4.1.1 H4IIE-*luc* reporter gene assay (Aryl-hydrocarbon receptor activation)

Sediment from both sampling sessions was extracted using the accelerated solvent extraction method and as mentioned in section 3.3.2.2 the elemental sulphur was removed from the sediment extracts. Only sediment extracts were tested in this assay because the dioxin-like compounds, compounds of interest, tend to accumulate in the sediment. Most of the sediment extracts did not elicit a quantifiable response except for two that were sampled in the wet season (Figure 10) (supplementary table 1): Honeymoon Creek and Paul Kruger Bridge which elicited $BEQ_{20}$ values of $1.04x10^{-7}$ and $3.45x10^{-7}$ ng TCDD equivalents (eq)/g sediment respectively. The sediment samples were also weakly to not cytotoxic (Figure 11) when applying ISO 10993-5 for assessing viability. The grey lines in Figure 11 indicate the different levels of cytotoxicity (>80%: non-cytotoxic; 60–80%: weakly cytotoxic: 40–60%: moderately cytotoxic; and <40%: strongly cytotoxic). The only sample reaching cytotoxicity was the 2.2 g/mL dilution of the Paul Kruger Bridge from the wet season which is an anomaly because the more concentrated dilution was not cytotoxic (Figure 11).

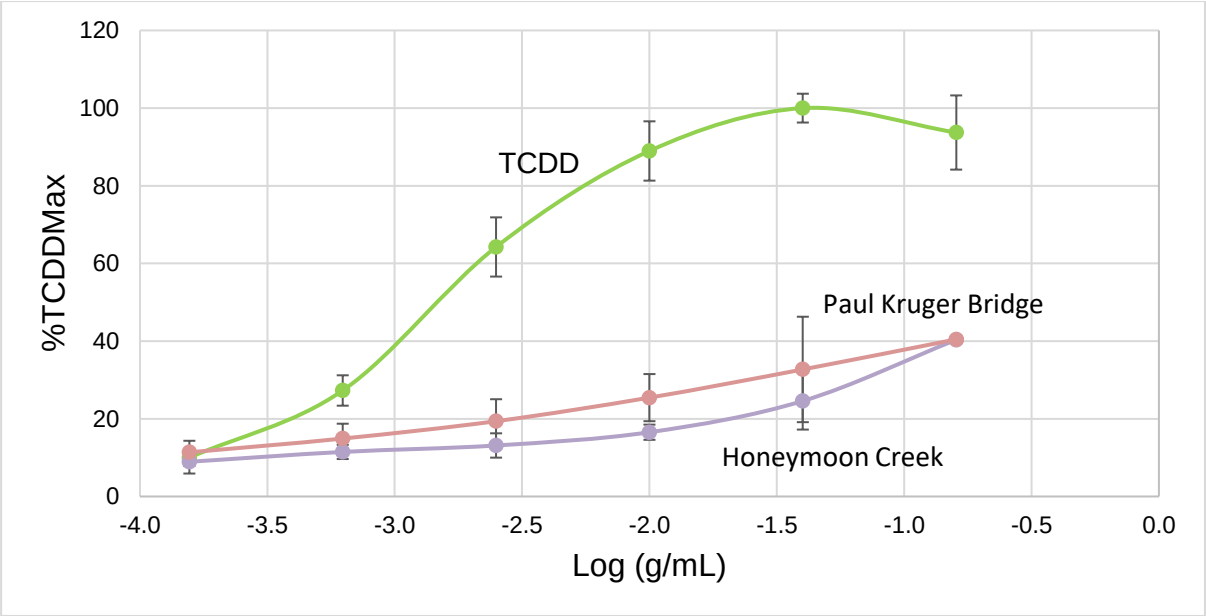



**Figure 10:** Aryl-hydrocarbon receptor activation responses of the reference compound, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and wet season sediment samples from Honeymoon Creek and Paul Kruger Bridge. The error bars indicate the standard deviation.

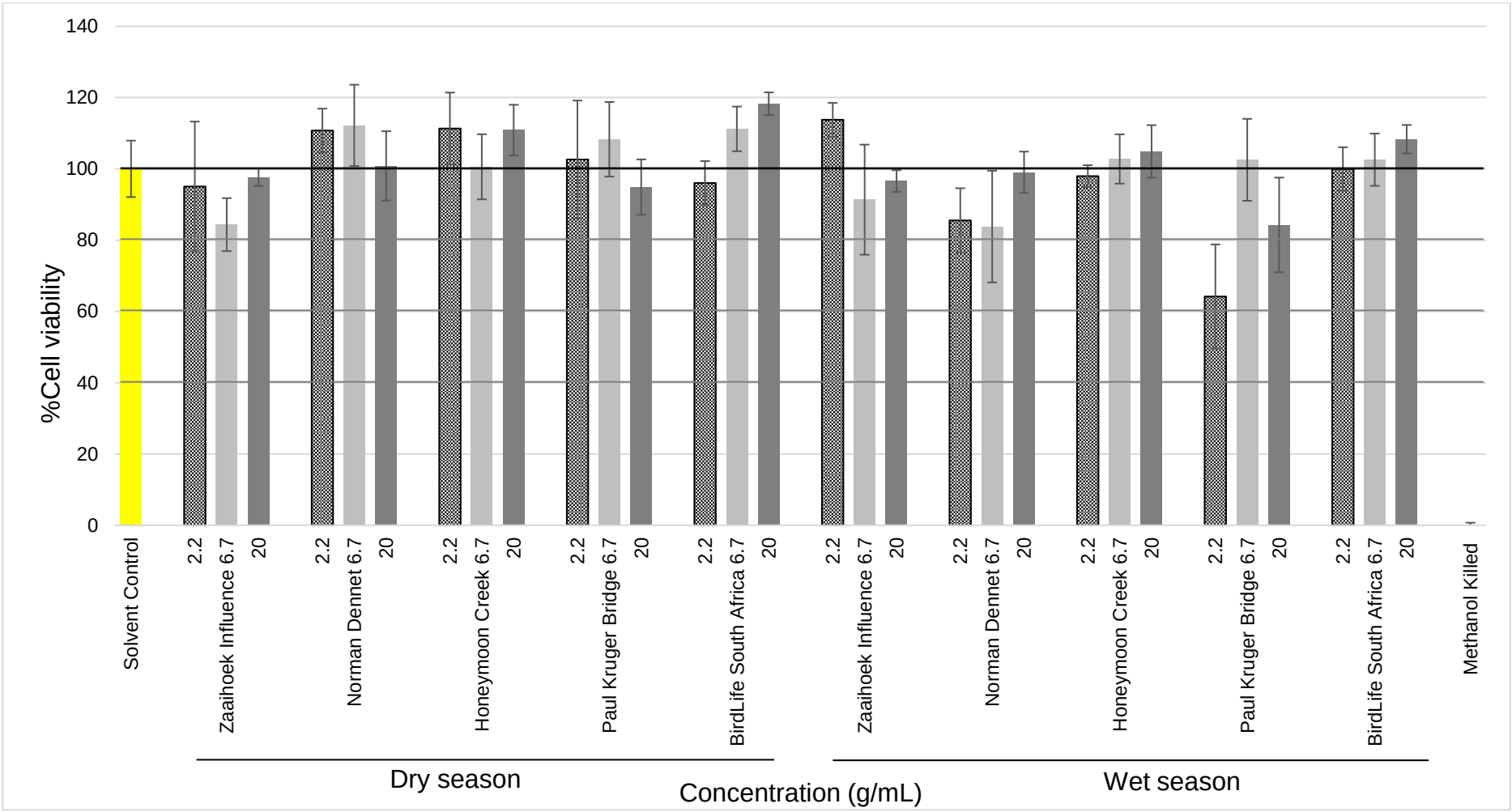



**Figure 11:** Percentage viability of the H4IIE-*luc* cells in response to the three highest concentrations of sediment sample extracts. Both the dry (September 2021) and wet (February 2022) seasons are presented. The black horizontal line indicates the absorbance of the control throughout the entire graph. Grey lines indicate cytotoxicity (>80%: non-cytotoxic; 60–80%: weakly cytotoxic: 40–60%: moderately cytotoxic; and <40%: strongly cytotoxic).

## 4.2 Endocrine disruption

### 4.2.1 MDA-kb2 reporter gene assay (androgen and glucocorticoid receptor activity)

Water and sediment samples were extracted to investigate potential androgen and glucocorticoid receptor (respectively AR and GR) activation as well as AR inhibition. The polar and non-polar compounds were extracted from the water using SPE and ultrasonication was used on the sediment (section 3.3.3.1). Evidence that cells were operational and the assays therefore functional, is seen in the dose-response graphs of the reference compounds: testosterone (AR agonist) (Figure 12), flutamide (AR antagonist) (Figure 13), and dexamethasone (GR agonist) (Figure 14).

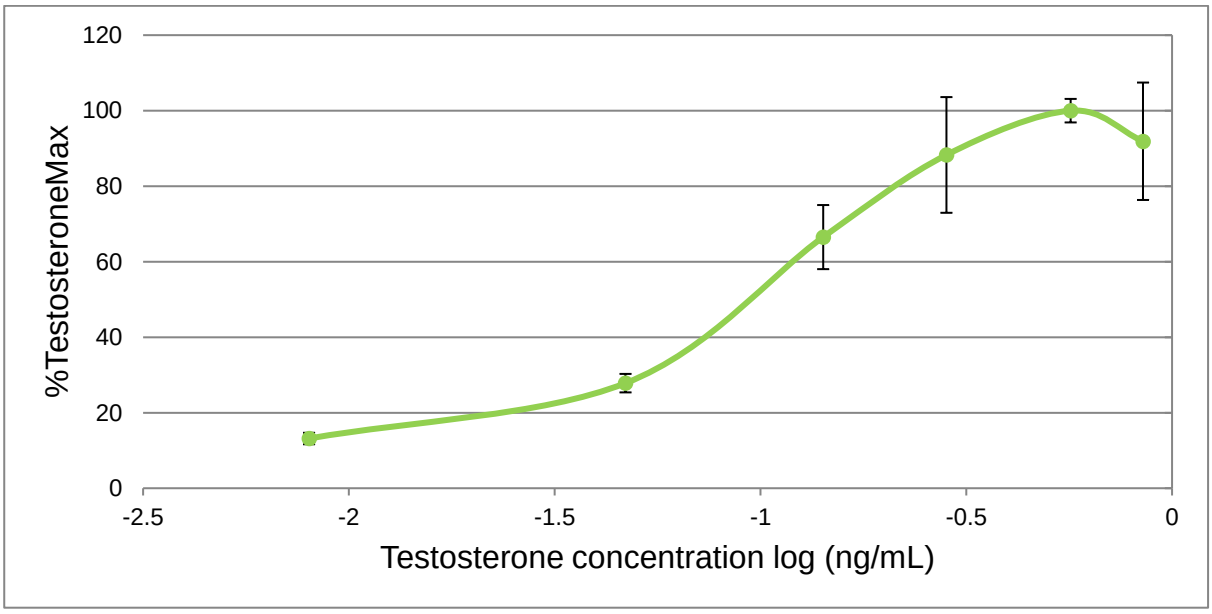



**Figure 12:** Dose-response curve of testosterone as the androgen agonist reference compound used in this study. The error bars indicate the standard deviation.

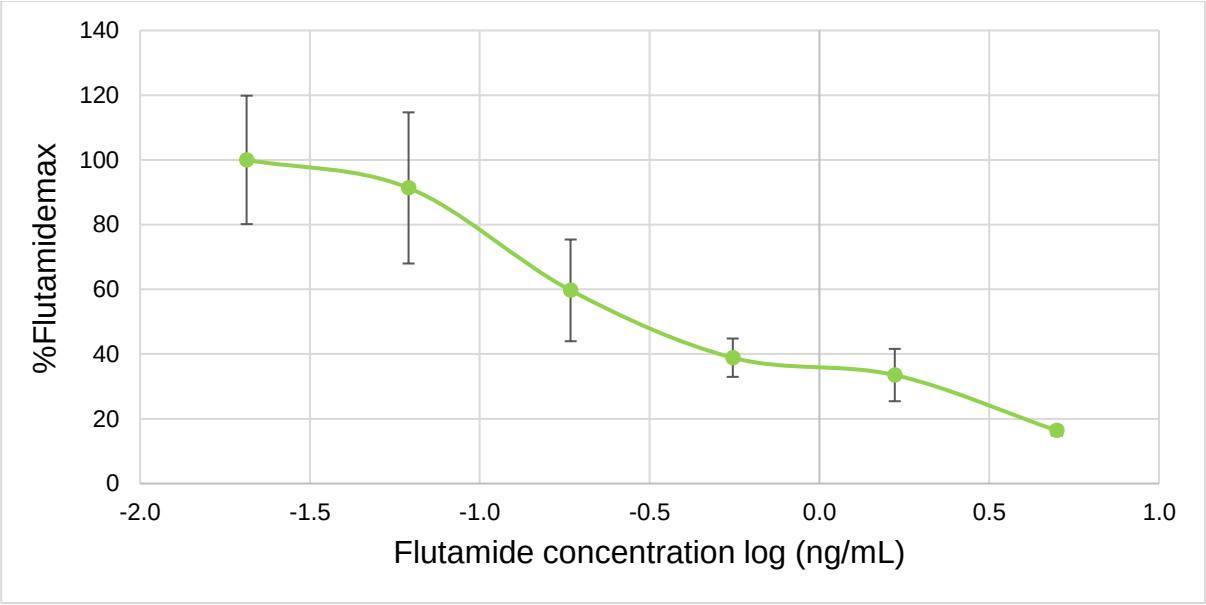



**Figure 13:** Flutamide dose-response curve. Flutamide was the AR antagonist reference compound used in this study. The error bars indicate the standard deviation.

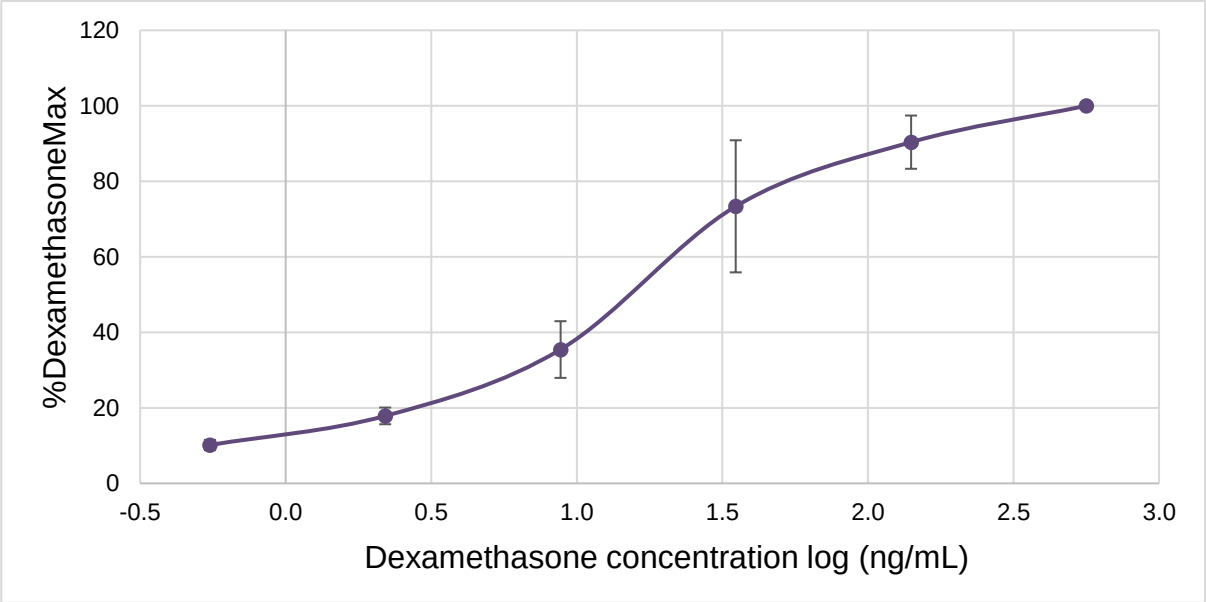



**Figure 14:** Dexamethasone dose-response curve. Dexamethasone was the glucocorticoid receptor agonist used in this study. The error bars indicate the standard deviation.

There was no GR activation in any of the samples tested possible explanations are discussed in chapter 5.

The only sample that elicited the AR agonist effect, was the WWTP effluent sampled in the dry season (Figure 15) (supplementary table 2). This response was equal to that

elicited by the testosterone reference and $REP_{20–80}$ could be calculated: 0.12, 0. 17, and 0.23 ng testosterone eq/µL since a complete dose-response curve was possible. Relative luminescence units expressed in terms of the %TestosteroneMax were statistically significant (Mann-Whitney (MW), $p<0.05$) for the three highest concentrations when compared to the solvent control (supplementary table 3). The highest concentration was weak to non-cytotoxic (60–80%) according to the ISO standard for viability guideline (ISO 10993-5, 2009), but after the first dilution the concentrations were not cytotoxic anymore thus the reaction can be considered as true (Figure 16).

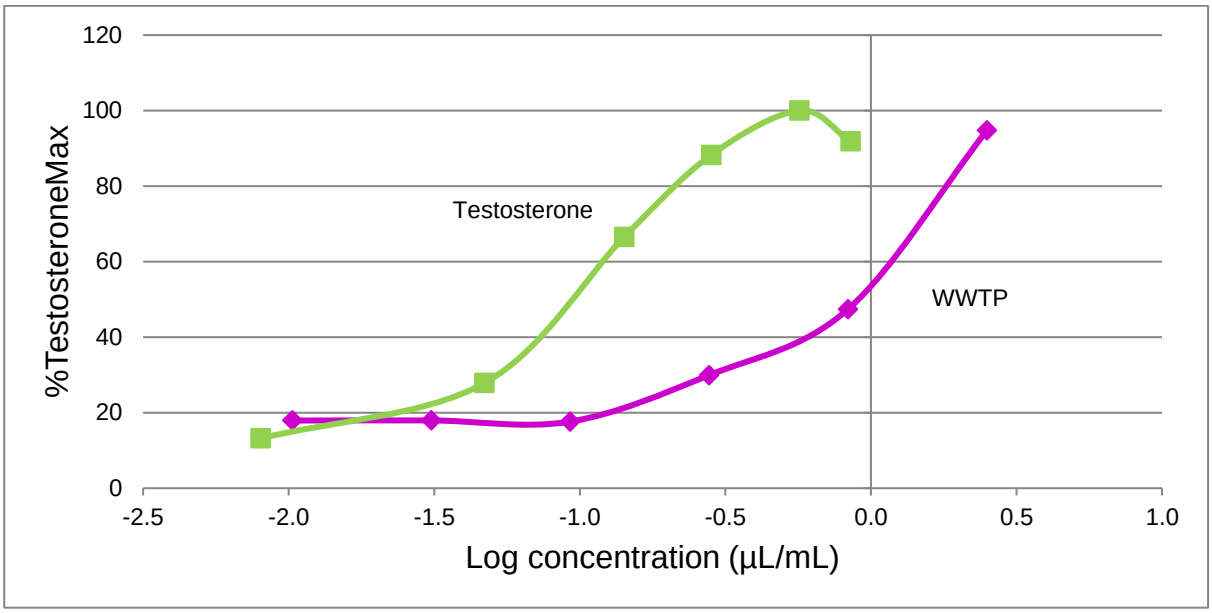



**Figure 15:** Androgen receptor activation caused by the water sampled at the WWTP's outlet (effluent), sampled during the dry season. The error bars indicate the standard deviation.

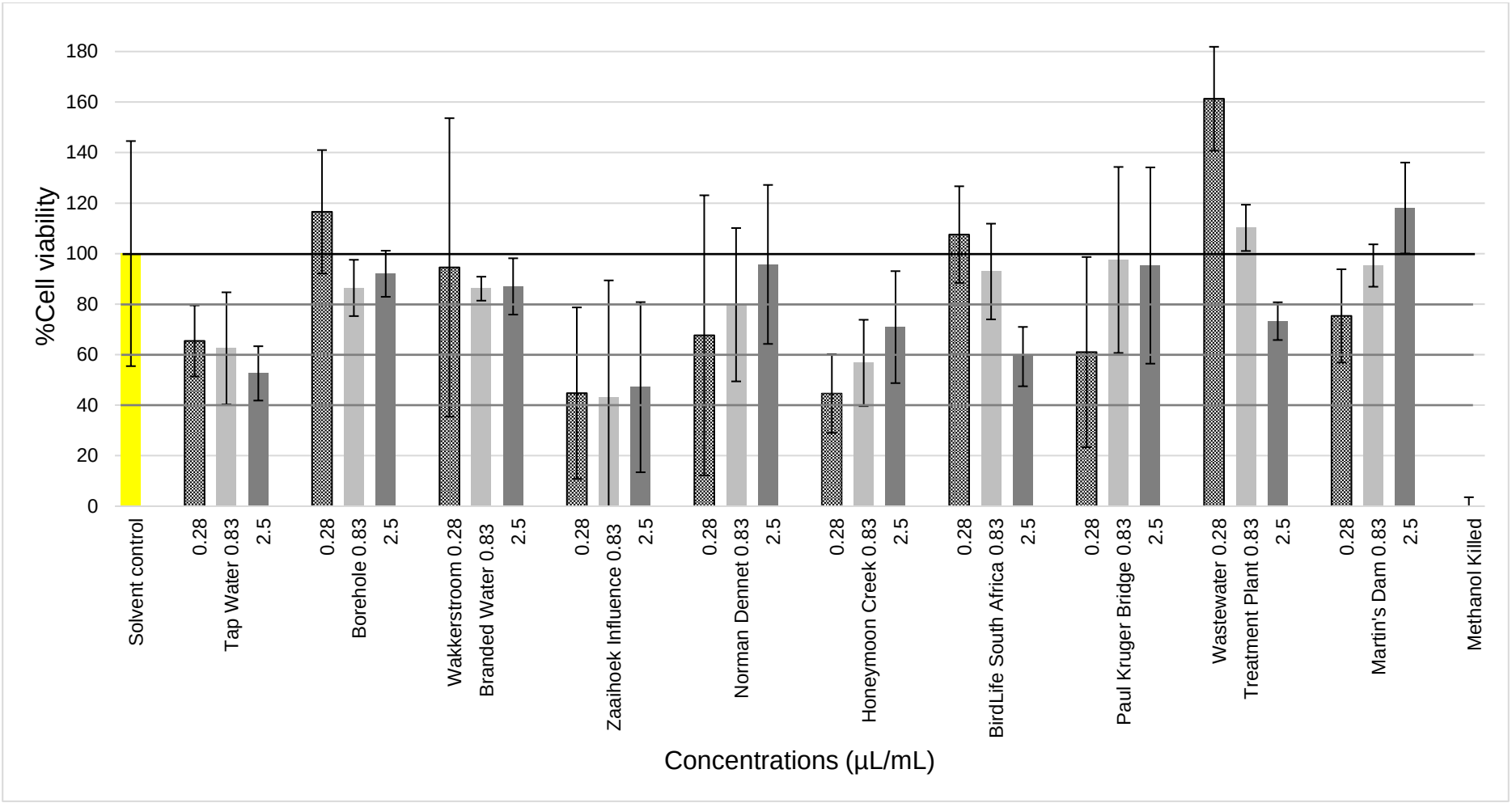
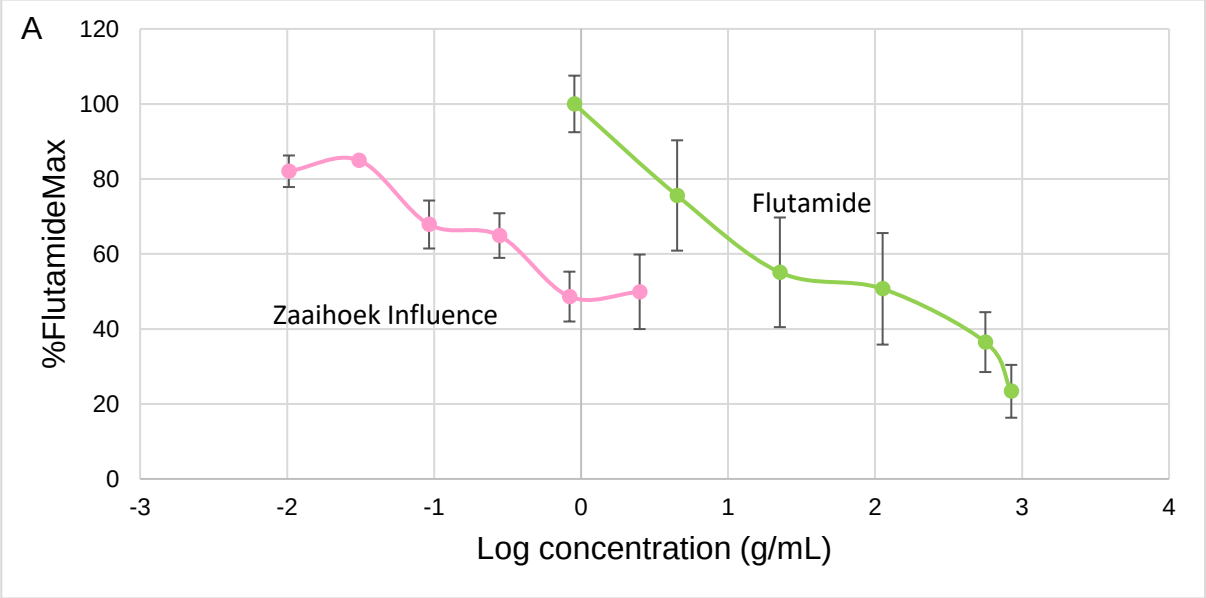



**Figure 16:** Viability results for MDA-kb2 cells exposed to the dry season water samples. The black horizontal line indicates the absorbance of the control throughout the entire graph. Grey lines indicate cytotoxicity (>80%: non-cytotoxic; 60–80%: weakly cytotoxic: 40–60%: moderately cytotoxic; and <40%: strongly cytotoxic).

Only two sediment samples collected during the dry season caused AR inhibition: Zaaihoek Influence (Figure 17A) and Norman Dennet (Figure 18A) (supplementary table 4). The effect concentration suppression ratio at 20% ($ECSR_{0.2}$) for the sample from the Zaaihoek Dam influence, was 0.0012 g/L (Figure 17B). Zaaihoek Influence's highest concentration was moderately to weakly cytotoxic (Figure 19) (ISO 10993-5, 2009), but after dilution, the sample was not cytotoxic anymore (Figure 19). For the AR inhibition responses, the second highest concentration was statistically and practically significant for the amount AR inhibited (supplementary table 5) while the third and last concentration was only practically significant when compared to the solvent control.

I calculated an $ECSR_{0.2}$ (0.0088 g/L) (Figure 18B) for Norman Dennet's but the highest concentration was weakly to moderately (60% and higher) cytotoxic in comparison to the solvent control (ISO 10993-5, 2009) (Figure 19). Due to the evidence of cytotoxicity, it is possible that this antagonistic effect was attributed to cytotoxicity. Also, for the AR inhibition responses, none of this site's concentrations were significantly different compared to the solvent control (MW, $p>0.05$) (supplementary table 5).



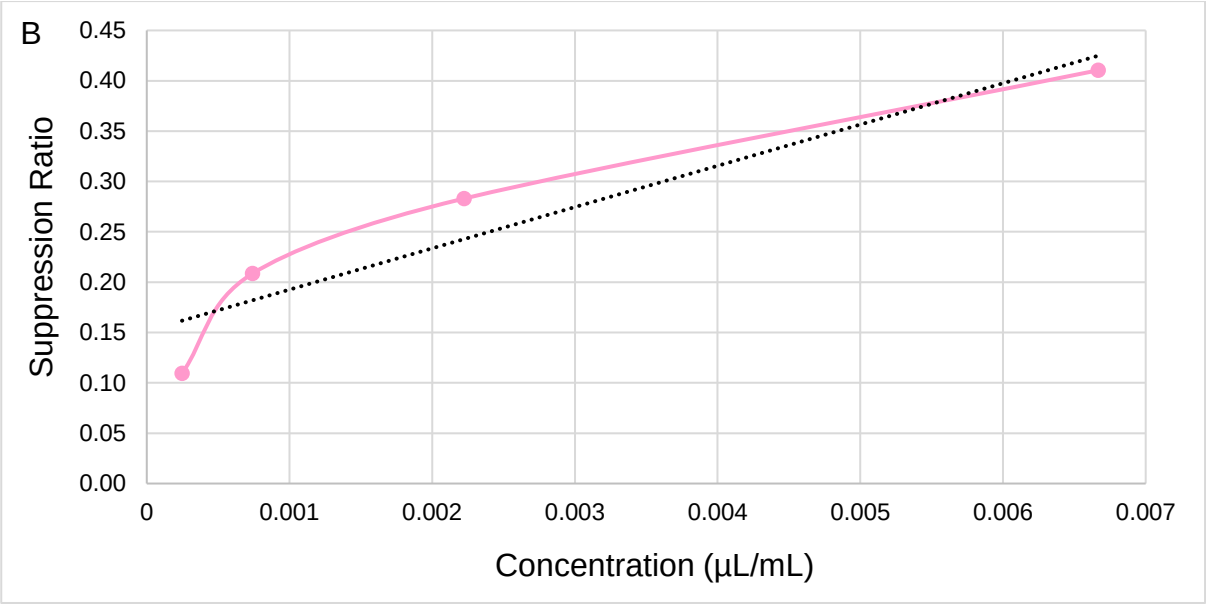
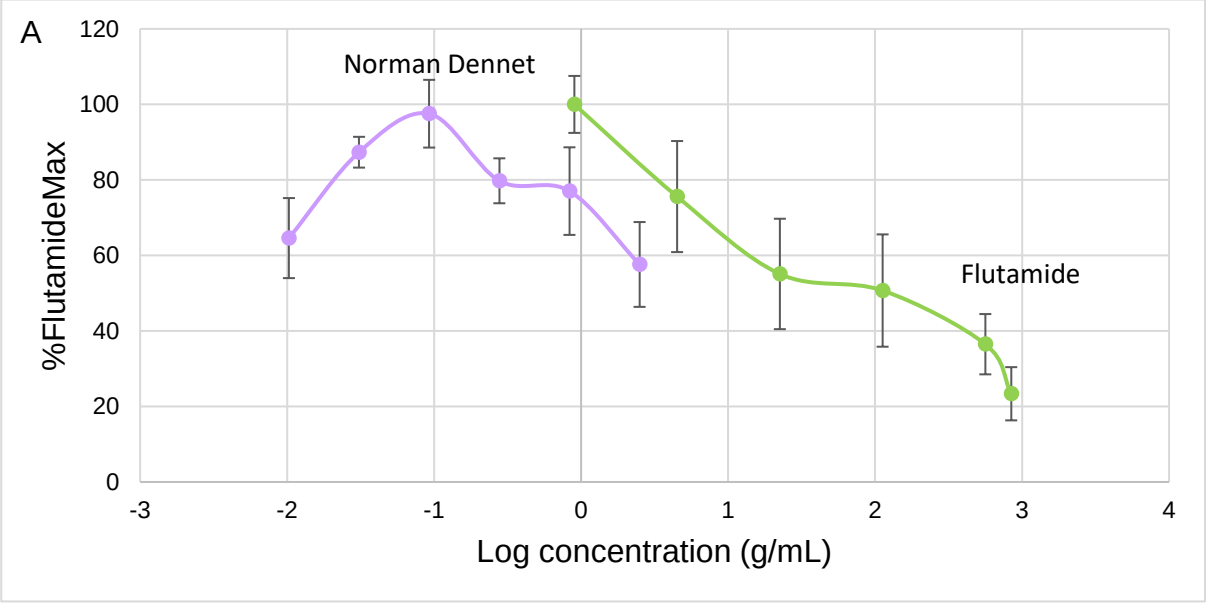



**Figure 17:** A – Zaaihoek Influence Sediment caused AR inhibition, dry season sample. Flutamide is the AR antagonist reference compound used in this study. The error bars indicate the standard deviation. B – Derivation of the concentrations causing suppression ratio of 20%.



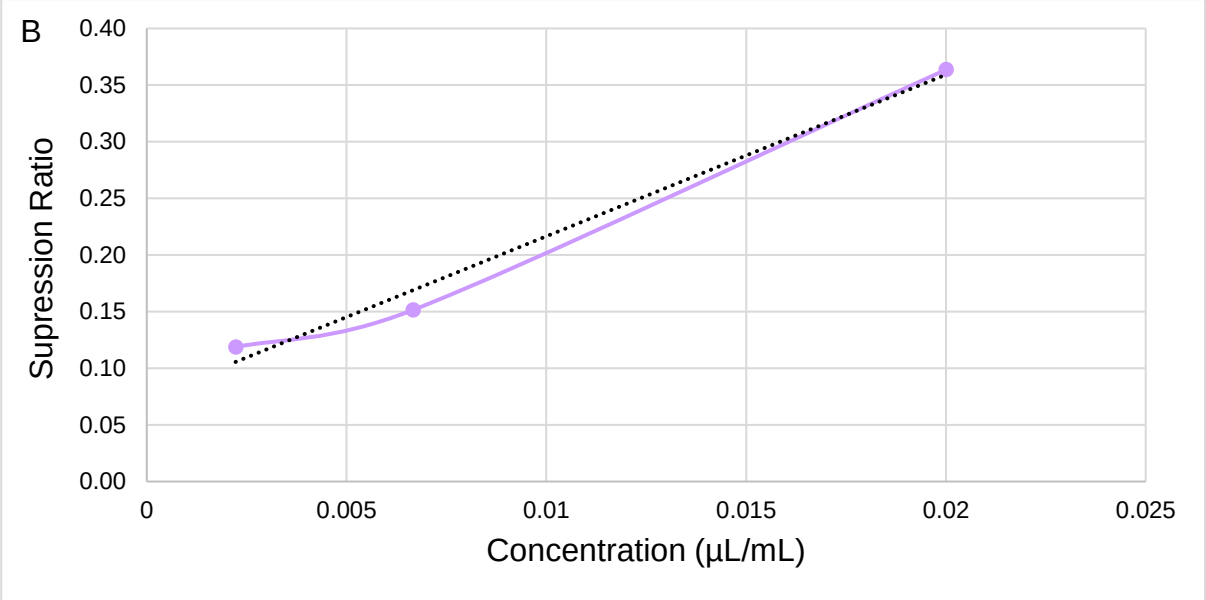



**Figure 18:** A – Norman Dennet Sediment sampled in the dry season caused AR inhibition. Flutamide is the AR antagonist reference compound used in this study. The error bars indicate the standard deviation. B – Derivation of the concentrations causing a suppression ratio of 20%.

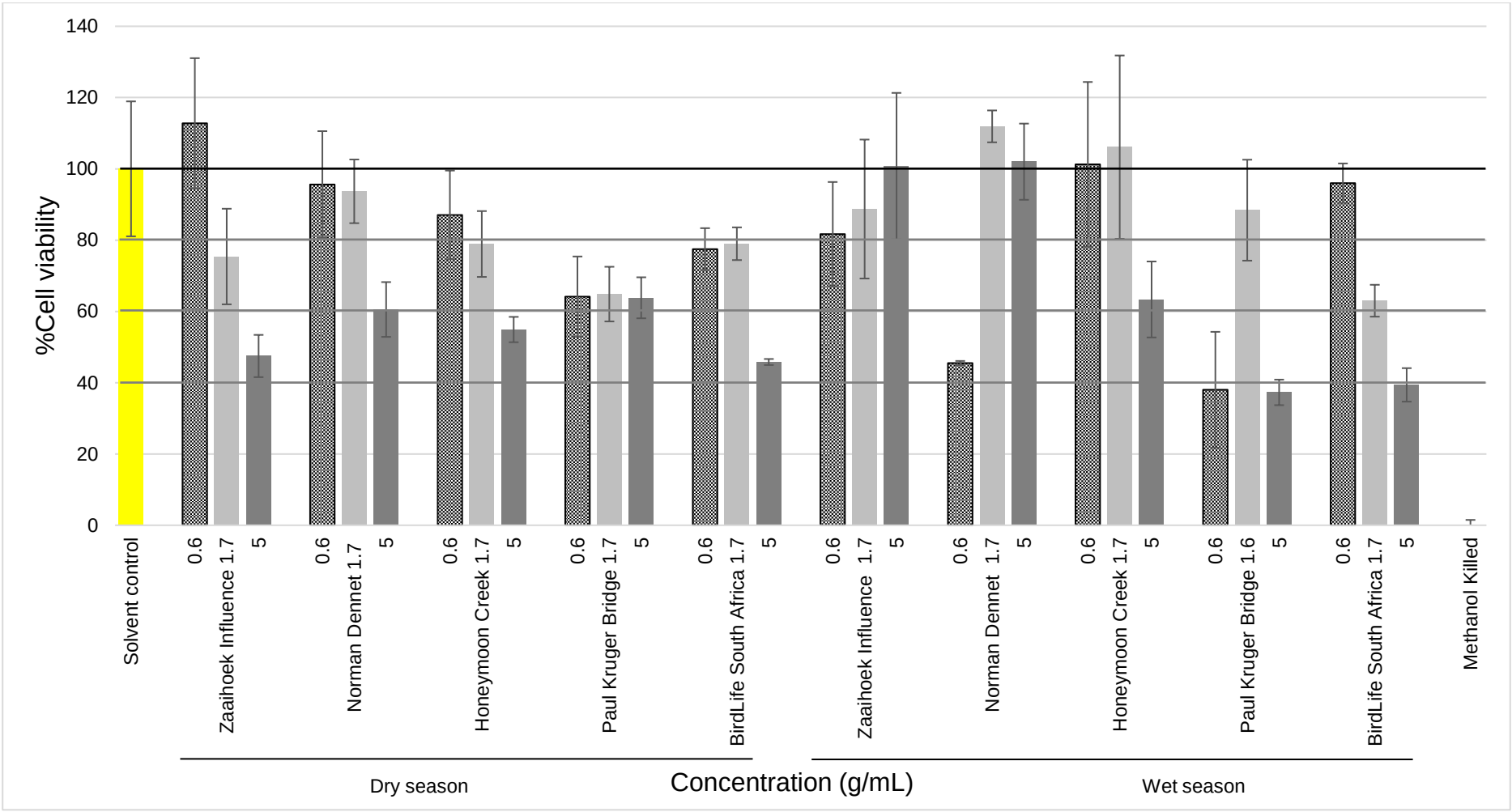



**Figure 19:** Cell viability assay results for sediment extracts given to the MDA-kb2 cell line (dry and wet season). The black horizontal line indicates the absorbance of the control throughout the entire graph. Grey lines indicate cytotoxicity (>80%: non-cytotoxic, 60–80%: weakly cytotoxic, 40–60%: moderately cytotoxic; and <40%: strongly cytotoxic).

Water samples from two sites collected at the end of the wet season were the only sites to show AR inhibition. Water collected at Honeymoon Creek had an $ECSR_{0.2}$ of 0.004 µL/L (Figure 20) and the viability assay showed that the highest concentration was not cytotoxic (Figure 21) but the other two concentrations tested were weakly cytotoxic (ISO 10993-5, 2009). Water from Honeymoon Creek might not actually have caused AR inhibition and the results seen in the reporter gene assay was due to cytotoxicity.

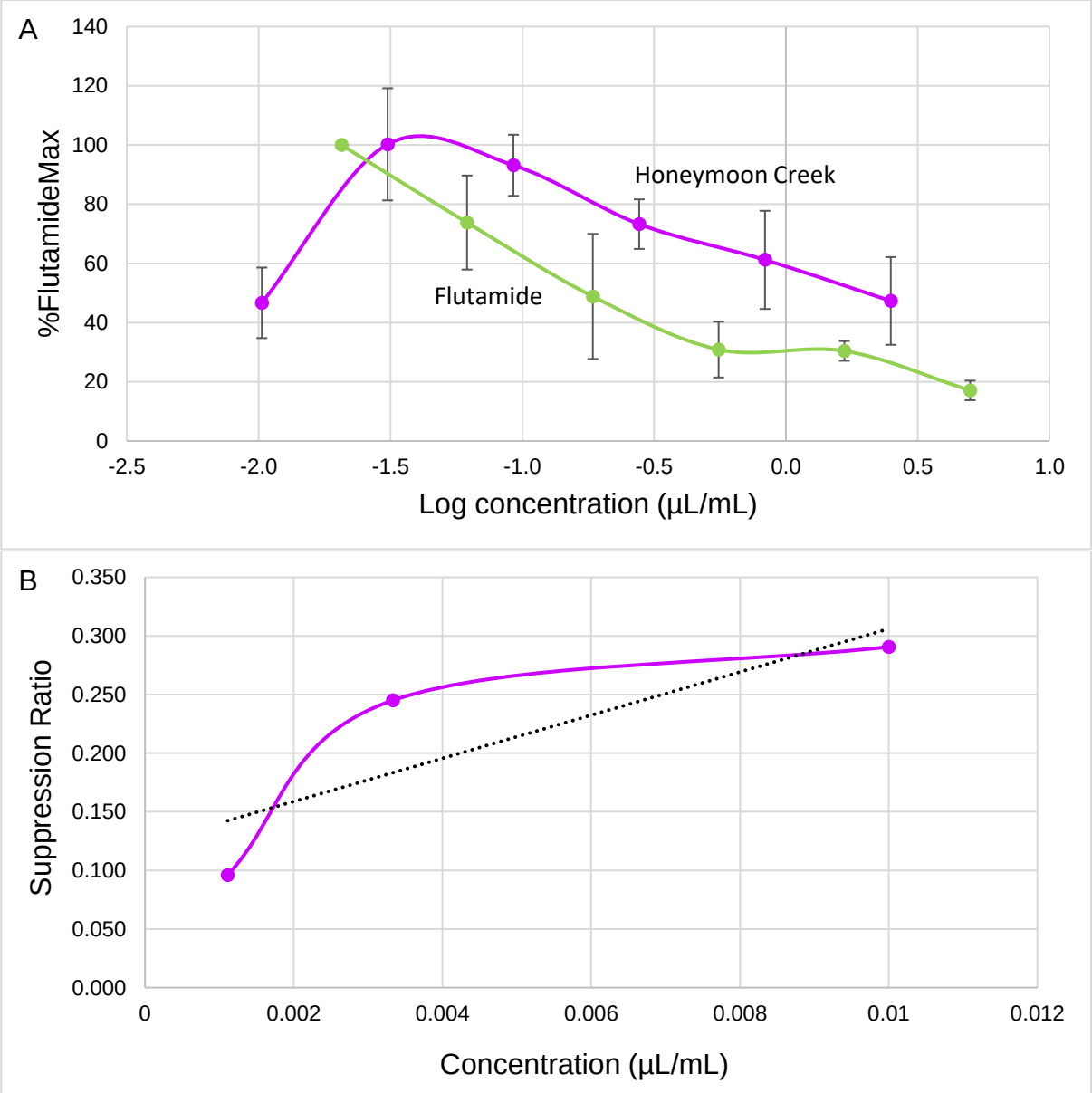



**Figure 20:** A **–** Honeymoon Creek water extract caused AR inhibition, sampled during wet season. Flutamide is the AR antagonist reference compound used in this study. The error bars indicate the standard deviation. B – Derivation of the concentrations causing suppression ratio of 20%.

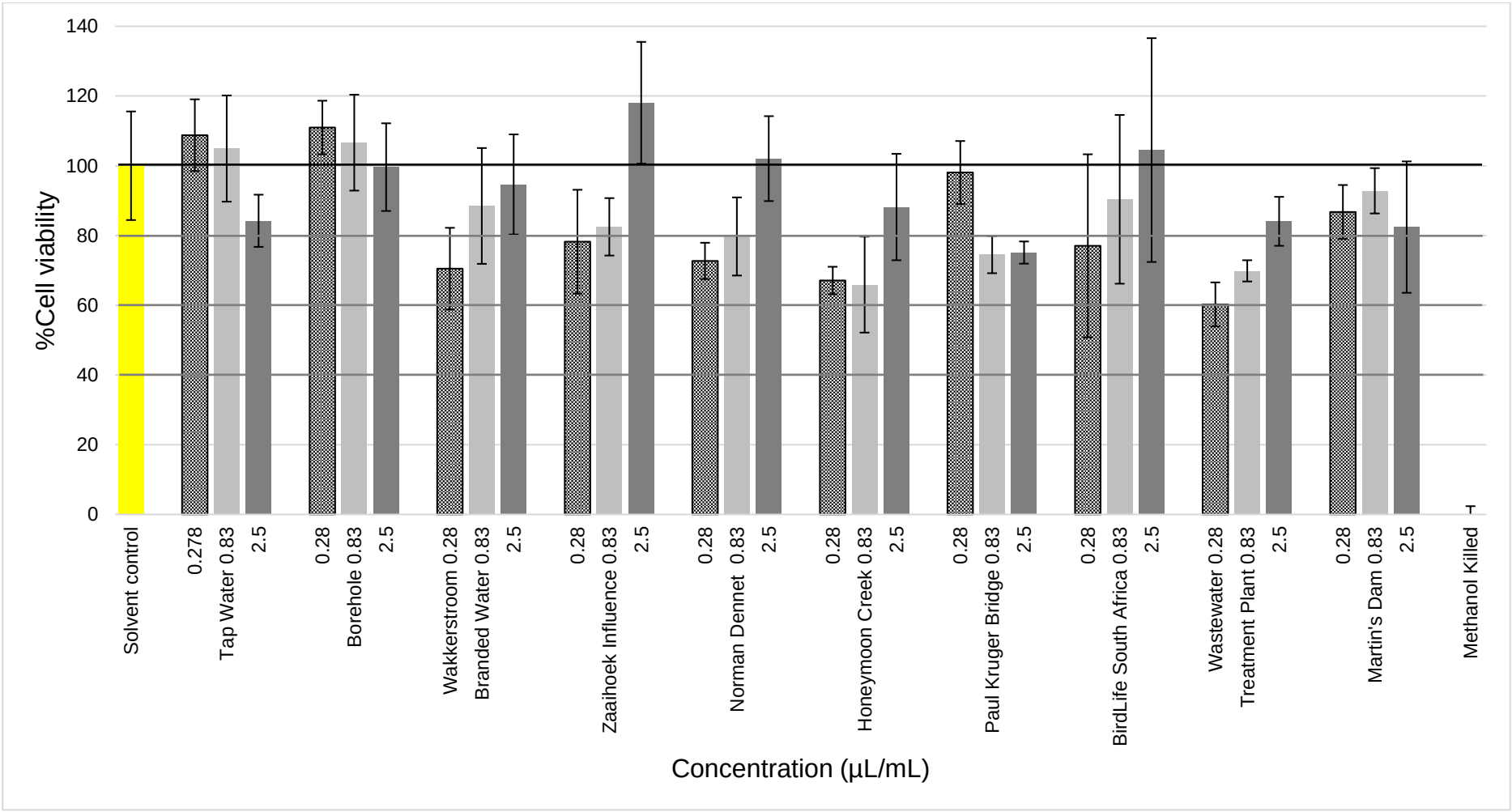



**Figure 21:** Results for cell viability assay for MDA-kb2 cells exposed to the wet season water samples. The black horizontal line indicates the absorbance of the control throughout the entire graph. Grey lines indicate cytotoxicity (>80%: non-cytotoxic; 60–80%: weakly cytotoxic: 40–60%: moderately cytotoxic; and <40%: strongly cytotoxic).

Water from BirdLife South Africa caused an $ECSR_{0.2}$ of 0.005 µL/L (Figure 22). The MTT viability assay's results indicated that the sample's concentrations were weakly to non-cytotoxic (ISO 10993-5, 2009) (Figure 21). In the AR inhibition response data, the highest concentration was statistically and practically significantly lower than the solvent control ($p<0.05$, $d\geq0.8$) (supplementary table 3).

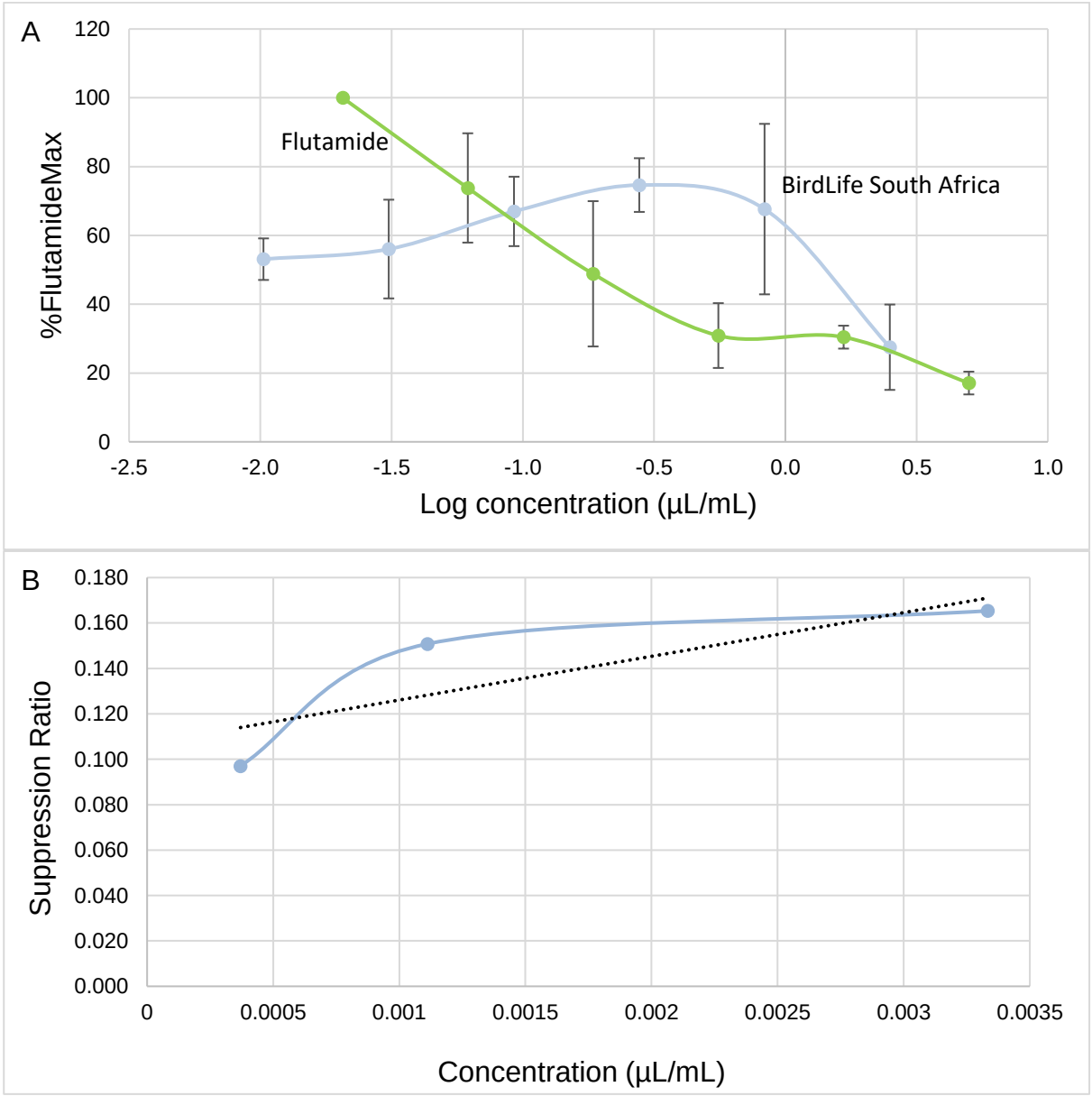



**Figure 22:** A **–** BirdLife South Africa water extract caused AR inhibition, wet season sample. Flutamide is the AR antagonist reference compound used in this study. The error bars indicate the standard deviation. B – Derivation of the concentrations causing suppression ratio of 20%.

### 4.3 Oxidative stress biomarkers

For these assays the extracts were given to duodenum (HuTu 80) and liver (H4IIE-*luc*) cells because these organs are involved in the absorption of compounds from the intestine and the decomposition of xenobiotics, respectively. The two cell lines received the same concentration water extract (0.28 µL/mL) and sediment extract (0.56 g/mL).

#### 4.3.1 Reactive oxygen species

Reactive oxygen species play an important role as cell signalling molecules but once there is an imbalance between the ROS production and the antioxidant defence it will cause oxidative stress (Auten & Davis, 2009). The sediment and water extracts were investigated for whether they would cause an increase in the amount of ROS produced in the cells. The results obtained for the different cell lines are reported separately.

Human duodenum cells (HuTu 80):

There were only two dry season samples that caused an increase in ROS which was statistically significant ($p<0.05$): water sample from Zaaihoek Influence and sediment sample from BirdLife South Africa (Figure 23). Water from the WWTP also raised the ROS, but this was only practically significant ($d\geq0.8$). Some sediment samples caused a statistically significant decrease in the ROS: Samples from Norman Dennet and Honeymoon Creek (Figure 23). Water samples from Martin's Dam decreased ROS when compared to the control but this was only practically significant (Figure 23) (supplementary table 6). The rest of the samples did not cause an effect on the ROS produced in the cells that was of any significance (Figure 23). None of the sites where both water and sediment were collected did both matrixes cause responses of significance, only the water sample from Zaaihoek Influence, sediment from Norman Dennet, Honeymoon Creek, and BirdLife South Africa ($p<0.05$ and $d\geq0.8$). Neither the sediment nor the water from Paul Kruger Bridge caused a response of significance (Figure 23).

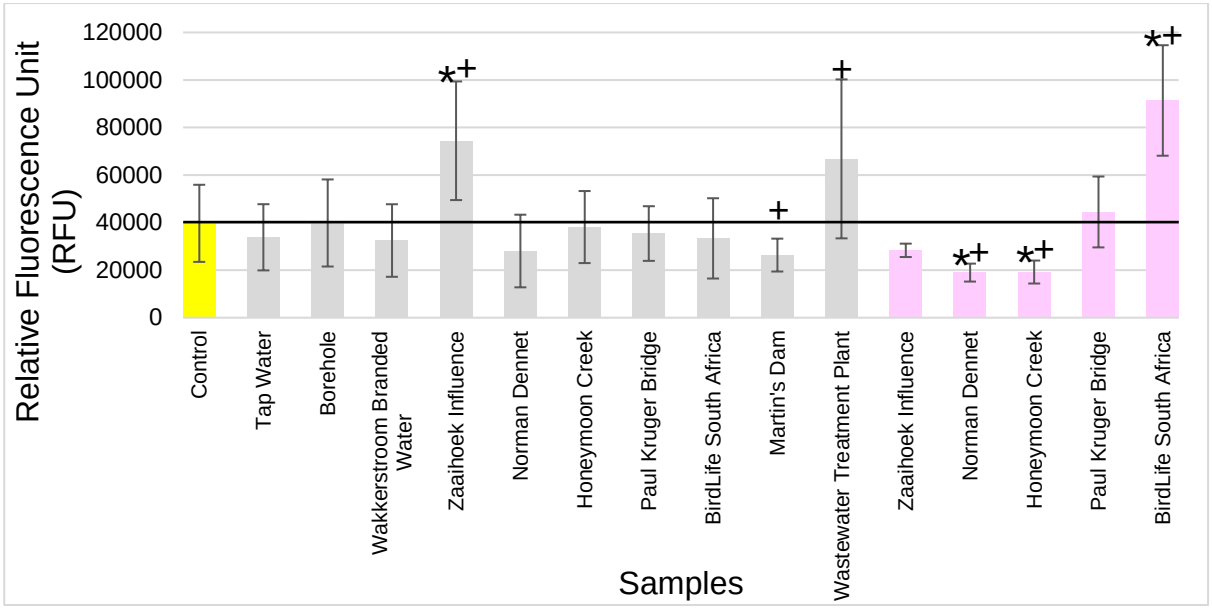



**Figure 23:** Reactive oxygen species results from HuTu 80 cells exposed to dry season sample extracts. Standard deviation in the results is indicated with error bars, * indicates statistically significant where p<0.05 using Mann Whitney, + indicates the practically significance with Cohen's d≥0.8. Significance was calculated using raw absorbance data. The black horizontal line indicates the RFU of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Four of the samples collected in the wet season increased the ROS which was statistically significant (MW, p<0.05) (supplementary table 6): the Tap Water, Borehole water, and Wakkerstroom Branded Water, and sediment sample from BirdLife South Africa (Figure 24). It is interesting to note that the effect caused by sediment from BirdLife South Africa was statistically significant because the effect was barely higher than the control. Some samples caused a decrease in the effect in ROS that was statistically significant: water samples from BirdLife South Africa, Paul Kruger Bridge, and the effluent from the Wastewater Treatment Plant, and sediment samples from Zaaihoek Influence, Norman Dennet, and Honeymoon Creek, and Paul Kruger Bridge (Figure 24). Most of these samples also were practically significant (d≥0.8) except the borehole and Wakkerstroom

Branded Water and sediment from Paul Kruger Bridge and BirdLife South Africa (Figure 24).

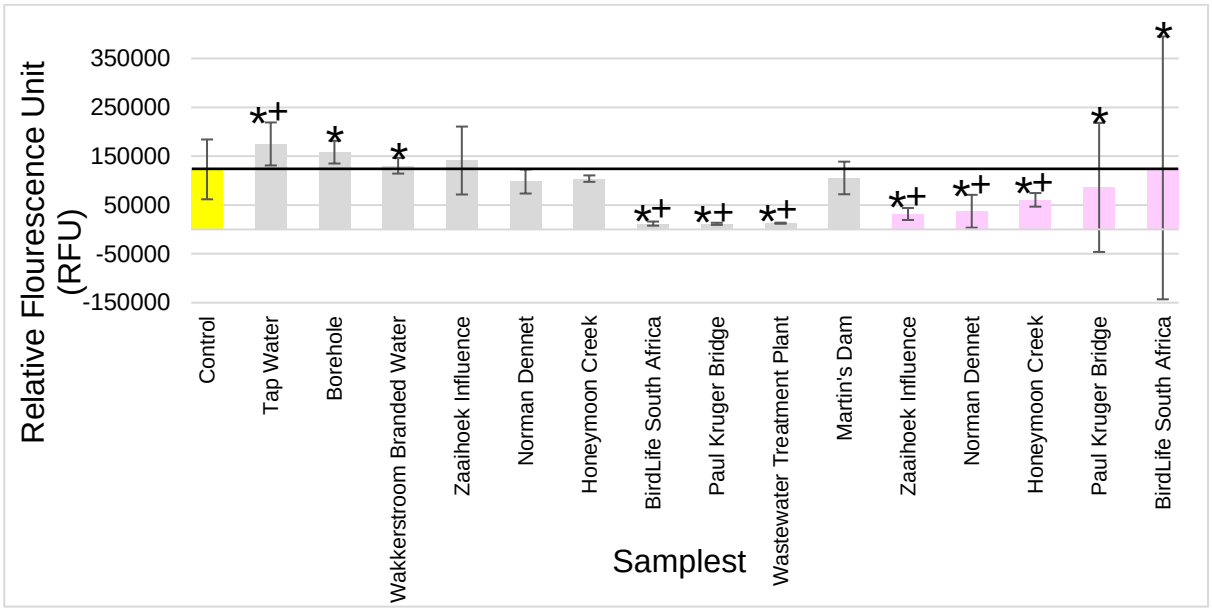



**Figure 24:** Reactive oxygen species results from HuTu 80 cells exposed to wet season sample extracts. Standard deviation in the results is indicated with error bars, * indicates statistically significant where p<0.05 using Mann Whitney, + indicates the practically significance with Cohen's d≥0.8. Significance was calculated using raw absorbance data. The black horizontal line indicates the RFU of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Rat liver cells (H4IIE-*luc*):

Extracts from the following samples caused a statistically significant increase in ROS when compared to the control (MW, p<0.05): water samples from Honeymoon Creek, Paul Kruger Bridge, BirdLife South Africa and Martin's Dam and the sediment sample from Norman Dennet (Figure 25) (supplementary table 7). Some samples also caused a statistical decrease in ROS: Water samples from Tap Water, Borehole, Wakkerstroom Branded Water, Zaaihoek Influence, and sediment samples from Honeymoon Creek and BirdLife South Africa (Figure 25). Most of the statistically significant differences were confirmed by the practical significance (Figure 25). The sediment sample from Zaaihoek Influence did cause an increase in ROS that was not statistically significant, but it was practically significant (d≥0.8). Except for two water samples, Norman Dennet and WWTP, and one sediment sample, Paul Kruger Bridge, all the samples caused an effect on the ROS produced by the cells. Four of the sediment samples that were collected at sites

where water samples were also collected caused a different effect than the water sample collected at the same site: Zaaihoek Influence; Norman Dennet; Honeymoon Creek; and BirdLife South Africa) (Figure 25).

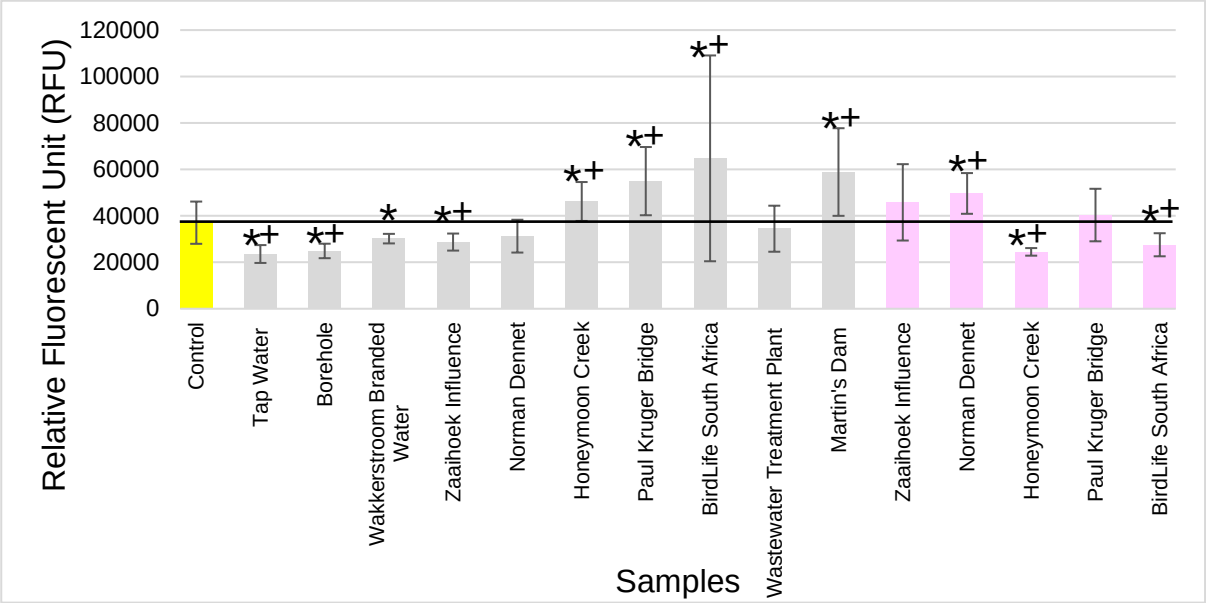



**Figure 25:** Reactive oxygen species results for H4IIE-*luc* cells exposed to dry season sediment and water extracts. Standard deviation in the results is indicated with error bars, * indicates statistically significant where $p<0.05$ using Mann Whitney, + indicates the practically significant with Cohen's $d\geq0.8$. Significance was calculated using raw absorbance data. The black horizontal line indicates the RFU of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

All the water samples, collected during the wet season, except for the Tap Water, caused an increase in ROS that was statistically significant (MW, $p<0.05$). These were also all practically significant except for Borehole water ($d\geq0.8$). All the sediment samples also caused an increase in the ROS except for one sample, Paul Kruger Bridge, and three of the four sediment samples were statistically significant, Zaaihoek Influence, Norman Dennet and BirdLife South Africa (Figure 26). The increased response from sediment sampled at Honeymoon Creek was not statistically significant but it was practically significant (Figure 26). The response seen at most of the sites is supported by their reactions being practically significant as well ($d\geq0.8$) (Figure 26) (supplementary table 7). Sediment and their corresponding water samples caused the same responses: increased ROS in the liver cells, except for Paul Kruger Bridge where the water sample caused an increase in ROS and the sediment sample caused the ROS to decrease in the cells.

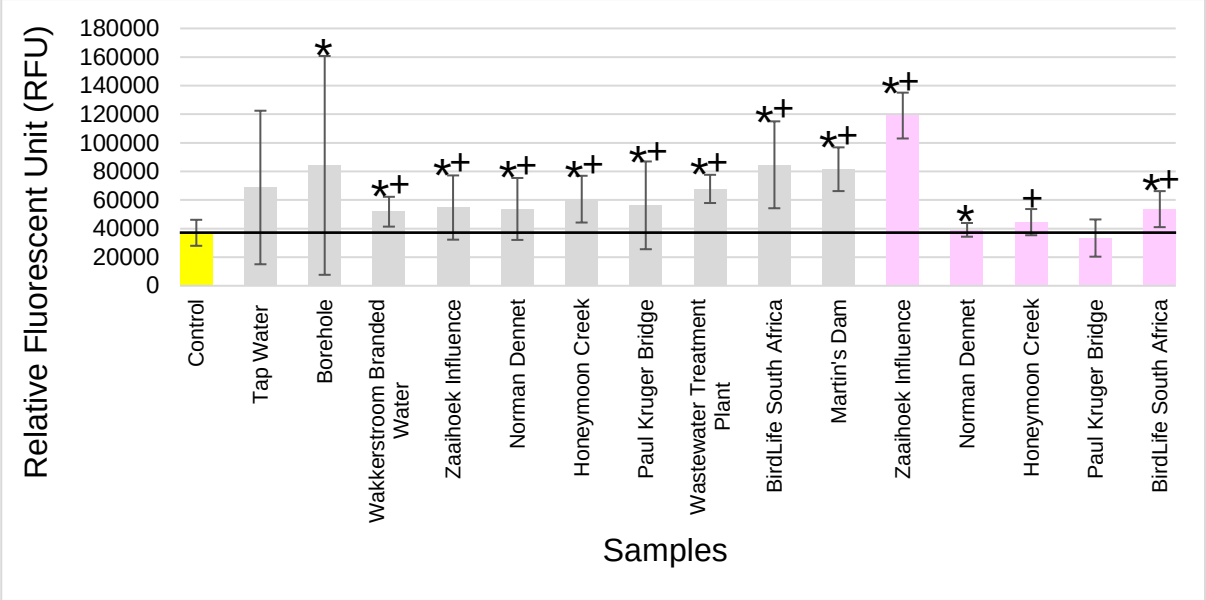



**Figure 26:** Reactive oxygen species results for H4IIE-*luc* cells exposed to wet season sample extracts. Standard deviation in the results is indicated with error bars, * indicates statistically significant where p<0.05 using Mann Whitney, + indicates the practically significance with Cohen's d≥0.8. Significance was calculated using raw absorbance data. The black horizontal line indicates the RFU of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Duodenum vs. liver cells:

Almost all the dry season samples given to the liver cells were either statistically or practically significant or both, except for water from Norman Dennet and WWTP and sediment from Paul Kruger Bridge whereas only six samples caused responses of significance in the duodenum cells (Figure 23). Only three samples caused an increase (p<0.05 and/or d≥0.8) in the ROS production in the duodenum cells, water from Zaaihoek Influence and WWTP and sediment from BirdLife South Africa while six samples caused an increase in the ROS production in the liver cells (Figure 25).

All the wet season samples caused an increase in ROS production in the liver cells (p<0.05 and/or d≥0.8) except for two samples that did not cause statistically or practically significant responses: Tap Water and sediment from Paul Kruger Bridge (Figure 26). In the duodenum cells an increase and decrease in ROS production were noted when exposed to the samples (p<0.05 and/or d≥0.8). Four water samples' responses were not of significance: Zaaihoek Influence, Norman Dennet, Honeymoon and Martin's Dam.

### 4.3.2 Protein Content

This assay measured the amount of protein present in each well because it was used as a proxy of cell number against which the SOD, CAT, LPO and AChE activities were expressed. Protein content was determined after exposure to the samples. A calibration curve was plotted using known concentrations of protein to determine the linear equation with which the protein content of cells exposed to samples was determined (Figure 27). The protein content was determined for each of the assays but is not reported separately.

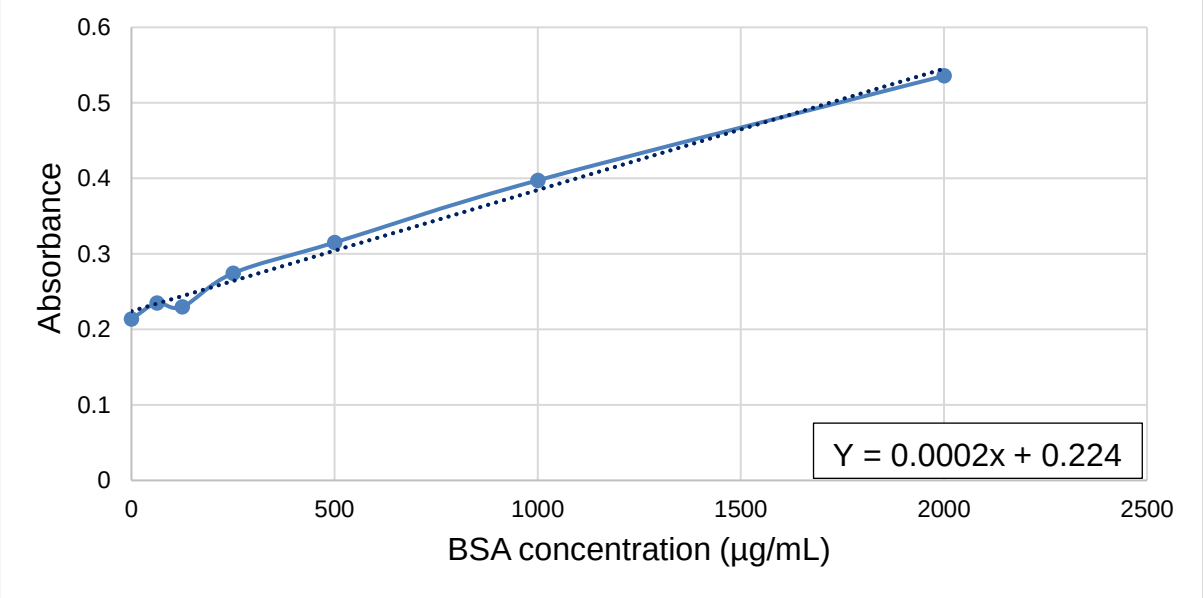



**Figure 27:** Protein content calibration curve with the linear equation to determine the x-value.

### 4.3.3 Superoxide dismutase

This enzyme forms part of the first line of defence against oxidative stress and begins the detoxification in the cell (Ighodaro & Akinloye, 2018).

Human duodenum cells (HuTu 80):

Only three samples collected in the dry season caused an increase in the SOD activity of the cells that were statistically significant ($p<0.05$): water samples from BirdLife South Africa and WWTP, and one sediment sample, that from Norman Dennet (Figure 28). The sediment sample from the Zaaihoek Dam influence also caused a raise in SOD but this was only practically significant ($d\geq0.8$). The Honeymoon Creek sediment sample was the

only sample that caused a decrease in the SOD activity that was statistically significant ($p<0.05$) (supplementary table 8). Apart from being statistically significant this sample was also practically significant ($d\geq0.8$) (Figure 28). From all fifteen samples only five samples' reactions were statistically significant. There was no resemblance between the reaction of the cells exposed to the water and sediment samples that was sampled at the same sites (Figure 28).

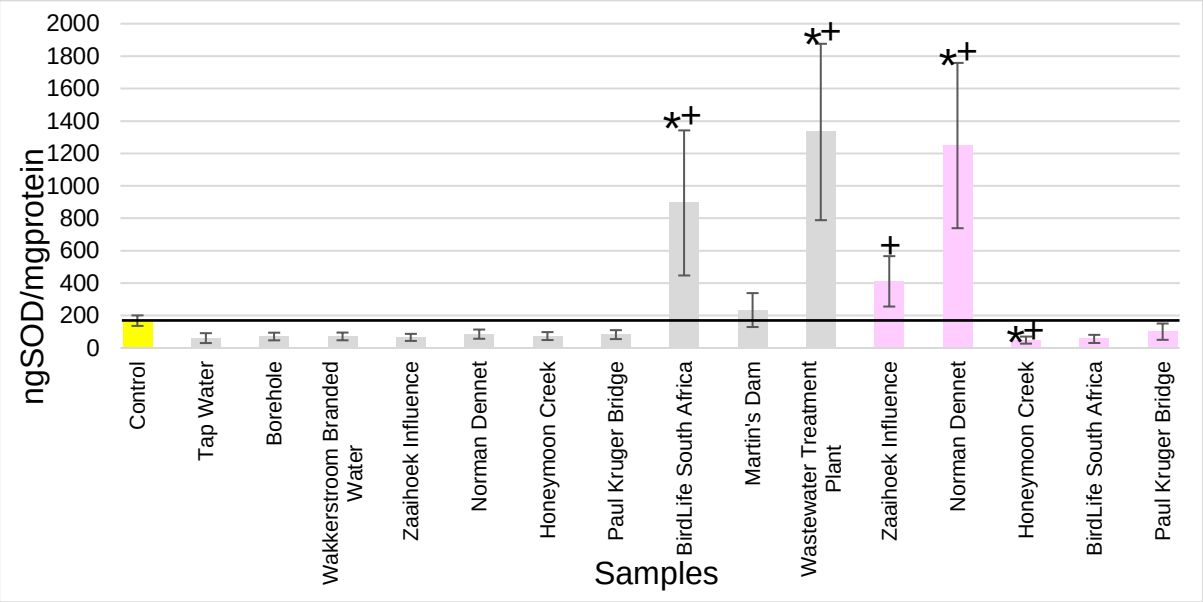



**Figure 28:** Superoxide dismutase results for HuTu 80 cells exposed to dry season sample. Standard deviation in the results is indicated with error bars, * indicates statistically significant where $p<0.05$ using Mann Whitney, + indicates the practically significance with Cohen's $d\geq0.8$. Significance was calculated using raw absorbance data. The black horizontal line indicates the ngSOD/mg protein of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Some of the samples collected in the wet season caused a statistically significant increase in the SOD activity in the cells ($p<0.05$) (Figure 29). None of the samples caused a decrease in enzyme activity which was statistically significant. The response caused by two water samples (Zaaihoek Influence and Martin's Dam) are supported by their responses also being practically significant ($d\geq0.8$) (supplementary table 8). Two of the samples caused an increase in the SOD activity, which was not statistically significant, but it was practically significant (Figure 29) (Tap Water and sediment from Paul Kruger Bridge). Most of the samples did not influence the SOD activity in the cells. None of the responses caused by the sediment samples were the same as water samples collected at the corresponding sediment site.

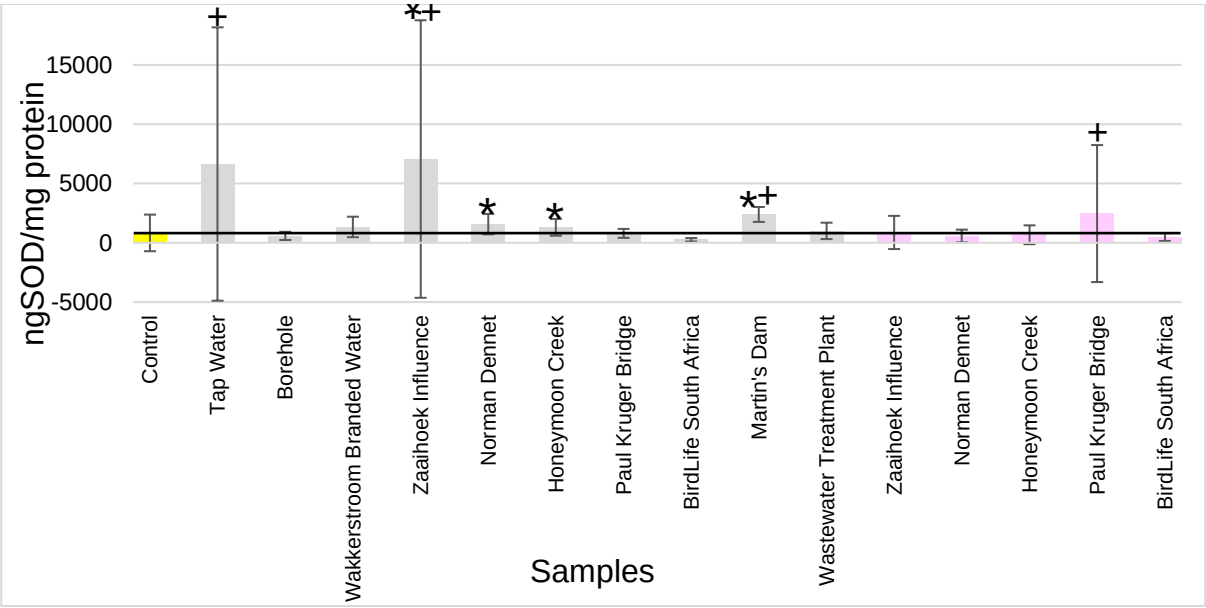



**Figure 29:** Superoxide dismutase results for HuTu 80 cells exposed to wet season samples. Standard deviation in the results is indicated with error bars, * indicates statistically significant where p<0.05 using Mann Whitney, + indicates the practically significance with Cohen's d≥0.8. Significance was calculated using raw absorbance data. The black horizontal line indicates the ngSOD/mg protein of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Rat liver cells (H4IIE-*luc*):

The water samples from the dry season did not significantly cause different responses in SOD from that elicited from the control sample except for the borehole water which cause an increased SOD activity (p<0.05). The only sediment sample that caused a significant increase in SOD activity (p>0.05) was from BirdLife SA (supplementary table 9). And only two sediment samples (Honeymoon Creek and Paul Kruger Bridge) decreased SOD significantly (p<0.05). None of the sediment samples with significant responses had corresponding responses from their water samples (Figure 30).

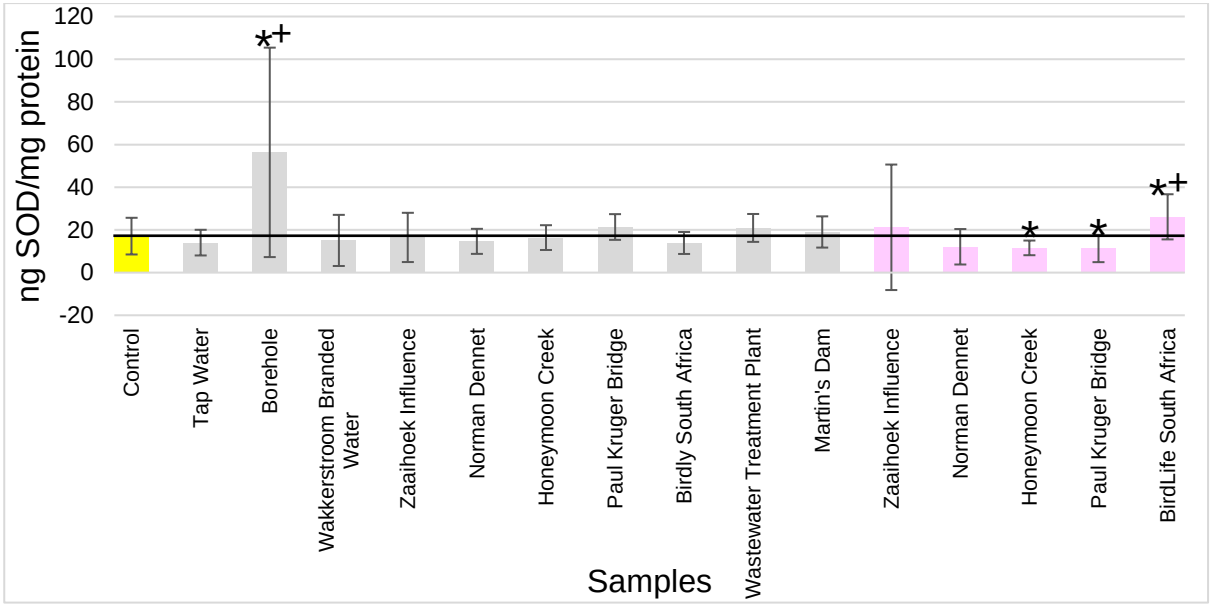



**Figure 30:** Superoxide dismutase results for H4IIE-*luc* cells exposed to dry season samples. Standard deviation in the results is indicated with error bars, * indicates statistically significant where p<0.05 using Mann Whitney, + indicates the practically significance with Cohen's d≥0.8. Significance was calculated using raw absorbance data. The black horizontal line indicates the ngSOD/mg protein of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Most of the water samples from the wet season, did not cause a response that differed significantly from the control. The exceptions were Paul Kruger Bridge and effluent from the WWTP (p<0.05) (Figure 31). The sediment samples caused responses that increased the SOD, but none of them were statistically significant. There were, however, two samples that caused an increase in SOD activity that was practically significant (sediment from Zaaihoek Influence and Norman Dennet) (d≥0.8) (Figure 31). Of the samples with significant responses there was no resemblance in response between the water and sediment samples collected at the same sites (Figure 31).

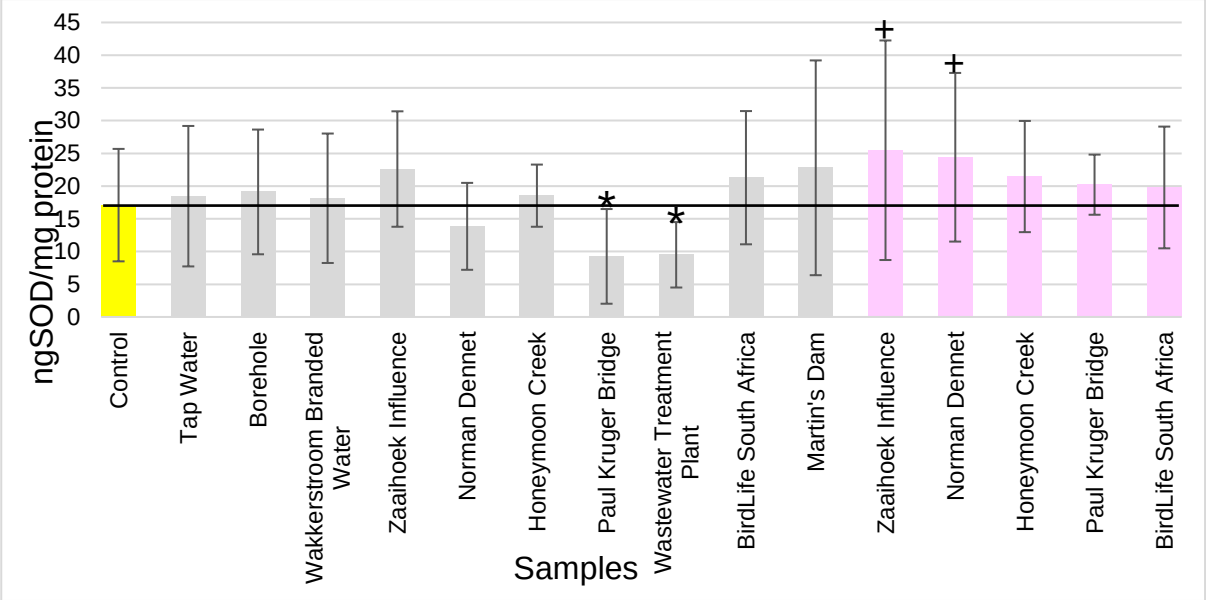



**Figure 31:** Superoxide dismutase results for H4IIE-*luc* cells exposed to wet season samples. Standard deviation in the results is indicated with error bars, * indicates statistically significant where p<0.05 using Mann Whitney, + indicates the practically significance with Cohen's d≥0.8. Significance was calculated using raw absorbance data. The black horizontal line indicates the ngSOD/mg protein of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Duodenum vs liver cells:

Samples collected in the dry season given to the duodenum cells caused five statistically and/or practically significant responses (Figure 28) while when the liver cells were exposed to the samples only four responses of significance were measured. Honeymoon Creek caused a decrease in the SOD activity in both cell lines but the response in the duodenum cells were both statistically and practically significant while in the liver cells it was only statistically significant (Figure 30).

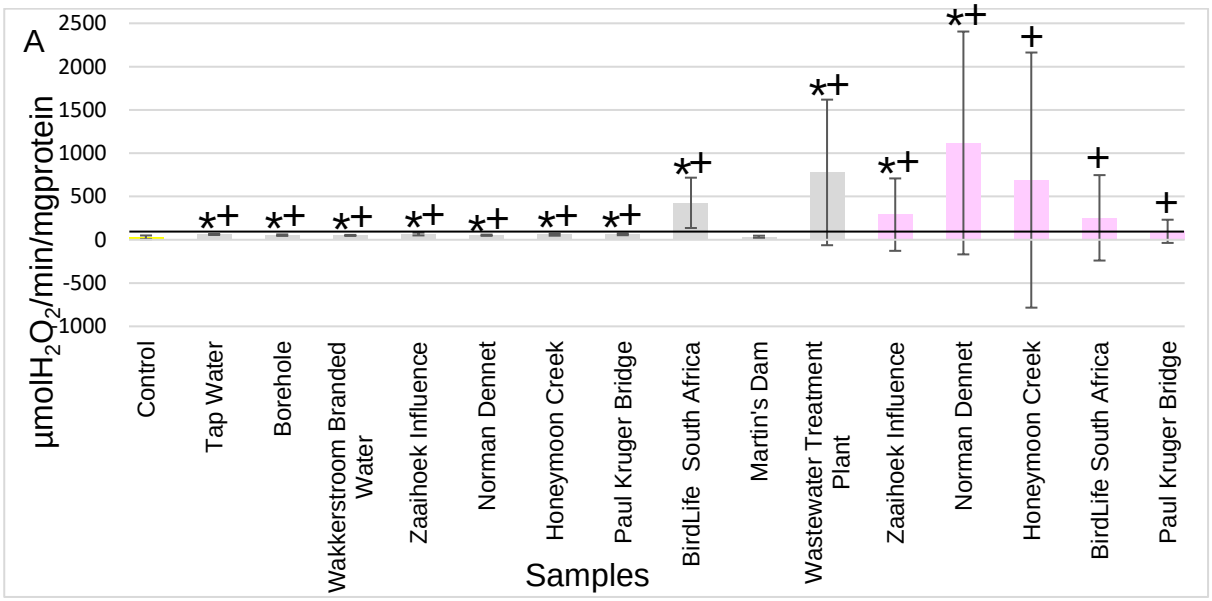
The wet season samples caused an increase in the SOD activity in the duodenum cells that was statistically and/or practically significant (five water samples and one sediment sample) whereas in the liver cells only four samples influenced the activity of SOD (p<0.05 and/or d>0.8) (two water samples and two sediment samples) (Figure 31). None of the samples caused a response of significance in both cell lines.

### 4.3.4 Catalase activity results

This enzyme is also part of the first line of defence antioxidants which is present in almost all living tissues and uses either manganese or iron as a cofactor during the hydrogen peroxide degradation process (Ighodaro & Akinloye, 2018). Catalase completes the detoxification process that SOD started. The sample extracts were given to the HuTu 80 and H4IIE-*luc* cell lines and the activity of catalase was measured after 24 h.

Human duodenum cells (HuTu 80):

All the samples from the dry season caused an increase in CAT and except for sediment samples from Honeymoon Creek, Paul Kruger Bridge, and BirdLife South Africa they were all statistically significant (MW, $p<0.05$) (Figure 32) (supplementary table 10). The latter were however practically significantly increased above the control cells. None of the samples caused a decrease in the enzyme's activity. In this instance, water and sediment samples from the same sites lead to increased CAT, if not with a $p<0.05$, then with $d\geq0.8$. (Figure 32).



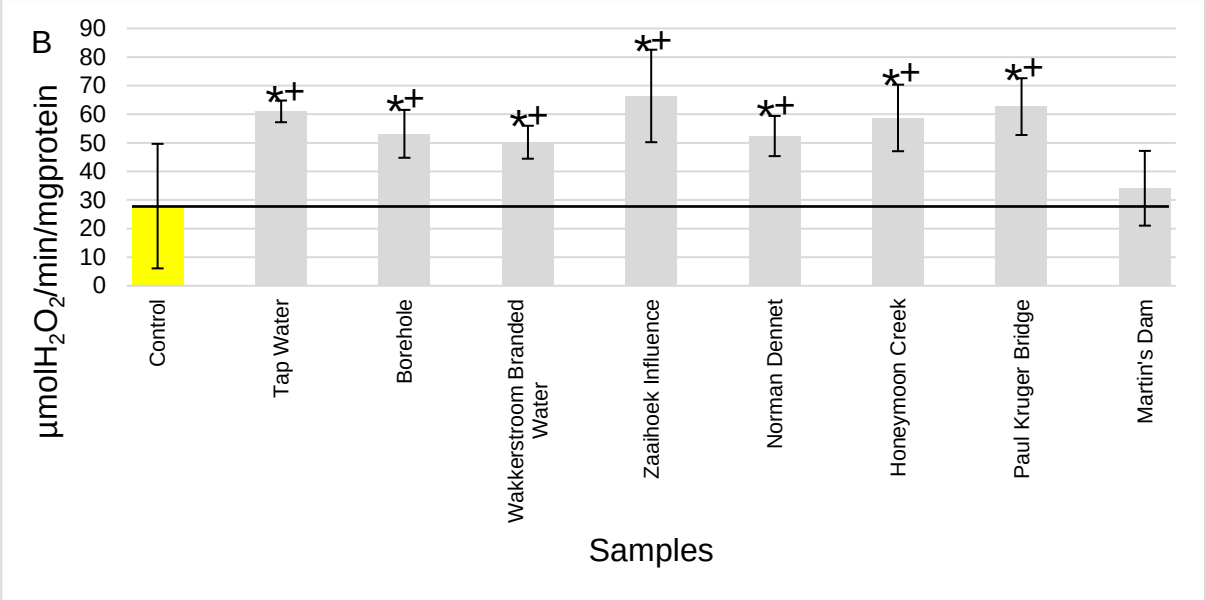



**Figure 32:** A - Catalase results for HuTu 80 cells exposed to dry season samples. Standard deviation in the results is indicated with error bars, * indicates statistically significant where $p<0.05$ using Mann Whitney, + indicates the practically significance with Cohen's $d \geq 0.8$. Significance was calculated using raw absorbance data. The black horizontal line indicates the µmol $H_2O_2$/min/mg protein of the control throughout the entire graph. B – Illustrating the results shown on Figure 23A which was barely visible due to scaling. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Analysis of CAT responses to samples from the wet season also led to increased responses for some samples but for only two of the samples it was statistically significant compared to the control cells ($p<0.05$): water from Martin's Dam and sediment from Norman Dennet's place. These however were close to the CAT levels of the control, and despite having statistically significant response, these were not confirmed by practical significance. The increased CAT responses from the Tap Water and water from the Zaaihoek influence as well as from sediment from Paul Kruger Bridge were all practically significant. None of the samples caused a decrease which was statistically significant (Figure 33). None of the water or corresponding sediment samples from the same sites caused correlating responses regarding the statistical or practical significance thereof (Figure 33).

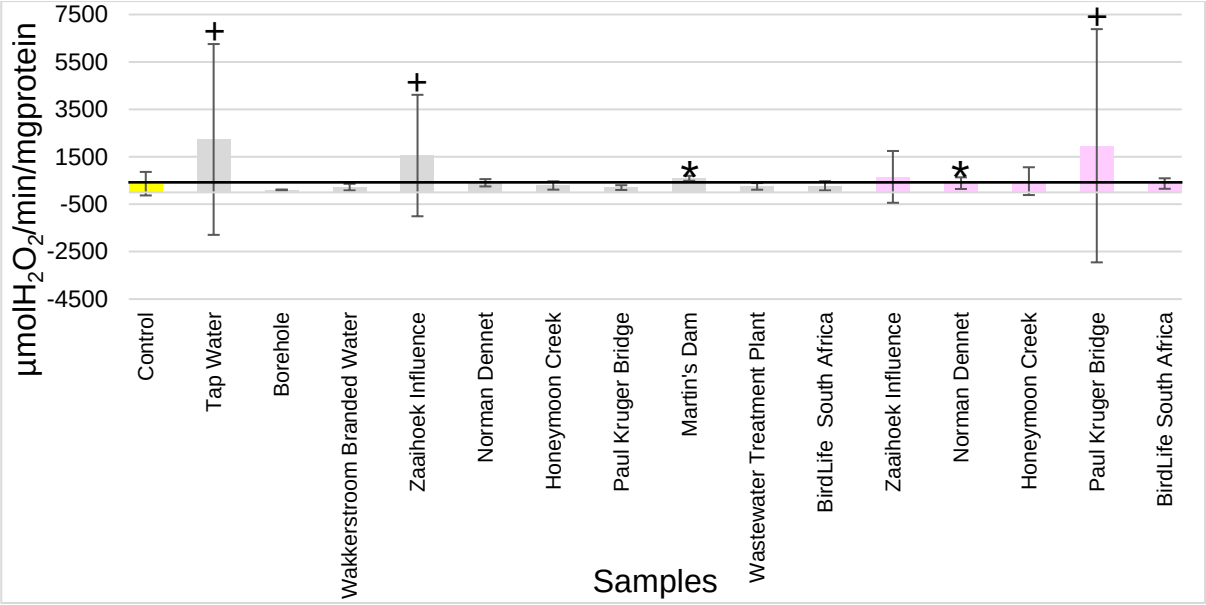



**Figure 33:** Catalase results for HuTu 80 cells exposed to wet season samples. Standard deviation in the results is indicated with error bars, * indicates statistically significant where $p<0.05$ using Mann Whitney, + indicates the practically significance with Cohen's $d\geq0.8$. Significance was calculated using raw absorbance data. The black horizontal line indicates the µmol $H_2O_2$/min/mg protein of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Rat liver cells (H4IIE-*luc*):

From all the samples from the dry season only two caused an increase which was statistically significant ($p<0.05$) compared to the control: borehole water and sediment sampled from BirdLife South Africa (Figure 34). Again, the latter was barely above that of the control, and although statistically significant, not practically significant. The Borehole was also practically significant ($d\geq0.8$, the + in Figure 34) (supplementary table 11). Most of the sediment samples caused a decrease in CAT levels of which three were statistically significant ($p<0.05$): sediment form Zaaihoek influence, Norman Dennet's Place, and Honeymoon Creek (Figure 34). Out of the fifteen samples only five was statistically significant. Sediment and water samples collected at the corresponding site did not elicit resembling responses because only four sediment samples did cause a response (Figure 34).

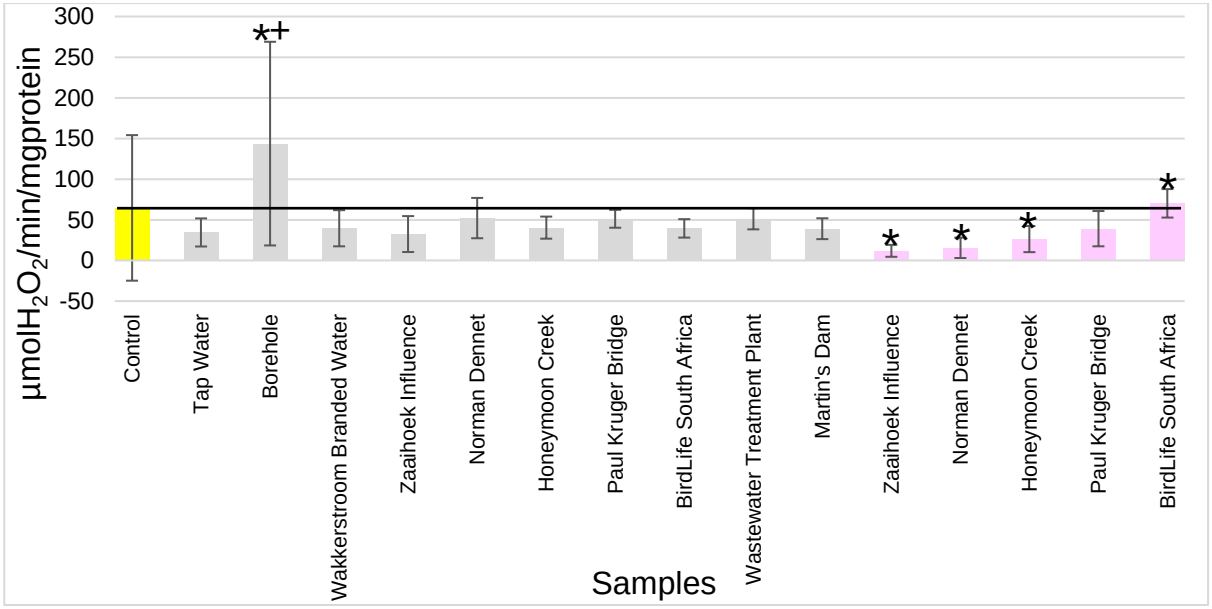



**Figure 34:** Catalase results for H4IIE-*luc* cells exposed to dry season samples. Standard deviation in the results is indicated with error bars, * indicates statistically significant where $p<0.05$ using Mann Whitney, + indicates the practically significance with Cohen's $d \geq 0.8$. Significance was calculated using raw absorbance data. The black horizontal line indicates the µmol $H_2O_2$/min/mg protein of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Only one sample from the wet season elicited an increase in CAT activity and this happened to be statistically significant ($p<0.05$) too: Wakkerstroom Branded Water (Figure 35). All other CAT responses were below that of the control: five water samples were statistically significantly different from the control, and they were water from Honeymoon Creek, Paul Kruger Bridge, effluent from the WWTP, and water from Martin's Dam. All sediment samples showed a lower than control value for CAT, but the sample from Honeymoon Creek was the only significant one ($p<0.05$) (Figure 35). None of the samples caused a response that was practically significant ($d \geq 0.8$). Honeymoon Creek water and sediment caused a similar response: statistically significant decrease in CAT activity compared to the control cells (Figure 35).

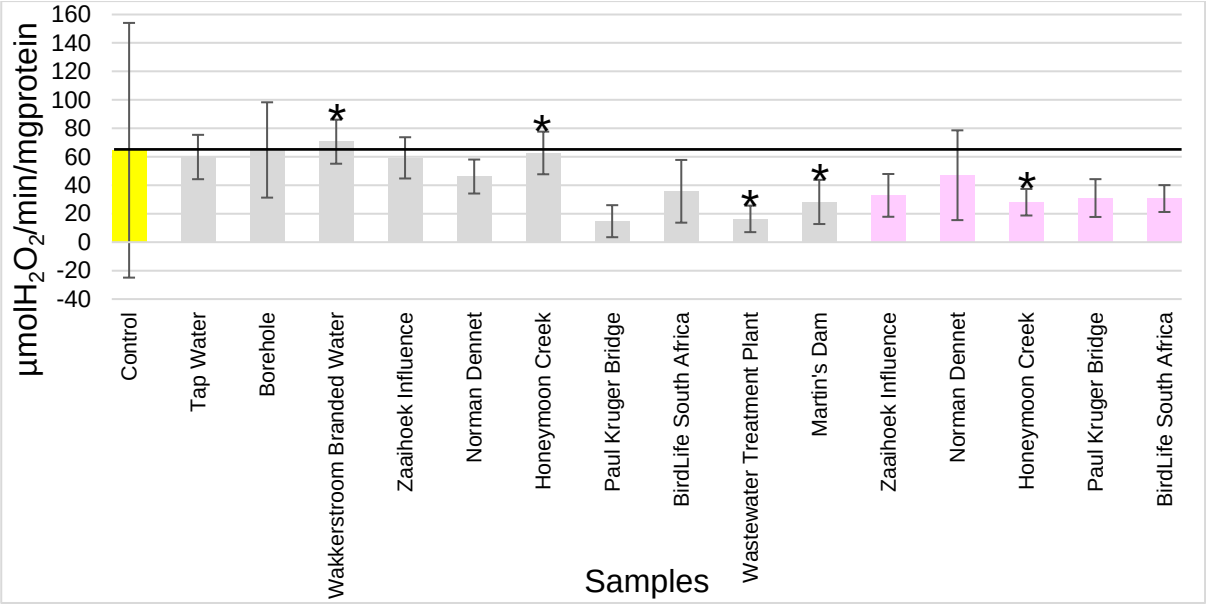



**Figure 35:** Catalase results for H4IIE-*luc* cells exposed to wet season samples. Standard deviation in the results is indicated with error bars, * indicates statistically significant where p<0.05 using Mann Whitney. Significance was calculated using raw absorbance data. The black horizontal line indicates the µmol $H_2O_2$/min/mg protein of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Duodenum vs liver cells:

Almost all the samples caused a statistically and/or practically significant response in the CAT activity in the duodenum cells, except for Martin's Dam water sample (Figure 32). Only five samples caused either an increase or decrease in the CAT activity that was of significance: Borehole water and sediment from Zaaihoek Influence, Norman Dennet, Honeymoon Creek, and BirdLife South Africa (Figure 34). BirdLife South Africa caused an increase in the enzyme's activity, in the duodenum cells the response was practically significant while in the liver cells the response was statistically significant. The remaining three sediment samples that caused a statistically significant decrease in the CAT activity in the liver cells, all caused an increase in the enzyme's activity in the duodenum cells but their responses were both statistically and practically significant except for Honeymoon Creek which was only practically significant in the duodenum cells.

Six of the fifteen wet season samples caused responses of significance (p<0.05) in the liver cells regarding the CAT activity (five water samples and one sediment sample) (Figure 35) while only five samples caused an increase in the activity of CAT in the duodenum cells (three water samples and two sediment samples). There were no

responses of practical significance in the liver cells while three of the samples caused only responses of practical significance in the duodenum cells: Tap Water and Zaaihoek Influence water and sediment from Paul Kruger Bridge. Martin's Dam is the only sample that caused responses of statistical significance in both cell lines but in the duodenum cells it caused an increase in CAT activity and a decrease in activity in the liver cells (Figure 35).

### 4.3.5 Lipid peroxidation

Lipid peroxidation is one of the consequences of uncontrolled oxidative stress because high levels of reactive oxygen species can cause damage to lipids (Ayala et al., 2022).

Both the cell lines, HuTu 80 and H4IIE-*luc*, which were exposed to the sample extracts for 24 h did not show any signs of lipid peroxidation. Once the absorbance values of the samples were substituted into the straight-line equation created by the calibration curve (Figure 36), the corresponding x-values were negative. This is an indication of a failed experiment because all cells would have either no (x=0) or some lipid content (x>0) which is not possible. The relative absorbance values are illustrated in Figures 37 and 38 where it is clear that they were not really different from the control, compared to the higher absorbance of the positive control (1,1,3,3-tetramethoxypropane (TMP)). The reference compound TMP worked well, but this control did not contain cells. The lack of proper response in the sample exposed cells is likely an indication of too few cells used during this assay. This flaw, and how it can be corrected is addressed in the discussion chapter (section 5.1).

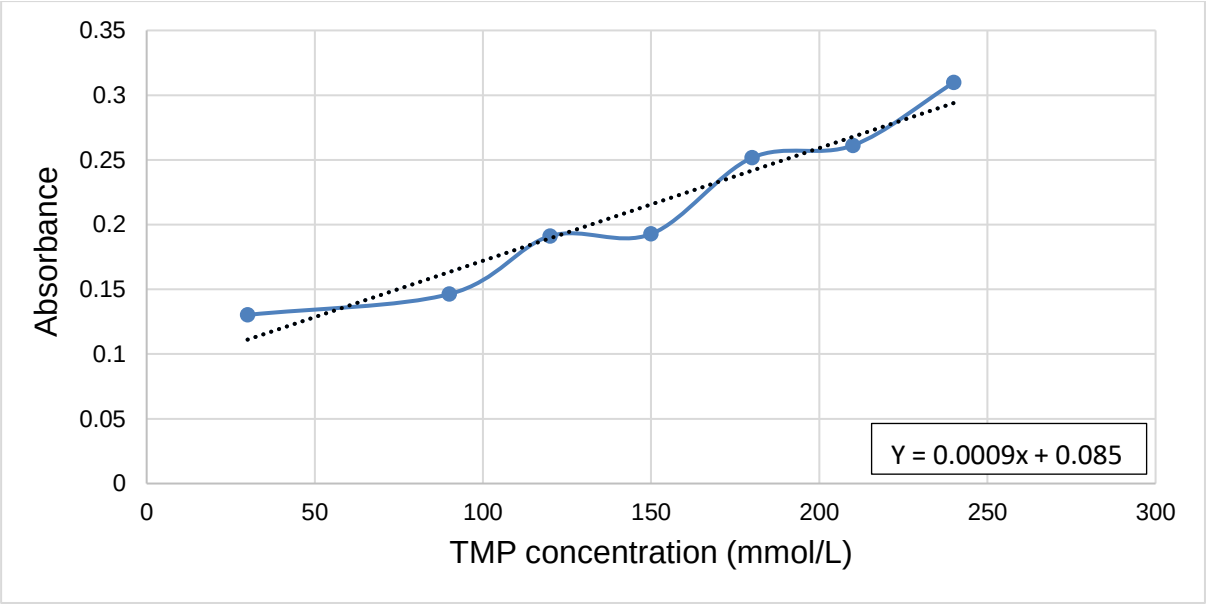



**Figure 36:** TMP calibration curve with the linear line equation to calculate the x-value (TMP - 1,1,3,3-tetramethoxypropane).

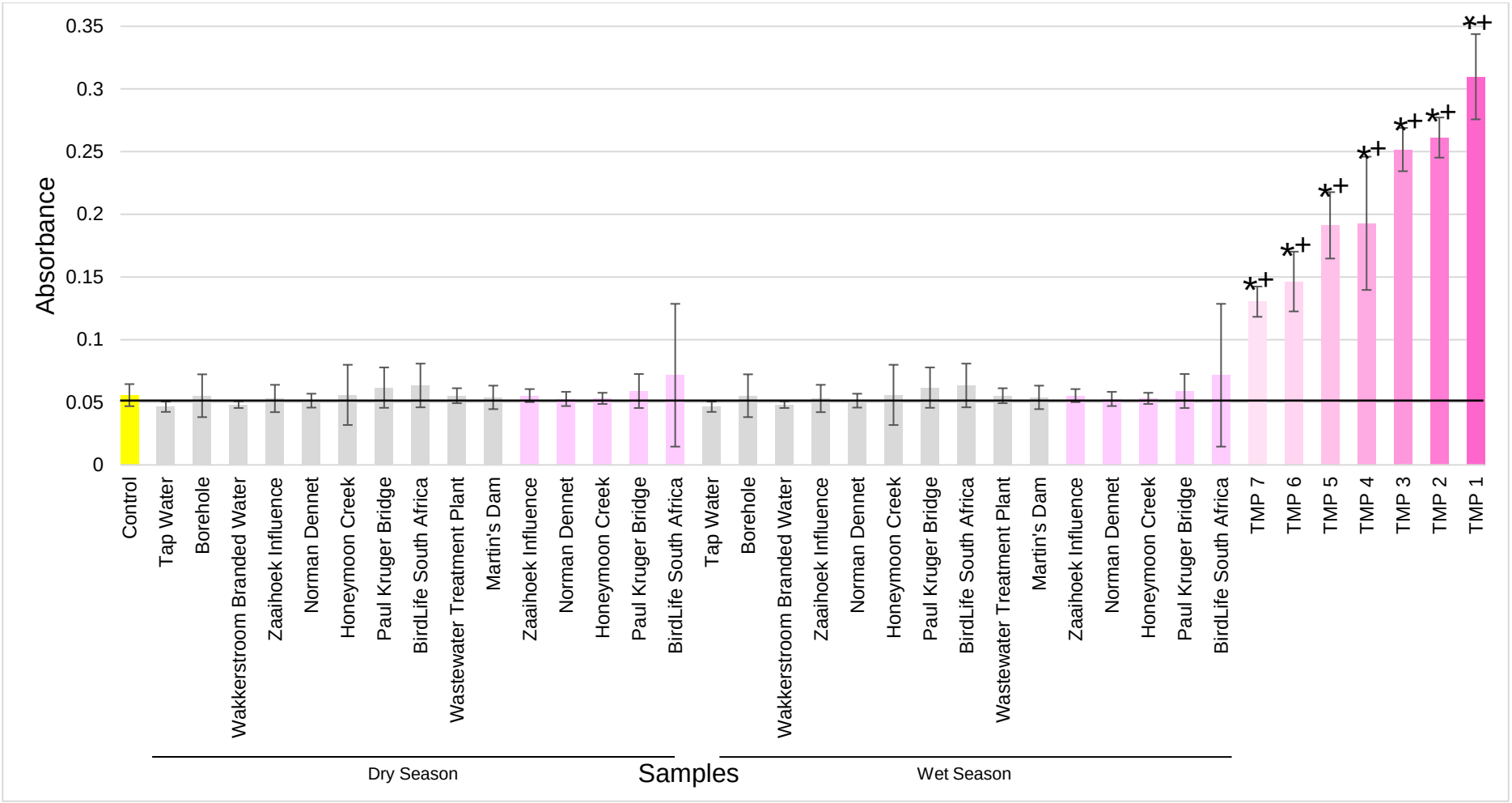



**Figure 37:** Lipid peroxidation content of HuTu 80 cells exposed to seasonal samples (TMP - 1,1,3,3-tetramethoxypropane). Absorbance values of the positive control were compared to the absorbance of control cells that did not receive any samples. Standard deviation in the results is indicated with error bars, * indicates statistically significant where $p<0.05$ using Mann Whitney, + indicates the practically significance with Cohen's $d \geq 0.8$. Significance was calculated using raw absorbance data. The black horizontal line indicates the absorbance of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

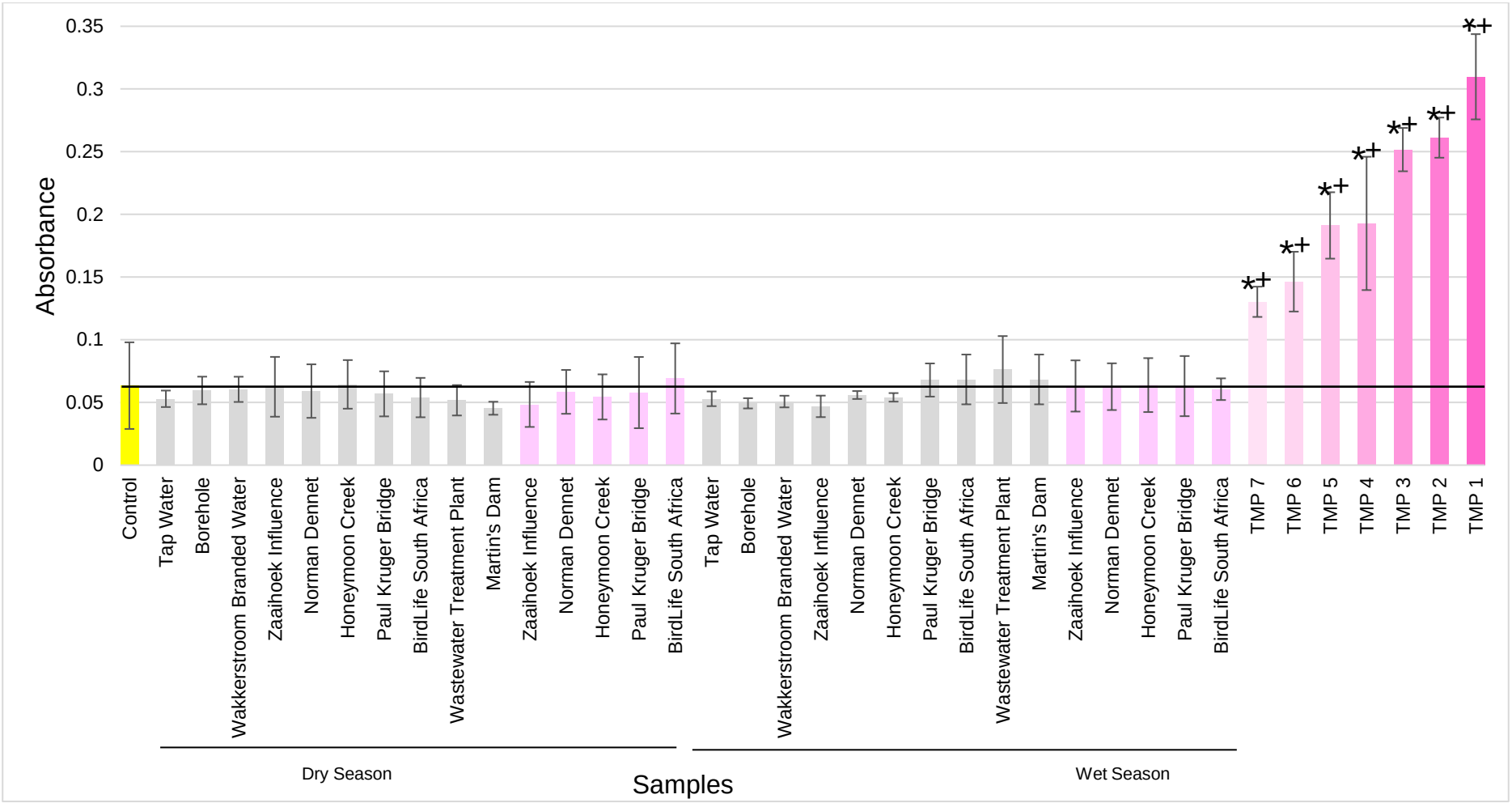



**Figure 38**Lipid peroxidation content of H4IIE-*luc* cells exposed to seasonal samples (TMP - 1,1,3,3-tetramethoxypropane). Absorbance values of the positive control were compared to the absorbance of control cells that did not receive any samples. Standard deviation in the results is indicated with error bars, * indicates statistically significant where $p<0.05$ using Mann Whitney, + indicates the practically significance with Cohen's $d \geq 0.8$. Significance was calculated using raw absorbance data. The black horizontal line indicates the absorbance of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

### 4.4 Acetylcholinesterase activity

In this study water and sediment extracts were given to the cells (HuTu 80 and H4IIE-*luc*) for 24 h before the activity of AChE were determined.

The activity AChE for the HuTu 80 cell line could not be calculated because once the slope was determined across the reaction time of six minutes, the results were negative. This caused the results expressed as absorbance/min/mg protein to also be negative and activity cannot take place against a negative gradient. This was an indication of a failed experiment and how to correct it is addressed in the discussion chapter. However, there was enough of a response from the liver cells that AChE activity could be calculated.

Rat liver cells (H4IIE-*luc*):

None of the samples from the dry season caused an increase or decrease in the AChE activity that was statistically significant ($p<0.05$) (supplementary table 12). Some of the samples caused an increase in the AChE activity that was practically significant ($d\geq0.8$, the + in Figure 39): Borehole water and water samples from Paul Kruger Bridge as well as the sediment sampled from Norman Dennet. None of the sediment and water samples collected at the same locations caused resembling statistical meaningful responses (Figure 39).

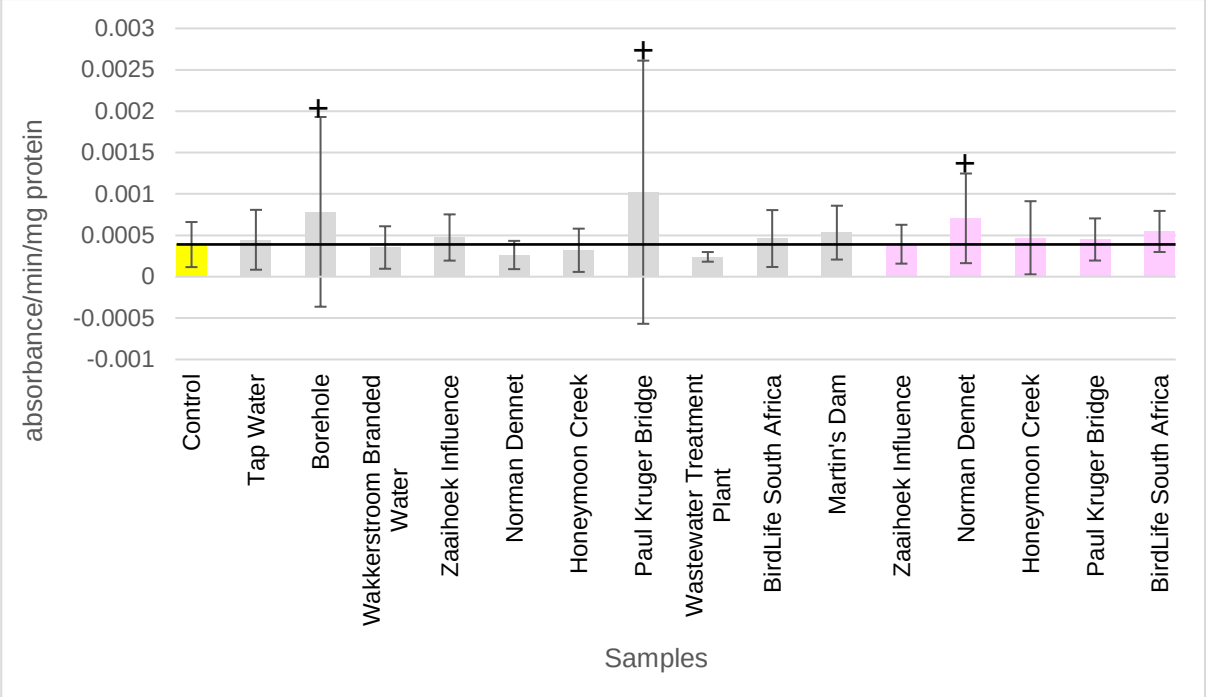



**Figure 39:** Acetylcholinesterase activity for dry season samples given to H4IIE-*luc* cell line. Standard deviation in the results is indicated with error bars, * indicates statistically significant where p<0.05 using Mann Whitney, + indicates the practically significance with Cohen's d≥0.8. Significance was calculated using raw absorbance data. The black horizontal line indicates the absorbance/min/mg protein of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

Two samples collected at the end of the wet season caused a statistically significant increase in the AChE activity in the cells (p<0.05): Water sampled from Martin's Dam and sediment sampled from Zaaihoek Influence (Figure 40). More samples caused a decrease in the AChE activity: There were two that were statistically significant (p<0.05) which included the borehole water and water from Norman Dennet's Place and others were only practically significant (d≥0.8): Tap Water, the Wakkerstroom Branded Bottled water, water from Norman Dennet's, Honeymoon Creek, BirdLife South Africa and sediment from Paul Kruger Bridge and BirdLife South Africa (Figure 40). The sediment and water collected at the same sites showed resembling statistical responses for the different matrixes from BirdLife South Africa. The water sample from Zaaihoek Influence caused a practically significant decrease in AChE activity while the sediment sample caused a statistically significant increase in the enzyme's activity (Figure 40).

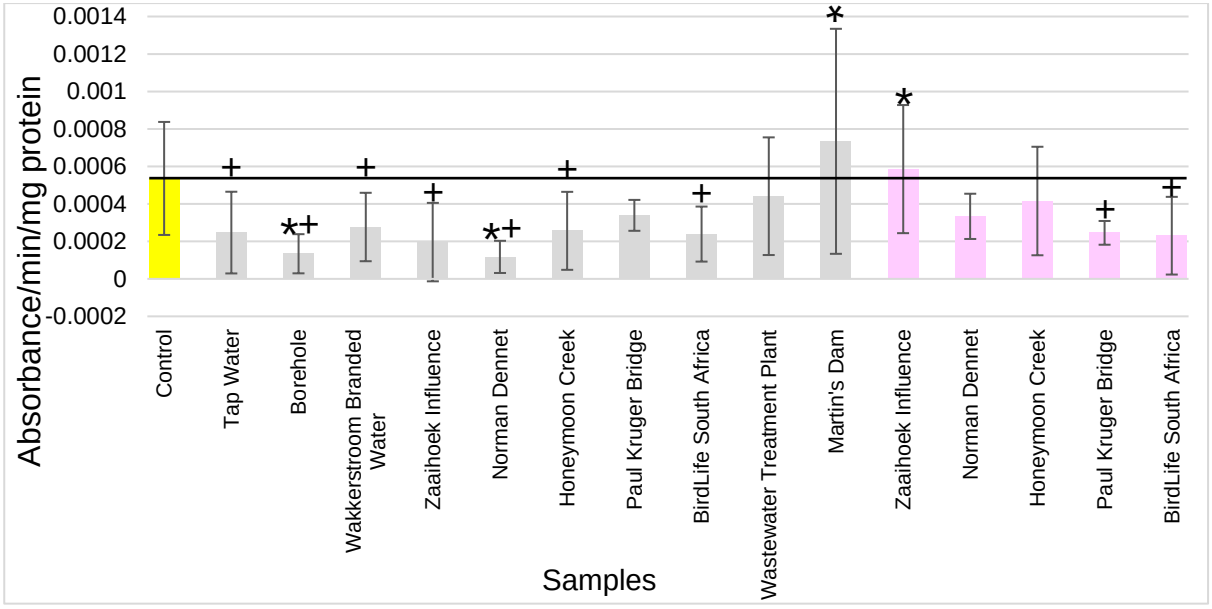



**Figure 40:** Acetylcholinesterase activity in H4IIE-*luc* cells exposed to wet season samples. Standard deviation in the results is indicated with error bars, * indicates statistically significant where p<0.05 using Mann Whitney, + indicates the practically significance with Cohen's d≥0.8. Significance was calculated using raw absorbance data. The black horizontal line indicates the absorbance/min/mg protein of the control throughout the entire graph. The grey bars represent the water samples while the pink bars are representing the sediment samples.

### 4.5 *In vivo* biological (mortality) assays

Water and sediment samples were given to the respective organisms within 24 h after collection. Investigating the effect of the samples on these organisms with mortality and growth inhibition as endpoints.

*Thamnocephalus platyurus*

This species was exposed to a dilution series of the wet season water samples (6.25, 12.5, 25, 50 and 100%) for 24 h before the percentage mortality was determined. Tap Water caused the highest percentage mortality across the concentration range (Figure 41). Water from Paul Kruger Bridge and BirdLife South Africa were the only two sites that did not cause any mortality across the concentration range (Figure 41). All the responses to the water samples were statistically significant (p<0.05) (supplementary table 13).

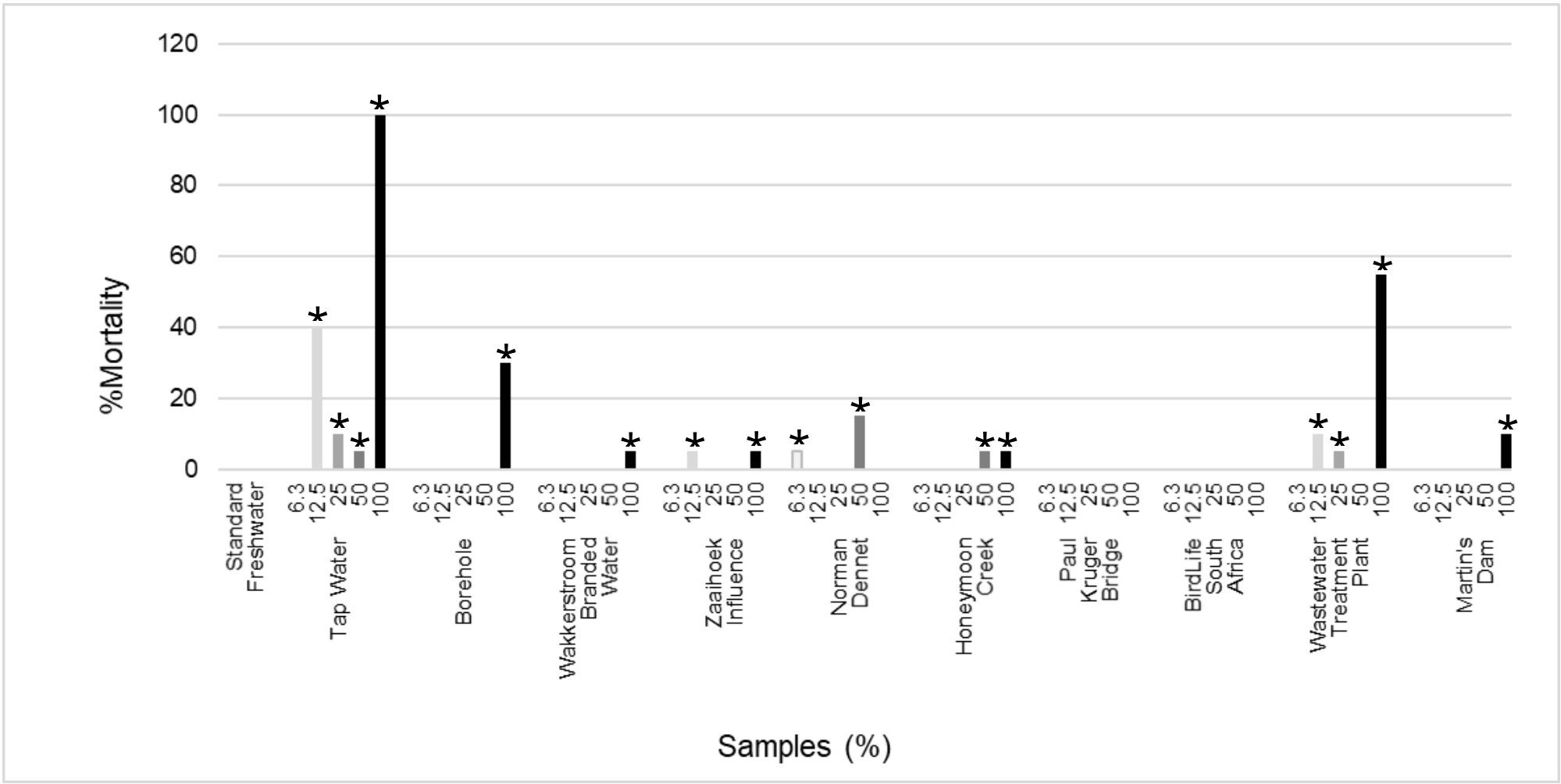



**Figure 41:** Percentage mortality of *Thamnocephalus platyurus* exposed to water samples collected in the wet season. * - indicates statistically significance. Significance was calculated using raw absorbance data.

*Heterocypris incongruens*

This ostracod species was exposed to the sediment, collected in the wet season, for 144 h before the percentage mortality and the growth inhibition was determined (Table 3). The mean percentage growth inhibition for organisms exposed to sediment from Paul Kruger Bridge and BirdLife South Africa could not be determined because there were no surviving ostracods after the exposure period (Table 3). Sediment collected at Zaaihoek Influence had the lowest mean percentage growth inhibition while sediment sampled at Norman Dennet had the highest mean percentage growth inhibition of the samples at which the ostracods did survive (Table 3).

**Table 3:** Results for *Heterocypris incongruens* exposed to sediment from the wet season.

| Site | Mean % growth inhibition in test sediment | Mortality (%) |
|---|---|---|
| Control | 0 | 0 |
| Zaaihoek Influence | 6 | 0 |
| Norman Dennet | 53 | 0 |
| Honeymoon Creek | 21 | 0 |
| Paul Kruger Bridge | – | 100 |
| BirdLife South Africa | – | 100 |

– Did not measure growth inhibition due to 100% mortality. Significance was calculated using raw absorbance data.

### 4.6 Chemical analysis

Water samples from both the dry and wet seasons were extracted and the analytes were separated using a UHPLC. The results were subjected to Bruker Compass DataAnalysis 4.3 software to inspect the peak shapes of the compounds detected. The software uses mass spectra and isotopic distribution patterns to find compounds – and these are referred to in table 4 and 5 as the number of compounds detected per sample. From there on, the accurate mass of each compound with its fragments was run in two databases: Chemical Entities of Biological Interest (ChEBI) and Kyoto Encyclopedia of Genes and Genomes (KEGG) to identify the compound. The Wakkerstroom Branded Water was not chemically analysed because the sample was not provided for the chemical analysis. Compounds from four water samples (Tap Water, Zaaihoek Influence, Honeymoon

Creek, and WWTP) collected in the dry season could be identified (Table 4). Compounds were identified in all the water samples from the wet season, except for the Tap Water (Table 4). Tropinone and oleamide were the compounds that occurred in most of the water samples (Table 4). Most of the samples contained either 49 or 50 total compounds except for water from the Zaaihoek Influence collected in the dry season sample which had 1 739 total compounds (Table 4).

**Table 4:** Compounds identified in water sampled in the dry season.

| Site | Number of compounds detected | Compounds identified in samples | |
|---|---|---|---|
| | | Molecular Formula | Compound name |
| Tap water | 49 | $C_{12}H_{22}N_2O_2$ | 1,8-Diazacyclotetradecane-2,9-dione |
| Borehole | 49 | – | – |
| Zaaihoek Influence | 1739 | $C_{12}H_{18}N_2O_2$ | 3-Hydroxymonoethylglycinexylidide |
| Norman Dennet | 49 | – | – |
| Honeymoon Creek | 49 | $C_{23}H_{41}N$ | Piptamine |
| Paul Kruger Bridge | 49 | – | – |
| BirdLife South Africa | 49 | – | – |
| Wastewater Treatment Plant | 49 | $C_{15}H_{23}N_5O_{10}P_2$ | N6-Isopentenyladenosine |
| | | $C_{14}H_{16}N_6O_2$ | Abacavir 5”-carboxylic acid |
| | | $C_{12}H_{17}NO$ | N,N-diethyl-m-toluamide |
| | | $C_8H_{10}FN_3O_3S$ | Emtricitabine |
| | | $C_{14}H_{20}N_2O_3$ | Feruloylputrescine |
| | | $C_{37}H_{48}N_4O_5$ | Iopinavir |
| Martin’s Dam | 49 | – | – |

– No results.

**Table 5:** Compounds identified in water sampled in the wet season..

| Site | Number of compounds detected | Compounds identified in samples | |
|---|---|---|---|
| | | Molecular Formula | Compound name |
| Tap water | 49 | – | – |
| Borehole | 50 | $C_{15}H_{22}O$ | Alpha-Vetivone |
| | | $C_8H_{13}NO$ | Tropinone |
| Zaaihoek Influence | 50 | $C_{18}H_{35}NO$ | Oleamide |
| | | $C_8H_{13}NO$ | Tropinone |
| Norman Dennet | 50 | $C_{44}H_{69}NO_{12}$ | Tacrolimus |
| | | $C_{18}H_{35}NO$ | Oleamide |
| | | $C_8H_{13}NO$ | Tropinone |
| Honeymoon Creek | 49 | $C_{18}H_{35}NO$ | Oleamide |
| | | $C_8H_{13}NO$ | Tropinone |
| Paul Kruger Bridge | 50 | $C_{37}H_{48}N_4O_5$ | Lopinavir |
| BirdLife South Africa | 50 | $C_{18}H_{35}NO$ | Oleamide |

| | | | |
|---|---|---|---|
| | | $C_8H_{13}NO$ | Tropinone |
| Wastewater Treatment Plant | 50 | $C_{12}H_{17}NO$ | N,N-Diethyl-m-toluamide |
| | | $C_{37}H_{48}N_6O_5S_2$ | Ritonavir |
| | | $C_{37}H_{48}N_4O_5$ | Lopinavir |
| | | $C_{18}H_{35}NO$ | Oleamide |
| Martin's Dam | 50 | $C_8H_{13}NO$ | Tropinone |

– No results.

## 4.7 References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Ayala, A., Muñoz, M.F., and Argüelles, S., 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity*, 2014. https://doi.org/10.1155/2014/360438

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# CHAPTER 5 DISCUSSION

“The important thing is to never

stop questioning.”

– Albert Einstein

### 5.1 The water quality of Wakkerstroom from the biological analysis' perspective

All the biological responses were summarised in Table 6 to enable a comprehensive interpretation of the drinking and environmental water quality of Wakkerstroom. This table distinguishes between the dry season and the wet season sampling events.

The cytotoxicity results are discussed first because it gives a response that is not necessarily linked to a specific mode of action, but it summarises the total 'well-being' of the cells after exposure. The AhR and AR reporter gene assays specifically investigate mechanisms of action mediated through cellular receptors and oxidative stress and AChE assays use enzyme activity to detect certain biological effects. Most of the remarks on cytotoxicity are based on the results of the human breast cancer (MDA-kb2) cells because they were the most sensitive of the two cell lines (rat river cells: H4IIE-*luc*) used (Table 6). There is also more data available for the cytotoxicity of the MDA-kb2 cells because they were exposed to both the sediment and water samples whereas the H4IIE-*luc* cells were only exposed to the sediment samples (Table 6).

In the first three columns (Table 6) the cytotoxicity results are presented by colour grading (green, yellow, orange or red) of the levels of cytotoxicity caused by a sample. None of the drinking water samples, Tap Water, Borehole, and Wakkerstroom Branded Water, were cytotoxic as indicated by the green colour except for Tap Water (dry season) which was moderately cytotoxic (orange) and Wakkerstroom Branded Water (wet season) that was weakly cytotoxic (yellow) (Table 6). The environmental samples (water and accompanying sediment samples) from the wetland and rivers, were mostly weakly cytotoxic (yellow) to moderately cytotoxic (orange), except for three samples that were cytotoxic (red): Zaaihoek Influence water sample (dry season), wet season sediment samples from Paul Kruger Bridge (upstream of wetland) and BirdLife South Africa (middle of wetland) (Table 6). On two occasions the environmental samples showed no cytotoxicity for the more sensitive cell line (MDA-kb2 which was used for testing AR activity), which was the wet season samples collected at Martin's Dam (water) and sediment from Zaaihoek Influence (Table 6).

A figure (Figure 42) was generated from Table 6, in which a *biological effect score* was calculated. This was done by counting each assay, except for cytotoxicity, in Table 6 which showed an effect (note that the sampling events were kept separately). The score depends on the number of successful assays for each sample. If a single assay could distinguish between agonism and antagonism for example: MDA-kb2, that would count as two biological effects which were determined. In the case where an assay could be expressed in two ways (growth inhibition and mortality), such as the ostracod (*Heterocypris incongruens*) test it was counted as a single response. When growth inhibition was >50% it was given one point, but the 21% growth inhibition was not regarded as worth counting thus it did not receive a point. Mortality results received one point, there was no cut-off percentage because all the mortality responses seen in *T. platyurus* were statistically significant compared to the control. Regarding the oxidative stress and AChE assays where two cell lines were used to investigate the same effect, one point was given when either one or both cell lines showed a response (Table 6). For example: Honeymoon Creek sediment, wet season, had three biological responses out of the eight biological effects tested and therefore the site received a score of 37.5% (Figure 42). A score was not allocated to cytotoxicity because often biological responses were seen, nonetheless for example Zaaihoek Influence water sample (dry season) was cytotoxic but oxidative stress responses could be measured (Table 6).

**Table 6:** Summary of the responses from all the assays caused by dry and wet season samples as well as the chemical compounds identified in the water samples.

| Site | *In vitro* | | | | | | | *In vivo* | | | Chemical analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Endocrine disruption | | Xenobiotic metabolism | Oxidative stress | | | Non-neuronal | *Thamnocephalus platyurus* | *Heterocypris inconguens* | | |
| | AR ↑ | AR ↓ | AhR ↑ | ROS | SOD | CAT | AChE activity | %Mortality | %Growth inhibition | %Mortality | Identified compounds |
| **Dry season** | | | | | | | | | | | |
| Tap Water | | | | ↓p,d | | | | | | | 1,8-Diazacyclotetradecane-2,9-dione |
| Borehole | | | | ↓p,d | ↑p,d | ↑p,d↑p,d | ↑d | | | | |
| Wakkerstroom | | | | | | | | | | | |
| Branded Water | | | | ↓p | | ↑ p,d | | | | | |
| Zaaihoek Influence | | | | ↑p,d↓p,d | | ↑ p,d | | | | | 3-Hydroxymonoethylglycinexylidide |
| Norman Dennet | | | | | | ↑ p,d | | | | | |
| Honeymoon Creek | | | | ↑p,d | | ↑ p,d | | | | | Piptamine |
| Paul Kruger Bridge | | | | ↑p,d | | ↑ p,d | ↑d | | | | |
| BirdLife South Africa | | | | ↑p,d | ↑p,d | ↑ p,d | | | | | |
| WWTP | ✓ | | | ↑d | ↑p,d | ↑ p,d | | | | | N6-Isopentenyladenosine, Abacavir 5”-carboxylic acid, N,N-diethyl-motoluamide, Emtricitabine, Feruloylputrescine, Iopinavir |
| Martin’s Dam | | | | ↓d↑p,d | | | | | | | |
| Zaaihoek Influence Sediment | | ✓ | | ↑d | ↑d | ↑ p,d ↓p | | | | | |
| Norman Dennet Sediment | | ✓ | | ↓p,d↑p,d | ↑p,d | ↑ p,d ↓p | ↑d | | | | |
| Honeymoon Creek Sediment | | | | ↓p,d↓p,d | ↓p,d↓p | ↑d↓p | | | | | |
| Paul Kruger Bridge Sediment | | | | | ↓p | ↑d | | | | | |
| BirdLife South Africa Sediment | | | | ↑p,d↓p,d | ↑p,d | ↑d↑p | | | | | |
| **Wet season** | | | | | | | | | | | |
| Tap Water | | | | ↑p,d | ↑d | ↑d | ↓d | ✓ | | | Alpha-Vetivone, Tropinone |
| Borehole | | | | ↑p↑p | | | ↓p,d | ✓ | | | |
| Wakkerstroom | | | | | | | | ✓ | | | |
| Branded Water | | | | ↑p↑p,d | | ↑p | ↓d | | | | |

| Site | *In vitro* | | | | | | | *In vivo* | | | Chemical analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Endocrine disruption | | Xenobiotic metabolism | Oxidative stress | | | Non-neuronal | *Thamnocephalus platyurus* | *Heterocypris inconguens* | | |
| | AR ↑ | AR ↓ | AhR ↑ | ROS | SOD | CAT | AChE activity | %Mortality | %Growth inhibition | %Mortality | Identified compounds |
| Zaaihoek Influence | | | | ↑p,d | ↑p,d | ↑d | ↓d | ✓ | | | Oleamide, Tropinone |
| Norman Dennet | | | | ↑p,d | ↑p | | ↓p,d | ✓ | | | Oleamide, Tropinone, Tacrolimus |
| Honeymoon Creek | | ✓ | | ↑p,d | ↑p | ↓p | ↓d | ✓ | | | Oleamide, Tropinone |
| Paul Kruger Bridge | | | | ↓p,d↑p,d | ↓p | ↓p | | × | | | Lopinavir |
| BirdLife South Africa | | ✓ | | ↓p,d↑p,d | | | ↓d | × | | | Oleamide, Tropinone |
| WWTP | | | | ↓p,d↑p,d | ↓p | ↓p | | ✓ | | | N,N-Diethyl-m-toluamide, Ritonavir, Lopinavir, Oleamide |
| Martin's Dam | | | | ↑p,d | ↑p,d | ↑p↓p | ↑p | ✓ | | | Tropinone |
| Zaaihoek Influence Sediment | | | | ↓p,d↑p,d | ↑d | | ↑p | | 6 | 0 | |
| Norman Dennet Sediment | | | | ↓p,d↑p | ↑d | ↑p | | | 53 | 0 | |
| Honeymoon Creek Sediment | | | ✓ | ↓p,d↑d | | ↓p | | | 21 | 0 | |
| Paul Kruger Bridge Sediment | | | ✓ | ↑p | ↑d | ↑d | ↓d | | N/A | 100 | |
| BirdLife South Africa Sediment | | | | ↑p↑p,d | | | ↓d | | N/A | 100 | |

Keys to colours and symbols used in Table 6

| | | | | |
|---|---|---|---|---|
| Water categories | Drinking water | | | Environmental samples |
| Levels of cytotoxicity | Not cytotoxic | Weakly cytotoxic | Moderately cytotoxic | Cytotoxic |
| ✓ | Activation or inhibition responses of receptors tested. Also indication of mortality detected in *T. platyurus* exposures to water samples. | | | |
| ↑↓ | Increased (↑) or decreased (↓) responses in the HuTu 80 cell line. | | | |
| ↑↓ | Increased (↑) or decreased (↓) responses in H4IIE-*luc* cell line. | | | |
| p | Statistically significant p-value ($p<0.05$) determined with Mann-Whitney U test. | | | |
| d | Practically significant Cohen's d-value (≥0.8). | | | |
| | Did not perform assay with that matrix | | | |
| | Results were not statistically and/or practically significant | | | |

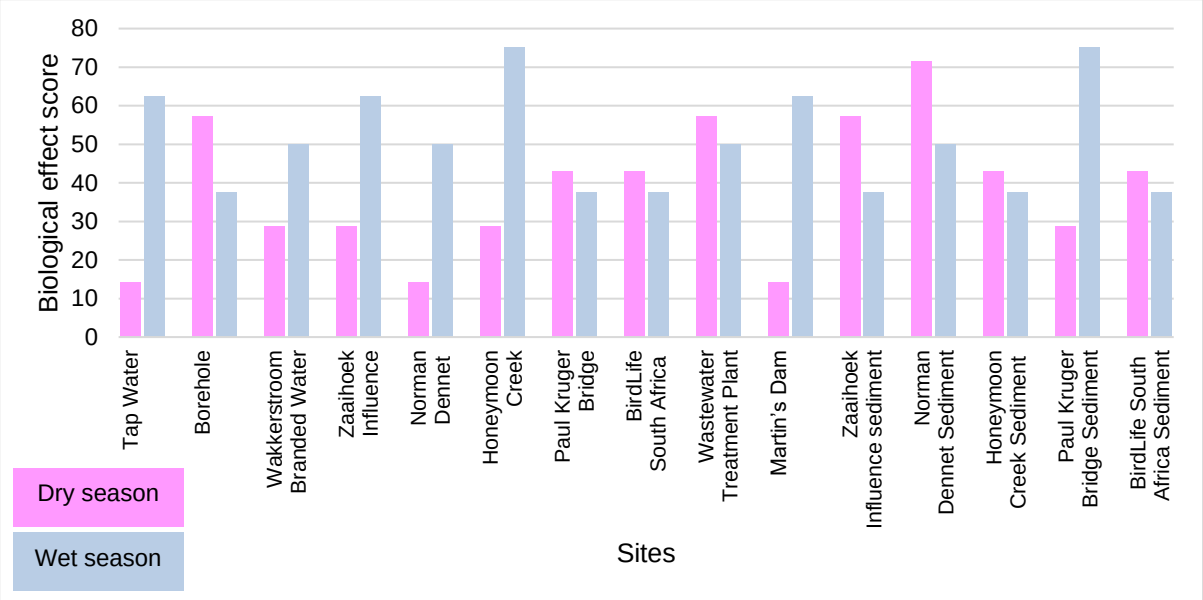


**Figure 42:** The biological effect score for all the sites, both seasons.

## 5.2 Overall biological effect scores

The biological effect scores were calculated based on the number biological effects seen per sample potentially caused by the chemical mixtures present in the sample extracts. Based on the biological effect scores (Figure 42) the dry and wet seasons' sites were separately arranged in water quality decreasing order, solely based on the assays used in this study (Table 7).

**Table 7:** Comparison of water quality based on the sampling season.

| Water quality | | Dry season | | Wet season |
|---|---|---|---|---|
| Decreasing order | 1 | Tap Water<br>Norman Dennet water<br>Martin's Dam water | 1 | Borehole water<br>Paul Kruger Bridge water<br>BirdLife South Africa water<br>Zaaihoek Influence sediment<br>Honeymoon Creek sediment<br>BirdLife South Africa sediment |
| | 2 | Wakkerstroom Branded Water<br>Zaaihoek Influence water<br>Honeymoon Creek water<br>Paul Kruger Bridge Sediment | 2 | Wakkerstroom Branded Water<br>Norman Dennet water<br>Wastewater Treatment Plant<br>Norman Dennet sediment |
| | 3 | Paul Kruger Bridge water<br>BirdLife South Africa water<br>Honeymoon Creek sediment<br>BirdLife South Africa sediment | 3 | Tap Water<br>Zaaihoek Influence water<br>Martin's Dam water |
| | 4 | Borehole water<br>Wastewater Treatment Plant<br>Zaaihoek Influence sediment | 4 | Honeymoon Creek water<br>Paul Kruger Bridge sediment |
| | 5 | Norman Dennet sediment | | |

From the arrangements above it is interesting to see how the order of water quality at the sites differed between the two sampling seasons (Table 7). The Tap Water sample from the dry season had the lowest score whereas the wet season Tap Water sample received

the third lowest score. Dry season Borehole water had the second highest score while the wet season sample received the lowest score. Generally, the sediment collected in the dry season received overall higher scores whereas the wet season sediment samples mostly had lower scores except for sediment collected from Paul Kruger Bridge. For the dry season samples, the water samples generally received lower scores, while the sediment samples had higher scores. The wet season samples' overview is a bit different because the sediment samples received mostly the lower scores whereas the water samples had higher scores.

Tap Water and water from Martin's Dam had the same biological effect scores for both seasons (Table 7), which makes sense because Martin's Dam is the source of drinking water for the town. The dry season water sample collected at Zaaihoek Influence has the second lowest score which is a good sign because it is indicating that the wetland did retain the toxicants that entered the river from the WWTP, Paul Kruger Bridge and Martin's Dam. The responses seen in the Zaaihoek Influence sediment from the dry season which received the second highest biological effect score is most likely due to the pollutants adsorbed to the sediment.

The order of the wet season samples changed completely compared to the dry season samples. It might be due to the rain events that took place before the sampling event and the additional *in vivo* tests performed with the wet season samples (more assays performed with the wet season samples). The water samples collected from the wetland were the cleanest this time around (Paul Kruger Bridge and BirdLife South Africa) (Figure 42). Some of the sediment samples also received the lowest biological effect score: BirdLife South Africa, Honeymoon Creek, and Zaaihoek Influence (Figure 42). Water from Martin's Dam and the Tap Water received the higher biological effect scores which could be due to chemical compounds that were either transported to these sites from the surrounding hills or the chemical compounds got resuspended into the water column during the rain events (Geranmayeh et al., 2018).

### 5.3 Detailed discussion of biological effects

Tap Water and Martin's Dam water samples both showed the lowest biological effect scores for the dry season (Figure 42). Both these sites only showed a response for ROS production (Table 6). Interestingly both these sites are drinking water sources and they had a score of 62.5% for their wet season water samples and responses were seen for the oxidative stress assays, AChE activity, and *in vivo*, *T. platyurus*, assay (Table 6). The high mortality percentage in *T. platyurus* caused by the Tap Water might be due to the chemicals added during the disinfection processes or the disinfection by-products that were formed during these processes. Chlorination is the most common disinfection process used at drinking water treatment plants (Ding et al., 2019) and is likely done at the Wakkerstroom plant too, which is not a sophisticated plant. as it is a class D water treatment plant. According to the Water Services Act (no. 108 of 1997) all water treatment plants are classified based on a scoring system (DWA, 2013). In this scoring system points are allocated according to a specific criteria including the size of the population supplied, operating prosedures (raw water quality, chemical dosing, and raw water flow rate) (DWA, 2013). A classification certificate is awarded to a plant indicating the class of the plant based on the total amount points that was awarded. For a class D rating a score of between 30–49 was awarded to the Wakkerstroom drinking water treatment plant (DWA, 2013). At the water treatment plants it is necessary to maintain a sufficient disinfectant residual such as chlorine in the distribution system to prevent unwanted microbes to regrow in the water (Ding et al., 2019).

Disinfectants such as free chloramines, ozone, chlorine dioxide and chlorine have the potential to react with different constituents in the water including pharmaceuticals, detergents, pesticides, natural organic matter, iodide, and bromide. These reactions form products such as trihalomethanes, haloacetaldehydes, haloacetic acids, and N-nitrosodimetylamine that are cytotoxic, carcinogenic, and genotoxic (Ding et al., 2019; Gilca et al., 2020). According to Brunner et al. (2019) transformation products have the potential to exhibit increased toxicity in comparison to their parent compounds. Several studies have found that *T. platyurus* is specifically sensitive to sodium, chloride and formate ions (Melnyk et al., 2014; Sulej et al., 2014).

In both the dry and wet season Tap Water samples there were 49 different compounds present (section 4.6) but only one compound could be identified in the dry season sample: 1,8-diazacyclotetradecane-2,9-dione, which is a ketone. In Martin's Dam samples, for both dry and wet seasons, there were also 49 compounds (section 4.6) but only tropinone was identified in the wet season sample. Tropinone is a key intermediate in synthesising alkaloids such as atropine, hyoscyamine and cocaine (Piechowska et al., 2020).

Tap Water sampled in the wet season inhibited the AChE activity in the H4IIE-*luc* cells (Table 6). As mentioned in section 2.5.4 the inhibition of AChE activity is a common endpoint used in water quality assessment for specific neurotoxicity (Escher et al., 2021). According to Wessler and Kirkpatrick (2008) the signalling pathways for the non-neuronal cholinergic cells are comparable with cholinergic neurotransmission. Non-neuronal AChE is found in the epithelial cells in the intestine, airways, skin, placenta, placenta, vagina, immune cells, liver and cornea (Pérez-Aguilar et al., 2015; Wessler & Kirkpatrick, 2008). In this study there were statistically significant changes in the AChE activity in the H4IIE-*luc* cells when compared to the control and according to Wessler and Kirkpatrick (2008) the changes in AChE activity could cause a dysfunction of the non-neuronal cholinergic system (NNCS). This is a cause for concern because the dysfunction of the NNCS plays a role in the pathophysiology of diseases (Wessler & Kirkpatrick, 2008).

The activity AChE for the HuTu 80 cell line could not be calculated as explained before (section 4.4). There are two types of cholinesterase enzymes present in mammals namely: AChE and butyrylcholinesterase (BChE) (Forsgren et al., 2009). Even though AChE is present in the small intestine, the presence of BChE is more ubiquitous in the gastrointestinal tract (Severi et al., 2022). It is possible that the AChE activity in the small intestine cells (HuTu 80) were below limit of detection and BChE activity might be easier to detect.

Acetylcholinesterase activity is reportedly inhibited by metals such as mercury, zinc, cadmium, and copper (Frasco et al., 2005), organophosphorus and carbamate pesticides (Lionetto et al., 2013), as well as inhibitors that occur naturally such as fungal antibiotic (puromycin) and solanaceous glycoalkaloids that occurs in the Solanaceae plants such as potatoes, tomatoes, and eggplant (Krasowski et al., 1997). Citizens of Wakkerstroom

might have vegetable gardens which could explain the presence of these plants in the area and how it ended up in the water sources. Pesticides such as chlorpyrifos and parathion do not have the ability to directly inhibit AChE but cytochrome P450 metabolically activates these compounds allowing them to inhibit the enzyme (Lionetto et al., 2013).

The Norman Dennet dry season water sample also had one of the lowest biological effect scores (Figure 42) and this sample only showed a response in CAT activity (Table 6). The wet season water sample from Norman Dennet is one of the samples that had the second lowest score (Figure 42), responses were seen in oxidative stress and *in vivo*, *T. platyurus,* assay. The issues regarding *T. platyurus* and possible influences on their response was discussed previously in this section, thus it is not repeated here. The dry season water sample from Norman Dennet had 49 compounds but none of them could be identified whereas the wet season water sample from this site contained 50 compounds and three compounds were identified: tacrolimus, oleamide, and tropinone (Table 5). Oleamide is known as a sleep-inducing agent (Mendelson & Basile, 2001) and tacrolimus is usually prescribed to patients that had organ transplants to prevent the body from rejecting the new organ (Stewart, 2020). This compound decreases the activity of the immune system.

Norman Dennet's sediment sample received the highest biological effect score for the dry season, whereas the wet season sample was also one of the samples with the second lowest biological effect scores (Figure 42). The dry season sediment sample, from this site, inhibited the AR, showed oxidative stress and AChE activity responses (influences on the AChE were discussed above) (Table 6).

The AR inhibition results are summarised in table 8, it is also compared to the effect-based trigger value. The Norman Dennet sediment sample had an $ECSR_{0.2}$ value that exceeded the effect-based trigger value that Escher et al. (2018) established specifically for the MDA-kb2 cell line (Table 8). The effect-based trigger value indicates the acceptable risk for a mixture of compounds occurring in a sample (Escher et al., 2018). Exceeding the trigger value, the possibility exists that vertebrate organisms will

experience adverse effects upon exposure to the sample either by ingestion or by living in the aquatic environment where the sample was collected.

**Table 8:** Comparison of AR inhibition results to effect-based trigger value.

| Site | Season sampled | Matrix | $REP_{50}$ (ng F eq/g, ng F eq/µL) | $REP_{25}$ (ng F eq/g, ng F eq/µL) | $ECSR_{0.2}$ | Compared to effect-based trigger value (3500 ng/L) (Escher et al., 2018) |
|---|---|---|---|---|---|---|
| Zaaihoek Influence | Dry | Sediment | 0.44 | 0.63 | 3 000ng/L | < |
| Norman Dennet | Dry | Sediment | 1.10 | 1.29 | 24 000ng/L | > |
| Honeymoon Creek | Wet | Water | 1 242.4 | 249.7 | 4 240 ng/L | > |
| BirdLife South Africa | Wet | Water | 955.1 | 225.1 | 4 840 ng/L | > |

ng F eq/g – sediment, ng F eq/µL – water, F – flutamide, > – higher

The response by the Norman Dennet sample (Table 7) might be due to the livestock growth stimulants, that are commonly applied in the agricultural sector, and is adding to the androgens and oestrogens naturally occurring in the environment (Liu et al., 2012, Truter et al., 2017). There was cattle manure in the areas where these samples were collected from which stimulants might have leached and ended up in the water. Other possible antiandrogens that might have added to the responses seen in this study are crude oil spills (Vrabie et al., 2010), pesticides (Archer & van Wyk, 2015), flame retardants (Kojima et al., 2009), bisphenol A and phthalates (plastics) (Sohoni & Sumpter, 1998). To prevent or slow the growth of a fire, flame retardants are applied to the materials such as curtains, mattresses, seat covers and fillings, phones, household appliances, cables, wires, and bumpers (NIEHS, 2021). Bisphenol A (BPA) is used to produce polycarbonate plastic which in turn is used to make any hard plastic items such as re-useable bottles, baby bottles, storage containers and it is also present in the inner lining of food cans, sunscreens, nail polish, and toys. The BPA in these items is released during production, packaging, handling, and transport (Adeyi & Babalola, 2019). Landfills are overflowing with appliances, mattresses, broken electronics, plastics containing BPA, and other items that people throw away. These items start to weather due to constant exposure to heat, UV-radiation, and rain and with the rain the chemicals (i.e. BPA and flame retardants) are transported to the water sources (Arp et al., 2021). Phthalates are present in

pharmaceuticals and personal care products (such as shampoo) thus it is inevitable that phthalates will end up in the water (Wang & Qian, 2021).

Kinani et al. (2010) tested sediment samples collected from different rivers which are in the north of France for AR activity using MDA-kb2 cells. Androgen receptor activation was found in two of the five sites with a $REP_{25}$ of 0.40 and 3.6 ng dihydrotestosterone-eq/g sediment (Kinani et al., 2010). Sediment collected at three of the sites caused AR inhibition and they reported the $REP_{25}$ as 1 100, 7 400, and 32 500 ngF/g. The latter is sediment that was sampled at a site that was heavily impacted by chemical contamination and was under anthropogenic pressure (Kinani et al., 2010). Northern France has a population of more than 12 million (Statista, 2022) compared to the almost seven thousand residents of Wakkerstroom counted in the 2011 census (Frith, 2011), unfortunately more recent census data for Wakkerstroom are not available, thus the higher results reported by Kinani et al. (2010) might be explained by the denser population in the north of France causing higher anthropogenic pressure and the chemical contamination at higher concentrations.

Shuliakevich et al. (2022) tested water and sediment from the Wurm River, in west Germany, for AR inhibition during the dry season as well as the rainy season using AR-CALUX. The Wurm River receive water from a WWTP where the more advanced water treatment process, ozonation, is used to eliminate EDCs (Shuliakevich et al., 2022). Water collected in the dry season caused AR inhibition with $REP_{50}$ values of 0.0443±0.0113 ngFeq/µL while in the wet (rainy) season the $REP_{50}$ values for the same site were between 0.092±0.0373 ngFeq/µL. The sediment collected at the same site as the water had a $REP_{50}$ value of 4 800±1 800 ngFeq/g during the dry season and 8 700±10 900 ngFeq/g during the wet season (Shuliakevich et al., 2022). Shulaikevich et al (2022) reported similar findings to this study where endocrine disruptive activity increased in the water phase of the Wurm River, Germany, after extensive rains. When the AR inhibition results for the sediment of this study ($REP_{25}$ and $REP_{50}$ values) are compared to the values reported by Kinani et al. (2010) and Shuliakevich et al. (2022) they are much lower, which might support the possibility of this study location, Wakkerstroom, to become a future reference site.

Water samples from Wakkerstroom Branded Water and Zaaihoek Influence both got the second lowest score for their dry season samples and second and third lowest scores respectively for their wet season water samples (Figure 42). For the dry season both samples showed responses for ROS production and CAT activity (Table 6). Due to unforeseen circumstances, the Wakkerstroom Branded Water was not chemically analysed. The number of compounds present in the dry season Zaaihoek Influence water sample was 1 739 but unfortunately only one compound was identified: 3-hydroxymonoethylglycinexylidide (Table 4) which is a metabolite of lidocaine. Lidocaine is used to treat skin inflammation symptoms such as itching, pain, and burning. It is also used as a local or dental anaesthetic in small surgeries (Doran et al., 2018).

In the wet season water sample from Zaaihoek Influence there was 50 compounds present and two compounds could be identified: oleamide and tropinone again (Table 5). Zaaihoek Influence sediment dry season sample got the second highest biological effect score and the lowest score for the wet season sample (Figure 42). The dry season sediment sample inhibited the AR (discussed above) and showed oxidative stress and AChE activity (discussed above) responses. Zaaihoek Influence sediment is the sample with an $ECSR_{0.2}$ value that was not exceeding the effect-based trigger value (Table 8).

Both the Honeymoon Creek water sample and Paul Kruger Bridge sediment sample received the second lowest biological effect score for the dry season and for the wet season both sites got the highest scores (Figure 42). Honeymoon Creek water sample for the wet season inhibited the AR, showed oxidative stress and AChE responses and cause mortality of *T. platyurus* (discussed above). This site's $ECSR_{0.2}$ value is exceeding the effect-based trigger value and as explained earlier it is a cause for concern (Table 8). The AR inhibition could be explained by the visible cattle manure which was present in the area around the site which could explain that stimulants might have leached out of the manure and ended up in the water (Liu et al., 2012). In a study done by Thresher (2023) it was found that anti-androgens are among the hormones present in cattle manure. The dry season water sample contained 49 compounds and piptamine was identified as one of these compounds present in Honeymoon Creek water sample (Table 4). The antibiotic piptamine is produced by a mushroom *Piptoporus betulinus* Lu 9–1 (now known as *Fomitopsis betulina*) (Schlegel et al., 2000). This mushroom does not grow in

South Africa, but it is used as a natural medicine worldwide (Pleszczyńska et al., 2017). This mushroom has medicinal properties such as anti-parasitic, immunoenhancing (against several cancer types), painkiller abilities, and can be used as an antiseptic. It is likely that Wakkerstroom residents or tourists, visiting this town, use this mushroom as a traditional medicine (Grienke et al., 2014). In the wet season water sample, there were also 49 compounds present and again oleamide and tropinone were identified (Table 5).

The biological effect score for Honeymoon Creek sediment sample was the third lowest in the dry season and the lowest in the wet season (Figure 42). The dry season sample only showed oxidative stress responses while the wet season sample activated the AhR and caused oxidative stress responses. In this study the sediment extracts were acid washed and this supposedly removed all the non-persistent compounds such as polycyclic aromatic hydrocarbons (PAHs) from the extracts (Yoo et al., 2006). Note that even though the acid wash clean-up step was performed to remove the non-persistent dioxin-like compounds it is possible that not all these dioxin-like compounds were removed. It has been reported that dioxin-like compounds with low molecular mass such as benzo (a)fluoranthene and other PAHs compounds with 2–3 rings, were only 80% removed by the acid wash (Lamoree et al., 2004). Some of these could still have been present in the sample extract and responsible for the AhR response. Sediment sampled at Honeymoon Creek and Paul Kruger Bridge during the wet season both caused a result of 40% TCDDMAX and the calculated $REP_{20}$ was respectively $1.04x10^{-7}$ and $3.45x10^{-7}$ ngTCDDeq/g sediment. The lack of a full dose-response curve gained by both sites might be due to too few ligands present in the sample to activate all the receptors. The activation of AhR, in this study, might also have been caused by persistent dioxin-like compounds referring to those that take a long time to break down in the environment. The dioxin-like compounds could have been released into the environment through forest fires, the burning of household and industrial waste, the degradation of pentachlorophenol by sunlight, the chlorine bleaching of wood pulp, residential wood burning, and dioxin-like compounds that are formed during the chlorination process in WWTPs (Institute of Medicine, 2003).

The HuTu 80 cells were exposed to the Honeymoon Creek sediment extracts which caused a decrease in the ROS production and the SOD activity decreased as well while

the CAT activity increased in the cells (Table 3). Reactive oxygen species in the cells must have increased during the exposure time to stimulate the SOD activity which dismutated the superoxide anion and produced molecular oxygen and $H_2O_2$. The dismutation caused the produced ROS levels to decrease in the cells (Table 3). Superoxide dismutase activity decreased as the number of available superoxide anions, that need to be converted, decreased. Catalase is responsible for completing the detoxification that SOD started. The activity of CAT increased as the levels of $H_2O_2$ increased, this enzyme convert $H_2O_2$ into water and molecular oxygen. According to Ventura et al. (2015) it is also possible that the SOD activity was inhibited by high levels of $H_2O_2$ because this enzyme is sensitive to its own product.

The Paul Kruger Bridge wet season sediment sample activated the AhR, caused oxidative stress and AChE activity responses (discussed above) and 100% mortality of *Heterocypris incongruens*. For the AhR activation, many studies report the $REP_{50}$, thus I used the extrapolated $REP_{50}$ of my study. The predicted $REP_{50}$ calculated for the sediment sampled at Honeymoon Creek and Paul Kruger Bridge was respectively $3.7x10^{-6}$ and $1.1x10^{-5}$ ngTCDDeq/g. Yoo et al., (2006) tested sediment samples which they collected from two main locations in the west and south-eastern coast of Korea, Lake Shihwa and Masan Bay. They too treated their sediment extracts with acid as a clean-up step before commencing the assays with H4IIE-*luc* cells. The $REP_{50}$ for the samples collected at Masan Bay ranged from 0.017–0.275 ngTCDD/g and a much higher $REP_{50}$ was reported at Lake Shihwa, 0.868 ngTCDD/g (Yoo et al., 2006). Otte et al. (2013) sampled sediment in the estuary of River Elbe in Germany to test for AhR activation using H4IIE-*luc* cells. The mean $REP_{50}$ for the tested samples was $3.19x10^{-12} \pm 1.38 x10^{-12}$ ngTCDDeq/g.

Xu et al. (2015) tested sediment from the freshwater Taihu Lake in China using the H4IIE-*luc* cells. The $REP_{50}$ for this study ranged from 0.173–0.770 ngTCDDeq/g dry mass. Celma et al. (2021) reported the activation of the AhR when exposed to the water sampled from the Spanish Mediterranean coastline. They reported AhR activation of $REP_{10}$ between $2.48x10^{-9}$–$7.15x10^{-9}$ ngTCDDeq/g (Celma et al., 2021). They used HepG2 cells (human hepatocellular carcinoma) to investigate activation of the AhR whereas in this study rat hepatoma cells were used, and they exposed the cells to water extracts while

this study made use of sediment extracts. The $REP_{10}$ for this study for both sites that activated the AhR was $3.17x10^{-8}$ (Honeymoon Creek) and $1.1x10^{-7}$ ngTCDDeq/g. These results are more comparable with those reported by Celma et al., (2021) but the differences (matrix tested and the type of cells used) between the studies should be taken into consideration as it might be important variables. The $REP_{10}$ and the predicted $REP_{50}$ values of this study falls in the same range as the studies mentioned above except for the results reported by Xu et al (2015) and Yoo et al. (2006) which are higher than the results of this study. This is most probably due to Europe and Asia being two of the continents that are the most polluted with dioxins in the world (Dopico & Gómez, 2015). Note that in the study reported by Xu et al. (2015) the acid-wash clean-up step was not applied to their extracts thus their extracts contained all the non-persistent dioxin-like compounds present in the samples, and it might also explain the high responses that they reported.

The tendency of the wet season samples to cause more responses than samples from the dry season can be explained that large quantities of pollutants are transported during rainfall events and directly enters the receiving water, threatening the hydro-ecological environment (Yang et al., 2021). Thus, it makes sense that chemical compounds washed from the surrounding hills and higher areas into the river, wetland, and dam.

Extracts from the wet season Paul Kruger Bridge sediment sample killed all the ostracods. *Heterocypris incongruens* is considered as a true benthic organism because this ostracod species spends a part of its life swimming and another part in and on the sediments (Sevilla et al., 2013). Several interactions between the sediment constituents have the potential to attenuate and/or amplify the toxic effects obtained from exposure to the polluted sediment (Nolte et al., 2020). Pollutants accumulate in the sediment and could be re-released into the overflowing water through biological, chemical, and physical processes (Cloete et al., 2017). Site-specific contamination in sediment is not likely to change due to the slow mobility of the sediment, while with water the sample is only a snapshot of that site's potential effect of pollutants at the time of sampling only (Cloete et al., 2017).

It is interesting to note that the upstream site of the wetland (Paul Kruger Bridge) and BirdLife which is in the middle of the wetland, caused the highest percentages of mortality. Norman Dennet, which is located where the water is flowing out of the wetland just after the Honeymoon Creeks' stream enters the water leaving the wetland, caused the highest percentage of growth inhibition (Table 6). Zaaihoek Influence had the lowest percentage of growth inhibition, affecting the aquatic organisms the least. It is important to note that the Zaaihoek Influence sediment sample from the dry season caused AR inhibition, while the wet season sample affected the *H. incongruens* the least and this might create a contradicting overall view of the Zaaihoek Influence site. With that being said it is important to note that the chemical compounds that caused the AR inhibition might not have any effect on the *in vivo* tests and *vice versa*. It has been reported that *H. incongruens* is sensitive to PAHs (Čvančarová et al., 2013), naproxen and diclofenac (nonsteroidal anti-inflammatory drugs) (Pawłowska et al., 2021), and heavy metals (Cloete et al., 2017; Kudlak et al., 2011).

Paul Kruger Bridge water sample got the third lowest and the lowest biological effect scores for the dry and wet seasons respectively (Table 8). This water sample caused oxidative stress and AChE activity (discussed above) responses. The dry season water sample contained 49 compounds but none of them could be identified, and the wet season sample contained 50 compounds of which one could be identified as lopinavir (Table 5). Lopinavir is a human immunodeficiency virus anti-retroviral (HIV-ARV) medication (Oldfield et al., 2005). According to Satoh and Boyer (2019) South Africa are home to a total of 7.2 million HIV-positive individuals making South Africans the seropositive population of the world. The use of HIV-ARV medications is thus very common in South Africa, and it is most likely that there are citizens in Wakkerstroom that are using these medications which will explain how the HIV-ARV medications ended up in the water system. In fact, the presence of HIV-ARV drugs in the aquatic environment of South Africa had been reported (Swanepoel et al., 2015; Wood et al., 2015, Horn et al., 2022).

BirdLife South Africa's water and sediment samples have the same biological effect scores with the third lowest in the dry season and the lowest in the wet season (Figure 42). The sediment samples from both the dry and wet seasons only caused responses

for oxidative stress and AChE activity (discussed above). The dry season water sample only showed oxidative stress responses, while the wet season sample inhibited the AR and decreased the AChE activity. In the dry season water sample, there were 49 compounds present and 50 compounds in the wet season water sample from BirdLife South Africa (section 4.6). From the wet season water sample two compounds could be identified: again, oleamide and tropinone. The wet season BirdLife South Africa sample exceeded the $ECSR_{0.2}$ effect-based trigger value (Table 8).

Borehole water from the dry season received the second highest biological effect score while the wet season got the lowest score (Figure 42). The dry season sample caused oxidative stress and AChE responses whereas the wet season showed responses for those assays but also mortality of *T. platyurus*. Both the dry and wet season samples contained 50 compounds, but only two compounds could be identified for the wet season sample namely (section 4.6): alpha-vetivone and tropinone. Alpha-Vetivone is an organic compound that is isolated from the roots of the grass *Vetiveria zizanioides* (Kirici et al., 2011). This compound forms a major part of vetiver oil which is used to make high-value perfumes (Kim et al., 2005). Although this grass species is not found in South Africa, the essential oil is commercially available and is used in personal care products (Burger et al., 2017) which might explain how the compound was present in the water sample.

The effluent from the Wastewater Treatment Plant received the second highest biological effect score for the dry season and the second lowest score for the wet season (Figure 42). The dry season sample activated the AR and caused responses for oxidative stress while the wet season sample showed *T. platyurus* mortality and oxidative stress responses (discussed above).

The sample from the dry season contained 50 compounds and six of these could be identified: N6-Isopentenyladenosine, abacavir 5”-carboxylic acid, N,N-diethyl-m-toluamide, emtricitabine, feruloylputrescine, and lopinavir (Table 4). Abacavir 5”-carboxylic-acid is a metabolite of the HIV-ARV drug, abacavir (Fernandez & Munir, 2022). N,N-diethyl-m-toluamide is an active ingredient of insecticides. This insect repellent can be applied on skin and clothing to protect against mosquitoes, ticks, flies and fleas (Legeay et al., 2016). The purine, N6-Isopentenyladenosine, is present in mammalian

cells and is excreted in their urine which will explain the presence of this compound in the WWTP (Dassano et al., 2014). As seen in table 6 there was an increase in both the antioxidants in the cells that were exposed to this sample and according to Dassano et al. (2014) N6-Isopentenyladenosine showed to have antioxidant properties which could add to the activity of antioxidants in the cells. Emtricitabine is an HIV-ARV medication (Oldfield et al., 2005). Lastly the natural compound, feruloyl putrescine, was identified. This compound is found in the juices and leaves of *Citrus paradisi* Macf., and grapefruit (Wheaton & Stewart, 1965). Both citrus and grapefruit are produced in the Mpumalanga province thus it is possible that this compound could add to the responses seen (Kasirivu, 2009; Sikuka, 2021) but it is more likely that the Wakkerstroom citizens ate the fruit and that feruloyl putrescine entered the water system through excretion. The wet season sample contained 50 compounds as well but only four compounds could be identified: N,N-diethyl-m-toluamide, ritonavir, lopinavir, and oleamide (Table 5). Ritonavir and lopinavir are both HIV-ARV medications.

According to Kasonga et al. (2021) WWTPs are so-called hot spots for EDCs. Kirk et al. (2002) reported that the androgenic activity present in the WWTPs is mainly due to domestic input. Androgen levels in humans are very high, higher than oestrogens that are commonly found in WWTPs: in adult males, the levels of plasma testosterone range between 3 000–10 000 ng/L and for adult females, the levels are much less ranging between 200–750 ng/L (Tietz, 1987). Chang et al. (2011) tested for androgens in WWTPs' samples using LC-ESI-MS/MS analysis, and they reported the presence of natural androgens: androsterone, androstenedione, and epiandrosterone. They also detected synthetic androgens in the WWTPs' influent: stanozolol and 19-nor-4-androstene-3,17-diol which are pharmaceuticals (Chang et al., 2011).

The wet season samples had overall more responses in the reporter gene assays compared to the dry season samples (Table 6). Water from the WWTP sampled in the dry season was the only sample that activated the AR (Table 6). The bioassay equivalents were 0.12, 0.17, and 0.23 ng testosterone-equivalents per µL for $REP_{20}$, $REP_{50}$ and $REP_{80}$ respectively. This is most likely due to the WWTP that is not designed to remove the EDCs (Truter et al., 2016; Wee & Aris, 2017). Truter et al. (2016) tested water from

the Olifants River in Mpumalanga Province, South Africa, and they reported a $REP_{50}$ of $3.44x10^{-6}$ ng/µL at the WWTP impacted site. Compared to the $REP_{50}$ of this study their reported $REP_{50}$ is much lower. It is important to take into account that Truter et al. (2016) used the Yeast Androgen Screen to investigate for androgenic activity and their sample was not collected directly at the effluent of the WWTP.

Leusch et al. (2006) reported a study where water samples from fifteen WWTPs were tested for AR activation by using a rainbow trout androgen receptor binding assay. They reported the androgen activity of the raw sewage influents, ranging between $1.92x10^{-3}$–$9.33x10^{-3}$ ng/µL testosterone equivalents, and the final effluents measured between $6.5x10^{-6}$–$7.36x10^{-3}$ ng/µL testosterone equivalents (Leusch et al., 2006). The final effluents' $REP_{50}$ is more comparable with this study than what Truter et al. (2016) reported but still it is evident that the $REP_{50}$ from Leusch et al. (2006) is lower than the one this study reported. It can be speculated that the treatment in the WWTP, where the effluent sample was collected for this study, is not efficient for removing all the androgens.

Zgórska et al. (2011) collected four wastewater samples from a hospital in Poland and performed the Thamnotox acute toxicity test. They reported a 100% mortality when the organisms were exposed to the raw water samples. Blinova (2000) exposed *T. platyurus*, *Daphnia magna*, *Selenastrum capricornutum* (green algae), *Lemna minor* (duckweed), and *Tetrahymena thermophila* (ciliated protozoan) to water samples collected at the Pühajogi and Kroodi Rivers and effluents from wastewater treatment plant, fishing industry, and the Estonian oil-shade industry in Tallinn. The short communication reported 100% mortalities where *T. platyurus* was exposed to the raw wastewater from the oil-shade industry and the fishing industry as well as 50% mortalities in the diluted fishing industry wastewater (Blinova, 2000). It was also reported that *T. platyurus* was more sensitive to environmental samples than *D. magna*.

None of the samples from either the dry or wet season caused GR activation (Table 6). Kakaley et al. (2021) also did not get any GR activation in their study where they tested pre-and post-distribution treated water in Chicago. They investigated GR activation by performing luminescence assays using the CV1 (African green monkey kidney) cell line which was transduced with human GR (Kakaley et al., 2021). Neale et al., 2019 reported

that they did not get any GR activation when they tested samples collected from water treatment plants using the GR-GeneBLAzer assay. Van der Linden et al. (2008) investigated water samples from different sources for GR activation using the GR-CALUX assay, in eight of the ten samples GR activation was detected. They reported 11 and 38 ng dexamethasone equivalents (Dex eq)/L for the samples collected at the sewage treatment plants while the samples from the industrial area had a GR activation of 243 ng Dex eq/L. Glucocorticoid receptor agonists mostly occur in medications: betamethasone (found in ointment used for skin irritation), beclomethasone (common in asthma inhalers) (Lu et al., 2006), and clobetasol propionate (anti-inflammatory) (Willi et al., 2018). Glucocorticoids are present in the environment because they are excreted through urine and faeces thus it was expected to find activation of the GR in the WWTP samples (Lu et al., 2006). It is possible that no activation responses were seen because the glucocorticoids in the samples were below the limit of detection (Schriks et al., 2013).

None of the samples given to the HuTu 80 and H4IIE-*luc* cells caused lipid peroxidation, as all the responses were too low to be quantified. In this project, the cells were seeded at 80 000 cells/mL in 24-well plates and exposed for 24 h, but with the studies mentioned below it is evident that the lack of lipid peroxidation results might be due to the seeding density being too low and the exposure time might have been too short. The cells were not exposed long enough to the extracts for lipid peroxidation to take place. Lipid peroxidation could not have been calculated for any of the samples (drinking water and environmental samples), both dry and wet seasons, given to the two cell lines.

Several studies where lipid peroxidation could be measured were as follows: human colon carcinoma cells (HCT116) seeded in 6-well plates with a density of 375 000 cells/mL and the exposure time was 24 h (Boussabbeh et al., 2016), human trophoblast cell line (HTR-8/SVneo) exposed for 24 h and seeding density 300 000 cells/mL in 12-well tissue culture plate (Gomez et al., 2020), human colonic cell line (Caco-2) seeded 125 000 cells/mL in 24-well tissue culture plates and exposed for 48 h (Ilboudo et al., 2014). It is evident that the seeding density and the duration of the exposure should be adjusted.

Overall, the number of responses increased for the wet season samples (Figure 42). This could possibly be due to compounds that are in suspension during heavy rain events but

also the *in vivo* assays were added for the wet season samples thus more responses were measured for the wet season samples. This lead to the biological effect score for the wet season samples to have a higher total markup score. These compounds were most likely also present during the dry season sampling event, but they settled to the bottom of the river, dam, and wetland. The water samples from Tap Water, Martin's Dam and Norman Dennet from the dry season are of the best quality compared to the other samples. There is no need to be concerned regarding the effects caused by Paul Kruger Bridge (just upstream of the wetland) and BirdLife South Africa (middle of wetland) (Table 6) because the function of the wetland is to retain toxicants (Turner et al., 2005). It was expected to obtain responses in the assays where the sediment was tested because pollutants are absorbed by the sediment (Geranmayeh et al., 2018). As explained above with WWTPs being the hot-spots for EDCs it was also expected to get responses (Kasonga et al., 2021). The AR activation assay only got one response out of the 30 samples investigated, AR inhibition got four responses out of the 30 samples and with the AhR two samples caused activation of this receptor out of ten samples tested. This overall result of the reporter gene assay is just proof that the water from Wakkerstroom is in a good condition.

### 5.4 Efficacy of biological analysis for water quality determination

The results from this study indicated that the bioassays are indeed sensitive enough to determine the quality of drinking as well as environmental water (Table 6). The battery assays used in this project provided information on whether persistent dioxin-like compounds were present in the sediment of the ecosystem, and whether chemical mixtures in both the water and sediment had the potential to activate or inhibit the androgen receptor. Oxidative stress assays seemed to be even more sensitive than the reporter gene assay based on the number of responses obtained. The *in vivo* assays are an important part of an effective test battery because the effects seen are based on the bioavailable chemical mixtures present in either the water or the sediment. This is in contrast to the *in vitro* assays where certain compounds were targeted during the extraction processes and the cells reacted only on the targeted chemical compounds present in the extracts.

In the European collaborative project SOLUTIONS, it is advised that effect-based methods should be integrated for water quality monitoring. Effect-based methods (EBMs) are bioanalytical tools used to detect and quantify the effects caused by chemical mixtures by testing for ecotoxicological endpoints (Brack et al., 2019). These EBMs can use either *in vivo* or *in vitro* assays. In the publication by Brack et al. (2019) a battery of bioassays was recommended to support the River Basin Management Planning with an integrative solution-orientated strategy. To cover the major ecotoxicological endpoints to following should be employed: apical bioassays such as 96 h fish embryo acute toxicity test, 48 h *Daphnia* immobilisation test, and the 72 h algae inhibition of population growth test (Brack et al., 2019). *In vitro* assays that address specific modes of action such as mutagenicity, endocrine disruption, and the activation of cellular mechanisms should also be included the battery of bioassays (Brack et al., 2019).

In this study a 24 h immobilisation test was done using the *T. platyurus* species instead of the 48 h *Daphnia* immobilisation test. Several studies reported that *T. platyurus* is more sensitive than the *Daphnia* species (Heinlaan et al., 2008; Palma et al., 2008). The *in vivo* assays recommended by Brack et al. (2019) are where the organisms are exposed to the water collected in this study a test was included where *Heterocypris incongruens* species were exposed to sediment samples. This study investigated both the water and sediment, where applicable, to determine the overall water quality per site.

Endocrine disruption was investigated through testing for AR and GR activity and the activation of cellular mechanisms were measured with AhR activity in this study just as Brack et al. (2019) recommended. This study did not test for mutagenicity but with mutagenicity being grouped under reactive toxicity by Kruger et al. (2022), it is likely comparable with oxidative stress which is also placed in this group.

With the knowledge gained during this study the following assays are recommended for future water quality studies: i) testing for endocrine disruption using the MDA-kb2 cell line because the activity of two steroids can be measured, ii) investigating xenobiotic metabolism with AhR activity, iii) oxidative stress through testing for ROS, SOD, and CAT due to these assays being more sensitive compared to the reporter gene assay (solely based on the results from this study), iv) algal growth inhibition assay even though this

assay was not performed in this study but being primary producers the algae forms an important part of most of the food webs in the aquatic ecosystem, v) *T. platyurus* 24 immobilisation test because this species are more sensitive than the *Daphnia* species and the exposure time is shorter, vi) *Heterocypris incongruens* 6 day mortality and growth inhibition test because the toxicity of the sediment has an influence on the water quality and overall health of the aquatic environment, vii) 96 h fish embryo acute toxicity using *Danio rerio* because this species is in a higher trophic level than the *T. platyurus* and a standardised method is established.

It is important to take into consideration that this study is part of a bigger Water Research Commission project. In the bigger project the samples collected at the same sites, as in this study, are investigated for oestrogenic activity by using the T47DKB-*luc* and signs of oxidative stress using the AREc32 cell line. Several *in vivo* assays will also be performed with the samples including: *Allivibrio fisheri* bioluminescent test, *Pseudokirchneriella subcapitata* growth inhibition, *Spirodela polyrhiza* growth inhibition, *Daphnia magna* acute toxicity, *Poecilia reticulata* acute toxicity, and *T. platyurus* acute toxicity. The bigger project includes a more extensive chemical analysis performed with the water samples collected.

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# CHAPTER 6 CONCLUSION

What is research but a blind date with

knowledge?

– Will Harvey

Biological analysis proved to be efficient in determining the water quality of different types of water sources within the study area of Wakkerstroom. These analyses gave an overview of the ecosystem's water quality through testing for certain endpoints. Chemicals in the environment are present in mixtures, and with the use of biological analyses, these mixtures' reactions within the water and sediment samples can be taken into consideration. In addition to this, the biological analyses also provide information on possible health implications consumers might experience when ingesting the water and the possible effect on organisms that are living in the water. The results obtained from this study created a picture of the town's water quality but there are grey areas because it was not possible to test for all the endpoints such as mutagenicity, genotoxicity and immunotoxicity. The ecological community structures within the aquatic environment such as the South African Scoring System 5 for macroinvertebrates or the Fish Response Assessment Index were also not investigated. Yet again it is important to remember this study was only a small part of a much bigger project and it was possible to address the problem statement with the few assays performed.

The presence of compounds with different polarities in the samples was confirmed by the results obtained from the performed assays. This was merely a confirmation that compound mixtures are present in the aquatic environment, including drinking water, thus the importance of applying a battery of assays, that test for both polar and non-polar compounds, is emphasised for future water quality investigations.

Water and sediment collected from Wakkerstroom were investigated for endpoints such as percentage mortality, and percentage growth inhibition with *in vivo* assays. The crustacean (*Thamnocephalus platyurus*) was exposed to the raw water collected and dilutions thereof to determine the percentage mortality. It was interesting to note that the environmental water samples caused minimum mortality while the drinking water samples and water from the WWTP caused the highest mortality percentages. *Heterocypris incongruens* was exposed to the sediment sampled and it was the sediment samples collected from the wetland that caused the complete mortality of all the organisms. This was probably due to the wetland retaining the toxicants that the sediment adsorbed (Geranmayeh et al., 2018) or that settled to the bottom. It is therefore important to include

the *H. incongruens* toxicity test. These *in vivo* tests were only an add-on for this study but for a better overall view of the health of the aquatic environment, future studies should include exposures of organisms from the different trophic levels (primary producers, primary consumers and/or secondary consumers, and predators) to the samples.

The chemicals that fall into the classes of pharmaceuticals and personal care products as well as pesticides were extracted from the water samples and chemically analysed. Only a selected few compounds were identified using the KEGG and ChEBI libraries. Knowing the identity of some of these compounds could aid in explaining the responses seen in the biological assays performed in this study. Although in many other studies chemical analysis results are complementary to the biological assays, in this study very few compound profiles matched the library and could be identified, making the chemical information very limited to use together with the biological assay results. The biological assays with specific endpoints therefore provided the study with more meaningful results compared to the chemical analysis.

*In vitro* assays made it possible to measure the water quality of Wakkerstroom through testing for AR and AhR effects of water and sediment collected and the potential of these samples to cause oxidative stress and influence acetylcholinesterase activity. *In vivo* assays were used to test for endpoints including mortality and growth inhibition which allowed the study to determine the health of the aquatic ecosystem. The results proved that the overall quality of the water in Wakkerstroom is of good quality, and I am of the opinion that there is no need for concern for either the citizens or organisms that inhabits the aquatic ecosystem.

### 6.1 Recommendations

If a future study includes industrial and agricultural sites, Wakkerstroom has the potential to be used as a reference area. The next step would be to assess the water quality of these samples with second tier screening which would involve whole organism, organisms of higher organisation, exposure .

### 6.1.1 Xenobiotic metabolism

Include two separate extractions for every sample and perform the acid wash clean-up step, which removes most of the non-persistent dioxin-like compounds, with one of the two extracts. Expose the cells to both extracts and compare the results. If the difference between the results gained from both exposures are minimal, then it might not be necessary to do the acid wash clean-up step because with every transfer of the extract target compounds might stay behind.

### 6.1.2 Endocrine disruption

Instead of using testosterone as the reference compound for the AR activation assay rather use dihydrotestosterone because there are more studies to compare your results to. It is also important to keep in mind that dihydrotestosterone is more potent than testosterone and binds longer to the AR.

### 6.1.3 Oxidative stress and acetylcholinesterase activity

In future, when using cells to test for oxidative stress and non-neuronal choline activity it will be better to seed in 6-well plates because there will be more cells which will improve the detection of the ROS production, antioxidant activity, as well as the acetylcholinesterase activity.

If the aim of a study is to investigate whether a sample causes oxidative stress through exposing a cell line to the sample, it will not be recommended to perform the lipid peroxidation assay. This is based on the fact that lipid peroxidation is a consequence of oxidative stress, and measuring ROS, SOD and CAT already provides the answer to whether oxidative stress is caused by a sample or not.

### 6.1.4 Acute toxicity *in vivo* assays

When including these assays in a study it is important to ask an expert to help especially if it is the first time an individual will perform the *in vivo* assays.

Make sure there is someone in the laboratory to assist you with the tests, especially when you want to commence more than one *in vivo* assay with the same samples. Planning is

key because it is ideal to do the exposures within 12 h after sampling or at most 48 h after sampling.

## 6.2 Reference

Geranmayeh, P., Johannesson, K.M., Ulén, B. and Tonderski, K.S., 2018. Particle deposition, resuspension, and phosphorus accumulation in small, constructed wetlands. *Ambio*, 47(1), 134–145. https://doi.org/10.1007/s13280-017-0992-9

# ANNEXURES

**Supplementary table 1:** Results from H4IIE-*luc* cells exposed to sediment extracts expressed in terms of the 2,3,7,8 -Tetrachlorodibenzo-*p*-dioxin (TCDD)**.**

| Site | Concentrations (g/mL) | AhR ↑ 2021 %TCDDmax | AhR ↑ 2022 %TCDDmax |
|---|---|---|---|
| **Zaaihoek influence Sediment** | 20 | 12 | 14 |
| | 6.67 | 12 | 15 |
| | 2.22 | 12 | 14 |
| | 0.74 | 11 | 14 |
| | 0.25 | 10 | 12 |
| | 0.08 | 9 | 11 |
| **Norman Dennet Sediment** | 20 | 10 | 14 |
| | 6.67 | 10 | 14 |
| | 2.22 | 10 | 14 |
| | 0.74 | 10 | 12 |
| | 0.25 | 10 | 12 |
| | 0.08 | 10 | 11 |
| **Honeymoon Sediment** | 20 | 9 | 40 |
| | 6.67 | 10 | 25 |
| | 2.22 | 10 | 17 |
| | 0.74 | 10 | 13 |
| | 0.25 | 10 | 11 |
| | 0.08 | 10 | 9 |
| **BirdLife Sediment** | 20 | 12 | 14 |
| | 6.67 | 12 | 14 |
| | 2.22 | 11 | 13 |
| | 0.74 | 11 | 12 |
| | 0.25 | 10 | 11 |

| Site | Concentrations (g/mL) | AhR ↑ 2021 %TCDDmax | AhR ↑ 2022 %TCDDmax |
|---|---|---|---|
| | 0.08 | 10 | 11 |
| Paul Kruger Sediment | 20 | 13 | 40 |
| | 6.67 | 13 | 33 |
| | 2.22 | 13 | 25 |
| | 0.74 | 12 | 19 |
| | 0.25 | 11 | 15 |
| | 0.023 | 10 | 11 |

AhR – aryl hydrocarbon receptor

**Supplementary table 2:** Results for MDA-kb2 cell line exposed seasonal water samples expressed in terms of %TestosteroneMax (AR activation) and %FlutamideMax (AR inhibition).

| Site | Concentrations (µL/mL) | AR ↑ 2021 %Testosteronemax | AR ↓ 2021 %Flutamidemax | AR ↑ 2022 %Testosteronemax | AR ↓ 2022 %Flutamidemax |
|---|---|---|---|---|---|
| Solvent control | 2.5 | 12 | 81 | 13 | 106 |
| | 2.5 | 16 | 84 | 11 | 123 |
| | 2.5 | 15 | 107 | 11 | 99 |
| Tap Water | 2.5 | 10*+ | 146+ | 19*+ | 76+ |
| | 0.83 | 13+ | 140+ | 15 | 143*+ |
| | 0.28 | 12+ | 157+ | 17+ | 101+ |
| | 0.09 | 12 | 101+ | 14 | 94+ |
| | 0.03 | 11*+ | 154*+ | 15 | 120*+ |
| | 0.01 | 10*+ | 131+ | 17+ | 148*+ |
| Borehole | 2.5 | 12+ | 225*+ | 18*+ | 110 |
| | 0.83 | 12 | 145*+ | 17+ | 112*+ |
| | 0.28 | 14 | 154*+ | 16+ | 113*+ |

| Site | Concentrations (µL/mL) | AR ↑ 2021 %Testosteronemax | AR ↓ 2021 %Flutamidemax | AR ↑ 2022 %Testosteronemax | AR ↓ 2022 %Flutamidemax |
|---|---|---|---|---|---|
| | 0.09 | 13 | 138*+ | 16+ | 120*+ |
| | 0.03 | 14 | 117+ | 16+ | 127*+ |
| | 0.01 | 12 | 128+ | 16+ | 175*+ |
| **Wakkerstroom Branded Water** | 2.5 | 11*+ | 65+ | 8+ | 74 |
| | 0.83 | 13 | 64+ | 7*+ | 67 |
| | 0.28 | 14 | 76 | 8+ | 46 |
| | 0.09 | 13 | 68 | 7*+ | 57+ |
| | 0.03 | 12*+ | 54+ | 7*+ | 29+ |
| | 0.01 | 9*+ | 48*+ | 7*+ | 57+ |
| **Zaaihoek influence** | 2.5 | 9*+ | 49*+ | 8+ | 79 |
| | 0.83 | 12 | 47+ | 8+ | 61 |
| | 0.28 | 13 | 67 | 7*+ | 69 |
| | 0.09 | 14 | 70 | 7*+ | 86+ |
| | 0.03 | 13 | 74 | 8+ | 69+ |
| | 0.01 | 9*+ | 68+ | 7+ | 52+ |
| **Norman Dennet** | 2.5 | 12 | 38*+ | 8+ | 44*+ |
| | 0.83 | 12*+ | 45*+ | 8+ | 62 |
| | 0.28 | 13 | 58*+ | 7+ | 63 |
| | 0.09 | 13 | 54*+ | 7*+ | 56 |
| | 0.03 | 11*+ | 54*+ | 8+ | 54+ |
| | 0.01 | 12+ | 65*+ | 7*+ | 56 |
| **Honeymoon Creek** | 2.5 | 13 | 91 | 18*+ | 47 |

| Site | Concentrations (µL/mL) | AR ↑ 2021 %Testosteronemax | AR ↓ 2021 %Flutamidemax | AR ↑ 2022 %Testosteronemax | AR ↓ 2022 %Flutamidemax |
|---|---|---|---|---|---|
| | 0.83 | 11+ | 92 | 13 | 61 |
| | 0.28 | 13 | 120 | 13 | 73 |
| | 0.09 | 14 | 103+ | 10 | 93+ |
| | 0.03 | 12 | 82 | 9 | 100 |
| | 0.01 | 11+ | 107+ | 9 | 47*+ |
| **Paul Kruger Bridge** | 2.5 | 23*+ | 77 | 12 | 27*+ |
| | 0.83 | 19*+ | 58+ | 13 | 36*+ |
| | 0.28 | 15 | 72 | 15 | 36*+ |
| | 0.09 | 16 | 89 | 10 | 46*+ |
| | 0.03 | 17 | 92 | 9 | 47*+ |
| | 0.01 | 19*+ | 63+ | 10 | 49*+ |
| **BirdLife South Africa** | 2.5 | 10*+ | 66+ | 8+ | 31*+ |
| | 0.83 | 11*+ | 71 | 8+ | 68 |
| | 0.28 | 11+ | 102 | 8+ | 69 |
| | 0.09 | 10 | 96+ | 7*+ | 67 |
| | 0.03 | 14 | 104 | 7*+ | 56 |
| | 0.01 | 11*+ | 123+ | 8+ | 53+ |
| **Wastewater Treatment Plant** | 2.5 | 95*+ | 94 | 9 | 51+ |
| | 0.83 | 47*+ | 89 | 8+ | 44*+ |
| | 0.28 | 30*+ | 107 | 8+ | 48*+ |
| | 0.09 | 18+ | 94 | 7*+ | 49+ |
| | 0.03 | 18+ | 108 | 7+ | 51+ |

| Site | Concentrations (µL/mL) | AR ↑ 2021 %Testosteronemax | AR ↓ 2021 %Flutamidemax | AR ↑ 2022 %Testosteronemax | AR ↓ 2022 %Flutamidemax |
|---|---|---|---|---|---|
| | 0.01 | 18 | 97 | 8+ | 47 |
| Martin's Dam | 2.5 | 16 | 73 | 8+ | 46*+ |
| | 0.83 | 16 | 68 | 8+ | 42*+ |
| | 0.28 | 19*+ | 112 | 8+ | 52+ |
| | 0.09 | 16 | 93 | 8+ | 50+ |
| | 0.03 | 18+ | 85 | 6*+ | 41*+ |
| | 0.01 | 14 | 91 | 7*+ | 44*+ |

AR – Androgen receptor

**Supplementary table 3:** The results for MDA-kb2 cell line exposed to seasonal water extracts that caused statistical ($p<0.05$) and practical significance ($d>0.8$). These are indicated in red.

| Site | Concentration | AR ↑ Dry season | | AR ↓ Dry season | | AR ↑ Wet season | | AR ↓ Wet season | |
|---|---|---|---|---|---|---|---|---|---|
| | µL/mL | p-value | d-value | p-value | d-value | p-value | d-value | p-value | d-value |
| Tap Water | 2.5 | 0.021 | 1.444 | 0.149 | 1.222 | 0.015 | 1.650 | 0.312 | 0.824 |
| | 0.83 | 0.194 | 0.958 | 0.043 | 1.762 | 0.28 | 0.701 | 0.009 | 2.905 |
| | 0.28 | 0.061 | 0.857 | 0.014 | 2.340 | 0.054 | 1.222 | 0.083 | 1.137 |
| | 0.09 | 0.083 | 0.780 | 0.219 | 0.931 | 0.487 | 0.491 | 0.112 | 0.864 |
| | 0.03 | 0.043 | 1.053 | 0.009 | 2.241 | 0.189 | 0.792 | 0.014 | 2.639 |
| | 0.01 | 0.03 | 1.526 | 0.149 | 0.916 | 0.076 | 1.081 | 0.009 | 3.950 |
| Borehole | 2.5 | 0.083 | 0.846 | 0.014 | 3.588 | 0.034 | 1.412 | 0.564 | 0.282 |
| | 0.83 | 0.248 | 0.685 | 0.014 | 1.918 | 0.076 | 1.102 | 0.03 | 1.589 |
| | 0.28 | 0.564 | 0.307 | 0.043 | 1.648 | 0.153 | 0.882 | 0.03 | 1.642 |
| | 0.09 | 0.564 | 0.356 | 0.021 | 1.663 | 0.082 | 1.002 | 0.021 | 1.936 |

| Site | Concentration | AR ↑ Dry season | | AR ↓ Dry season | | AR ↑ Wet season | | AR ↓ Wet season | |
|---|---|---|---|---|---|---|---|---|---|
| | µL/mL | p-value | d-value | p-value | d-value | p-value | d-value | p-value | d-value |
| | 0.03 | 0.885 | 0.182 | 0.061 | 2.289 | 0.153 | 0.861 | 0.009 | 3.107 |
| | 0.01 | 0.885 | 0.031 | 0.149 | 0.841 | 0.105 | 1.050 | 0.03 | 3.211 |
| **Wakkerstroom Branded Water** | 2.5 | 0.043 | 1.084 | 0.149 | 0.890 | 0.562 | 0.809 | 0.386 | 0.525 |
| | 0.83 | 0.194 | 0.557 | 0.112 | 0.953 | 0.018 | 1.089 | 0.885 | 0.315 |
| | 0.28 | 0.942 | 0.225 | 0.47 | 0.527 | 0.176 | 0.968 | 0.194 | 0.725 |
| | 0.09 | 0.564 | 0.420 | 0.248 | 0.791 | 0.018 | 1.089 | 0.112 | 1.048 |
| | 0.03 | 0.043 | 1.219 | 0.061 | 1.036 | 0.01 | 1.168 | 0.083 | 1.260 |
| | 0.01 | 0.009 | 1.957 | 0.009 | 1.488 | 0.017 | 1.109 | 0.083 | 1.362 |
| **Zaaihoek Influenc** | 2.5 | 0.009 | 1.702 | 0.009 | 1.455 | 0.562 | 0.819 | 0.47 | 0.191 |
| | 0.83 | 0.194 | 0.685 | 0.061 | 1.258 | 0.297 | 0.928 | 0.828 | 0.080 |
| | 0.28 | 0.312 | 0.611 | 0.773 | 0.448 | 0.043 | 1.049 | 0.885 | 0.215 |
| | 0.09 | 0.665 | 0.103 | 0.665 | 0.420 | 0.013 | 1.119 | 0.194 | 0.511 |
| | 0.03 | 0.312 | 0.583 | 0.665 | 0.600 | 0.354 | 0.898 | 0.885 | 0.215 |
| | 0.01 | 0.009 | 1.893 | 0.248 | 0.814 | 0.069 | 1.089 | 0.112 | 0.941 |
| **Norman Dennet** | 2.5 | 0.149 | 0.744 | 0.009 | 1.784 | 0.511 | 0.819 | 0.03 | 1.261 |
| | 0.83 | 0.043 | 0.961 | 0.03 | 1.394 | 0.202 | 0.949 | 0.665 | 0.518 |
| | 0.28 | 0.348 | 0.530 | 0.021 | 1.163 | 0.076 | 1.029 | 0.665 | 0.480 |
| | 0.09 | 0.194 | 0.636 | 0.03 | 1.290 | 0.037 | 1.078 | 0.248 | 0.748 |
| | 0.03 | 0.043 | 1.078 | 0.03 | 1.307 | 0.132 | 0.979 | 0.149 | 0.842 |
| | 0.01 | 0.149 | 0.882 | 0.03 | 1.073 | 0.034 | 1.108 | 0.194 | 0.753 |
| | 2.5 | 0.427 | 0.560 | 0.773 | 0.018 | 0.031 | 1.332 | 0.194 | 0.703 |

| Site | Concentration | AR ↑ Dry season | | AR ↓ Dry season | | AR ↑ Wet season | | AR ↓ Wet season | |
|---|---|---|---|---|---|---|---|---|---|
| | µL/mL | p-value | d-value | p-value | d-value | p-value | d-value | p-value | d-value |
| Honeymoon Creek | 0.83 | 0.07 | 1.020 | 0.885 | 0.054 | 0.699 | 0.301 | 0.368 | 0.546 |
| | 0.28 | 0.194 | 0.632 | 0.083 | 1.055 | 0.757 | 0.211 | 0.885 | 0.035 |
| | 0.09 | 0.885 | 0.256 | 0.47 | 0.430 | 0.817 | 0.449 | 0.149 | 0.808 |
| | 0.03 | 0.149 | 0.723 | 0.564 | 0.258 | 0.757 | 0.620 | 0.564 | 0.412 |
| | 0.01 | 0.061 | 1.043 | 0.149 | 1.254 | 0.616 | 0.689 | 0.03 | 1.161 |
| Paul Kruger Bridge | 2.5 | 0.021 | 2.904 | 0.248 | 0.751 | 0.969 | 0.111 | 0.009 | 2.010 |
| | 0.83 | 0.03 | 2.347 | 0.149 | 0.836 | 0.699 | 0.371 | 0.009 | 1.614 |
| | 0.28 | 0.47 | 0.337 | 0.47 | 0.671 | 0.203 | 0.792 | 0.009 | 1.627 |
| | 0.09 | 0.112 | 0.697 | 0.564 | 0.366 | 0.817 | 0.548 | 0.021 | 1.391 |
| | 0.03 | 0.083 | 0.761 | 0.47 | 0.422 | 0.316 | 0.619 | 0.043 | 1.138 |
| | 0.01 | 0.043 | 1.409 | 0.248 | 0.970 | 0.817 | 0.489 | 0.043 | 1.544 |
| BirdLife South Africa | 2.5 | 0.021 | 1.551 | 0.312 | 0.887 | 0.354 | 0.899 | 0.009 | 1.828 |
| | 0.83 | 0.043 | 1.068 | 0.312 | 0.702 | 0.28 | 0.879 | 0.368 | 0.618 |
| | 0.28 | 0.061 | 1.142 | 0.47 | 0.409 | 0.217 | 0.929 | 0.885 | 0.223 |
| | 0.09 | 0.386 | 0.232 | 0.248 | 0.897 | 0.041 | 1.088 | 0.773 | 0.301 |
| | 0.03 | 0.773 | 0.069 | 0.47 | 0.489 | 0.008 | 1.168 | 0.773 | 0.396 |
| | 0.01 | 0.043 | 1.043 | 0.083 | 1.157 | 0.142 | 0.960 | 0.149 | 0.889 |
| Wastewater Treatment Plant | 2.5 | 0.009 | 27.538 | 0.773 | 0.134 | 0.589 | 0.758 | 0.112 | 0.978 |
| | 0.83 | 0.009 | 12.763 | 0.665 | 0.386 | 0.202 | 0.949 | 0.014 | 1.283 |
| | 0.28 | 0.009 | 5.323 | 0.312 | 0.594 | 0.202 | 0.960 | 0.043 | 1.107 |
| | 0.09 | 0.083 | 1.108 | 0.773 | 0.105 | 0.031 | 1.080 | 0.061 | 1.056 |

| Site | Concentration | AR ↑ Dry season | | AR ↓ Dry season | | AR ↑ Wet season | | AR ↓ Wet season | |
|---|---|---|---|---|---|---|---|---|---|
| | µL/mL | p-value | d-value | p-value | d-value | p-value | d-value | p-value | d-value |
| | 0.03 | 0.061 | 1.218 | 0.312 | 0.629 | 0.058 | 1.029 | 0.083 | 0.964 |
| | 0.01 | 0.248 | 0.687 | 0.885 | 0.020 | 0.097 | 1.009 | 0.194 | 0.713 |
| **Martin's Dam** | 2.5 | 0.47 | 0.464 | 0.248 | 0.639 | 0.153 | 0.978 | 0.043 | 1.199 |
| | 0.83 | 0.194 | 0.441 | 0.248 | 0.790 | 0.487 | 0.848 | 0.014 | 1.341 |
| | 0.28 | 0.043 | 1.604 | 0.194 | 0.746 | 0.076 | 0.989 | 0.061 | 0.973 |
| | 0.09 | 0.312 | 0.428 | 0.773 | 0.080 | 0.097 | 0.919 | 0.071 | 1.026 |
| | 0.03 | 0.061 | 1.110 | 0.885 | 0.193 | 0.007 | 1.218 | 0.014 | 1.298 |
| | 0.01 | 0.885 | 0.116 | 0.885 | 0.001 | 0.008 | 1.198 | 0.014 | 1.288 |

AR – Androgen receptor

**Supplementary table 4:** Results for MDA-kb2 cell line exposed seasonal sediment samples expressed in terms of %TestosteroneMax (AR activation) and %FlutamideMax (AR inhibition).

| Site | Concentrations (g/mL) | AR ↑ 2021 %Testosteronemax | AR ↓ 2021 %Flutamidemax | AR ↑ 2022 %Testosteronemax | AR ↓ 2022 %Flutamidemax |
|---|---|---|---|---|---|
| **Zaaihoek influence Sediment** | 5 | 12 | 50 | 17 | 63 |
| | 1.67 | 13 | 49 | 16 | 114 |
| | 0.56 | 13 | 65 | 15 | 105 |
| | 0.19 | 13 | 68 | 15 | 95 |
| | 0.06 | 14 | 85 | 13 | 99 |
| | 0.02 | 16 | 82 | 16 | 104 |
| **Norman Dennet Sediment** | 5 | 18 | 58 | 15 | 40 |

| Site | Concentrations (g/mL) | AR ↑ 2021 %Testosteronemax | AR ↓ 2021 %Flutamidemax | AR ↑ 2022 %Testosteronemax | AR ↓ 2022 %Flutamidemax |
|---|---|---|---|---|---|
| | 1.67 | 13 | 77 | 15 | 94 |
| | 0.56 | 13 | 80 | 15 | 114 |
| | 0.19 | 14 | 98 | 13 | 118 |
| | 0.06 | 14 | 87 | 14 | 92 |
| | 0.02 | 16 | 65 | 16 | 10 |
| **Honeymoon Sediment** | 5 | 8 | 77 | 12 | 78 |
| | 1.67 | 9 | 73 | 10 | 99 |
| | 0.56 | 8 | 66 | 9 | 113 |
| | 0.19 | 8 | 76 | 7 | 115 |
| | 0.06 | 8 | 74 | 9 | 98 |
| | 0.02 | 7 | 83 | 9 | 42 |
| **Paul Kruger Bridge Sediment** | 5 | 9 | 41 | 8 | 23 |
| | 1.67 | 7 | 54 | 9 | 74 |
| | 0.56 | 9 | 102 | 8 | 92 |
| | 0.19 | 8 | 81 | 7 | 106 |
| | 0.06 | 7 | 106 | 8 | 100 |
| | 0.02 | 7 | 115 | 9 | 9 |
| **BirdLife South Africa Sediment** | 5 | 9 | 78 | 9 | 63 |
| | 1.67 | 7 | 72 | 8 | 95 |
| | 0.56 | 8 | 104 | 9 | 99 |
| | 0.19 | 7 | 95 | 8 | 103 |
| | 0.06 | 8 | 131 | 9 | 96 |

| Site | Concentrations (g/mL) | AR ↑ 2021 %Testosteronemax | AR ↓ 2021 %Flutamidemax | AR ↑ 2022 %Testosteronemax | AR ↓ 2022 %Flutamidemax |
|---|---|---|---|---|---|
| | 0.02 | 7 | 86 | 7 | 92 |

AR – Androgen receptor

**Supplementary table 5:** The results for MDA-kb2 cell line exposed to seasonal sediment extracts that caused statistical ($p<0.05$) and practical significance ($d>0.8$). These are indicated in red.

| Site | Concentrations | AR ↑ Dry season | | AR ↓ Dry season | | AR ↑ Wet season | | AR ↓ Wet season | |
|---|---|---|---|---|---|---|---|---|---|
| | (g/mL) | p-value | d-value | p-value | d-value | p-value | d-value | p-value | d-value |
| Zaaihoek influence Sediment | 5 | 0.969 | 0.142 | 0.039 | 1.762 | 0.045 | 1.201 | 0.121 | 2.048 |
| | 0.83 | 0.728 | 0.221 | 0.039 | 1.550 | 0.165 | 0.882 | 0.039 | 2.4073 |
| | 0.28 | 0.817 | 0.282 | 0.197 | 0.841 | 0.297 | 0.762 | 0.02 | 1.842 |
| | 0.09 | 0.728 | 0.340 | 0.796 | 0.427 | 0.354 | 0.631 | 0.092 | 1.165 |
| | 0.03 | 0.463 | 0.521 | 0.796 | 0.125 | 0.757 | 0.222 | 0.197 | 1.165 |
| | 0.01 | 0.07 | 1.071 | 0.439 | 0.856 | 0.142 | 0.992 | 0.121 | 2.100 |
| Norman Dennet Sediment | 5 | 0.037 | 1.381 | 0.071 | 1.289 | 0.203 | 0.762 | 0.071 | 2.459 |
| | 0.83 | 0.757 | 0.331 | 0.796 | 0.110 | 0.28 | 0.772 | 0.302 | 0.689 |
| | 0.28 | 0.671 | 0.261 | 0.897 | 0.073 | 0.28 | 0.672 | 0.02 | 2.422 |
| | 0.09 | 0.375 | 0.541 | 0.302 | 0.588 | 0.877 | 0.151 | 0.02 | 2.653 |
| | 0.03 | 0.487 | 0.521 | 0.439 | 0.666 | 0.537 | 0.391 | 0.302 | 0.979 |
| | 0.01 | 0.132 | 0.931 | 0.302 | 0.860 | 0.076 | 1.021 | 0.02 | 4.456 |
| Honeymoon Creek Sediment | 5 | 0.512 | 0.800 | 0.439 | 0.837 | 0.44 | 0.401 | 0.796 | 0.046 |
| | 0.83 | 0.616 | 0.719 | 0.606 | 0.319 | 0.817 | 0.410 | 0.302 | 0.647 |
| | 0.28 | 0.177 | 0.869 | 0.197 | 0.748 | 0.817 | 0.689 | 0.039 | 2.341 |
| | 0.09 | 0.177 | 0.958 | 0.796 | 0.748 | 0.041 | 1.049 | 0.02 | 2.477 |

| Site | Concentrations (g/mL) | AR ↑ Dry season | | AR ↓ Dry season | | AR ↑ Wet season | | AR ↓ Wet season | |
|---|---|---|---|---|---|---|---|---|---|
| | | p-value | d-value | p-value | d-value | p-value | d-value | p-value | d-value |
| | 0.03 | 0.699 | 0.750 | 0.796 | 0.412 | 0.699 | 0.729 | 0.439 | 1.335 |
| | 0.01 | 0.009 | 1.159 | 0.796 | 0.759 | 0.817 | 0.559 | 0.302 | 2.349 |
| **Paul Kruger Bridge Sediment** | 5 | 0.817 | 0.590 | 0.039 | 1.881 | 0.089 | 1.009 | 0.02 | 3.549 |
| | 0.83 | 0.037 | 1.050 | 0.02 | 1.486 | 0.616 | 0.768 | 0.606 | 0.205 |
| | 0.28 | 0.537 | 0.729 | 0.071 | 1.456 | 0.054 | 0.988 | 0.121 | 0.969 |
| | 0.09 | 0.231 | 0.919 | 0.197 | 0.979 | 0.028 | 1.138 | 0.02 | 1.870 |
| | 0.03 | 0.023 | 1.129 | 0.197 | 0.927 | 0.231 | 0.888 | 0.039 | 1.494 |
| | 0.01 | 0.064 | 1.029 | 0.197 | 1.468 | 0.671 | 0.768 | 0.02 | 4.501 |
| **BirdLife South Africa Sediment** | 5 | 0.699 | 0.699 | 0.439 | 0.610 | 0.315 | 0.779 | 0.071 | 1.139 |
| | 0.83 | 0.037 | 1.070 | 0.439 | 0.434 | 0.089 | 0.950 | 0.197 | 0.963 |
| | 0.28 | 0.082 | 1.009 | 0.02 | 1.557 | 0.562 | 0.678 | 0.071 | 1.421 |
| | 0.09 | 0.023 | 1.109 | 0.197 | 0.882 | 0.487 | 0.858 | 0.121 | 1.701 |
| | 0.03 | 0.082 | 0.999 | 0.071 | 2.251 | 0.203 | 0.719 | 0.071 | 1.246 |
| | 0.01 | 0.028 | 1.089 | 0.606 | 0.431 | 0.037 | 1.140 | 0.302 | 0.859 |

AR – Androgen receptor

**Supplementary table 6:** The reactive oxygen species (ROS) results for HuTu 80 cells exposed to seasonal extracts that caused statistical ($p<0.05$) and practical significance ($d>0.8$). These are indicated in red.

| Site | ROS dry-season | | ROS wet-season | |
|---|---|---|---|---|
| | p- value | d-value | p- value | d-value |
| Tap Water | 0.15 | 0.362 | 0.001 | 0.851 |
| Borehole | 0.797 | 0.008 | 0.001 | 0.576 |
| Wakkerstroom branded water | 0.237 | 0.446 | 0.004 | 0.120 |
| Zaaihoek Influence | 0.001 | 2.141 | 0.149 | 0.296 |
| Norman Dennet | 0.08 | 0.718 | 0.432 | 0.404 |
| Honeymoon Creek | 0.643 | 0.097 | 0.195 | 0.309 |
| Paul Kruger Bridg | 0.607 | 0.265 | 0.001 | 1.820 |
| BirdLife South Africa | 0.328 | 0.390 | 0.001 | 1.812 |
| Martin's Dam | 0.090 | 0.824 | 0.432 | 0.287 |
| Wastewater treatment plant | 0.150 | 1.672 | 0.001 | 1.799 |
| Zaaihoek Influence Sediment | 0.758 | 0.700 | 0.001 | 1.491 |
| Norman Dennet Sediment | 0.001 | 1.277 | 0.002 | 1.399 |
| Honeymoon Creek Sediment | 0.001 | 1.263 | 0.022 | 1.018 |
| Paul Kruger Bridge Sediment | 0.607 | 0.295 | 0.043 | 0.602 |
| BirdLife South Africa Sediment | 0.001 | 3.186 | 0.014 | 0.051 |

**Supplementary table 7:** The reactive oxygen species (ROS) results for H4IIE-*luc* cells exposed to seasonal extracts that caused statistical ($p<0.05$) and practical significance ($d>0.8$). These are indicated in red.

| Site | ROS dry-season | | ROS wet-season | |
|---|---|---|---|---|
| | p- value | d-value | p- value | d-value |
| Tap Water | 0.001 | 1.484 | 0.547 | 3.485 |
| Borehole | 0.001 | 1.339 | 0.001 | 5.183 |
| Wakkerstroom branded water | 0.018 | 0.756 | 0.001 | 1.622 |
| Zaaihoek Influence | 0.007 | 0.918 | 0.036 | 1.942 |
| Norman Dennet | 0.112 | 0.638 | 0.007 | 1.834 |
| Honeymoon Creek | 0.022 | 1.003 | 0.001 | 2.587 |
| Paul Kruger Bridg | 0.001 | 1.968 | 0.018 | 2.112 |
| BirdLife South Africa | 0.001 | 3.046 | 0.001 | 5.231 |
| Martin's Dam | 0.001 | 2.399 | 0.001 | 4.890 |
| Wastewater treatment plant | 0.371 | 0.286 | 0.001 | 3.376 |
| Zaaihoek Influence Sediment | 0.149 | 0.962 | 0.001 | 9.018 |
| Norman Dennet Sediment | 0.002 | 1.384 | 0.596 | 0.227 |
| Honeymoon Creek Sediment | 0.001 | 1.382 | 0.071 | 0.819 |
| Paul Kruger Bridge Sediment | 0.547 | 0.362 | 0.701 | 0.406 |

| | | | | |
|---|---|---|---|---|
| BirdLife South Africa | 0.006 | 1.045 | 0.001 | 1.820 |

**Supplementary table 8:** The superoxide dismutase (SOD) activity results for HuTu 80 cells exposed to seasonal extracts that caused statistical ($p<0.05$) and practical significance ($d>0.8$). These are indicated in red.

| Site | SOD dry-season | | SOD wet-season | |
|---|---|---|---|---|
| | p- value | d-value | p- value | d-value |
| Tap Water (TW) | 0.263 | 0.759 | 0.417 | 3.764 |
| Borehole (B) | 0.121 | 0.670 | 0.738 | 0.162 |
| Wakkerstroom branded water (WBW) | 0.129 | 0.685 | 0.144 | 0.327 |
| Zaaihoek Influence (ZI) | 0.129 | 0.727 | 0.015 | 4.037 |
| Norman Dennet (ND) | 0.16 | 0.587 | 0.005 | 0.482 |
| Honeymoon Creek (CK) | 0.121 | 0.671 | 0.025 | 0.306 |
| Paul Kruger Bridge (PKB) | 0.139 | 0.607 | 0.241 | 0.024 |
| BirdLife South Africa (BSA) | 0.013 | 5.116 | 0.176 | 0.374 |
| Martin's Dam (MD) | 0.659 | 0.460 | 0.006 | 1.009 |
| Wastewater treatment plant (WWTP) | 0.013 | 8.199 | 0.126 | 0.111 |
| Zaaihoek Influence Sediment (ZIS) | 0.406 | 1.708 | 0.84 | 0.025 |
| Norman Dennet Sediment (NDS) | 0.02 | 7.609 | 0.712 | 0.166 |
| Honeymoon Creek Sediment (HCS) | 0.027 | 0.846 | 0.863 | 0.101 |
| Paul Kruger Bridge Sediment (PKBS) | 0.377 | 0.479 | 0.791 | 1.058 |
| BirdLife South Africa Sediment (BSAS) | 0.223 | 0.794 | 0.941 | 0.225 |

**Supplementary table 9:** The superoxide dismutase (SOD) activity results for H4IIE-*luc* cells exposed to seasonal extracts that caused statistical ($p<0.05$) and practical significance ($d>0.8$). These are indicated in red.

| Site | SOD dry-season | | SOD wet-season | |
|---|---|---|---|---|
| | p- value | d-value | p- value | d-value |
| Tap Water | 0.403 | 0.355 | 0.871 | 0.159 |
| Borehole | 0.009 | 4.569 | 0.459 | 0.235 |
| Wakkerstroom branded water | 0.319 | 0.234 | 0.714 | 0.122 |
| Zaaihoek Influence | 0.512 | 0.053 | 0.111 | 0.642 |
| Norman Dennet | 0.568 | 0.285 | 0.25 | 0.377 |
| Honeymoon Creek | 0.898 | 0.081 | 0.469 | 0.168 |
| Paul Kruger Bridg | 0.087 | 0.498 | 0.003 | 0.912 |
| BirdLife South Africa | 0.319 | 0.374 | 0.217 | 0.488 |
| Martin's Dam | 0.419 | 0.227 | 0.48 | 0.664 |

| Site | SOD dry-season | | SOD wet-season | |
|---|---|---|---|---|
| | p- value | d-value | p- value | d-value |
| Wastewater treatment plant | 0.223 | 0.447 | 0.008 | 0.883 |
| Zaaihoek Influence Sediment | 0.217 | 0.447 | 0.150 | 0.976 |
| Norman Dennet Sediment | 0.051 | 0.581 | 0.084 | 0.851 |
| Honeymoon Creek Sediment | 0.045 | 0.641 | 0.104 | 0.508 |
| Paul Kruger Bridge Sediment | 0.039 | 0.675 | 0.136 | 0.364 |
| BirdLife South Africa Sediment | 0.011 | 1.053 | 0.353 | 0.314 |

**Supplementary table 10:** The catalase (CAT) results for HuTu 80 cells exposed to seasonal extracts that caused statistical ($p<0.05$) and practical significance ($d>0.8$). These are indicated in red.

| Site | CAT dry-season | | CAT wet-season | |
|---|---|---|---|---|
| | p- value | d-value | p- value | d-value |
| Tap Water | 0.006 | 1.522 | 0.685 | 3.762 |
| Borehole | 0.005 | 1.161 | 0.077 | 0.536 |
| Wakkerstroom branded water | 0.01 | 1.026 | 0.935 | 0.289 |
| Zaaihoek Influence | 0.0001 | 1.769 | 0.168 | 2.395 |
| Norman Dennet | 0.004 | 1.126 | 0.06 | 0.077 |
| Honeymoon Creek | 0.001 | 1.416 | 0.455 | 0.154 |
| Paul Kruger Bridg | 0.0001 | 1.598 | 0.972 | 0.333 |
| BirdLife South Africa | 0.001 | 18.300 | 0.825 | 0.180 |
| Martin's Dam | 0.169 | 0.287 | 0.035 | 0.457 |
| Wastewater treatment plant | 0.001 | 34.440 | 0.686 | 0.230 |
| Zaaihoek Influence Sediment | 0.004 | 12.050 | 0.544 | 0.578 |
| Norman Dennet Sediment | 0.001 | 50.067 | 0.037 | 0.046 |
| Honeymoon Creek Sediment | 0.186 | 30.372 | 0.461 | 0.216 |
| Paul Kruger Bridge Sediment | 0.377 | 3.200 | 0.339 | 3.223 |
| BirdLife South Africa Sediment | 0.243 | 10.354 | 0.21 | 0.012 |

**Supplementary table 11:** The catalase (CAT) results for H4IIE-*luc* cells exposed to seasonal extracts that caused statistical ($p<0.05$) and practical significance ($d>0.8$). These are indicated in red.

| Site | CAT dry-season | | CAT wet-season | |
|---|---|---|---|---|
| | p- value | d-value | p- value | d-value |
| Tap Water | 0.223 | 0.337 | 0.085 | 0.053 |
| Borehole | 0.032 | 0.884 | 0.15 | 0.002 |
| Wakkerstroom branded water | 0.399 | 0.280 | 0.009 | 0.067 |
| Zaaihoek Influence | 0.106 | 0.360 | 0.079 | 0.060 |

| Site | CAT dry-season | | CAT wet-season | |
|---|---|---|---|---|
| | p- value | d-value | p- value | d-value |
| Norman Dennet | 0.621 | 0.140 | 0.779 | 0.206 |
| Honeymoon Creek | 0.652 | 0.271 | 0.047 | 0.021 |
| Paul Kruger Bridg | 0.39 | 0.149 | 0.001 | 0.557 |
| BirdLife South Africa | 0.485 | 0.281 | 0.319 | 0.322 |
| Martin's Dam | 0.454 | 0.286 | 0.040 | 0.406 |
| Wastewater treatment plant | 0.433 | 0.149 | 0.001 | 0.539 |
| Zaaihoek Influence Sediment | 0.001 | 0.590 | 0.119 | 0.354 |
| Norman Dennet Sediment | 0.001 | 0.548 | 0.805 | 0.196 |
| Honeymoon Creek Sediment | 0.022 | 0.428 | 0.045 | 0.408 |
| Paul Kruger Bridge Sediment | 0.454 | 0.285 | 0.108 | 0.375 |
| BirdLife South Africa Sediment | 0.011 | 0.063 | 0.087 | 0.379 |

**Supplementary table 12:** The acetylcholinesterase activity (AChE act) results for H4IIE-*luc* cells exposed to seasonal extracts that caused statistical ($p<0.05$) and practical significance ($d>0.8$). These are indicated in red.

| Site | AChE act dry-season | | AChE act wet-season | |
|---|---|---|---|---|
| | p- value | d-value | p- value | d-value |
| Tap Water | 0.600 | 0.212 | 0.467 | 0.958 |
| Borehole | 0.726 | 1.451 | 0.036 | 1.333 |
| Wakkerstroom branded water | 0.727 | 0.131 | 0.906 | 0.8620 |
| Zaaihoek Influence | 0.546 | 0.314 | 0.169 | 1.127 |
| Norman Dennet | 0.327 | 0.463 | 0.025 | 1.389 |
| Honeymoon Creek | 0.858 | 0.254 | 0.784 | 0.927 |
| Paul Kruger Bridg | 0.654 | 2.323 | 0.239 | 0.653 |
| BirdLife South Africa | 0.465 | 0.268 | 0.783 | 0.985 |
| Martin's Dam | 0.221 | 0.529 | 0.03 | 0.3230 |
| Wastewater treatment plant | 0.485 | 0.546 | 0.135 | 0.315 |
| Zaaihoek Influence Sediment | 0.808 | 0.015 | 0.018 | 0.165 |
| Norman Dennet Sediment | 0.090 | 1.164 | 0.221 | 0.671 |
| Honeymoon Creek Sediment | 0.718 | 0.301 | 0.221 | 0.400 |
| Paul Kruger Bridge Sediment | 0.895 | 0.226 | 0.812 | 0.963 |
| BirdLife South Africa Sediment | 0.947 | 0.581 | 0.432 | 1.01 |

**Supplementary table 13:** Results for the *Thamnocephalus platyurus* exposed to water samples (wet season). Results that was statistically significant is indicated in red.

| Site | Concentration | % Mortality | p<0.05 |
|---|---|---|---|
| Standard Freshwater | 100% | 0 | |
| | 100% | 0 | |
| | 100% | 0 | |
| | 100% | 0 | |
| | 100% | 0 | |
| Tap Water | 100% | 100 | 0.025 |
| | 50% | 5 | 0.025 |
| | 25% | 10 | 0.025 |
| | 12.5% | 40 | 0.025 |
| | 6.25% | 0 | 1 |
| Borehole | 100% | 30 | 0.025 |
| | 50% | 0 | 1 |
| | 25% | 0 | 1 |
| | 12.5% | 0 | 1 |
| | 6.25% | 0 | 1 |
| Wakkerstroom Branded Water | 100% | 5 | 0.025 |
| | 50% | 0 | 1 |
| | 25% | 0 | 1 |
| | 12.5% | 0 | 1 |
| | 6.25% | 0 | 1 |
| Zaaihoek Influence | 100% | 5 | 0.025 |
| | 50% | 0 | 1 |
| | 25% | 0 | 1 |
| | 12.5% | 5 | 0.025 |
| | 6.25% | 0 | 1 |
| Norman Dennet | 100% | 0 | 1 |
| | 50% | 15 | 0.025 |
| | 25% | 0 | 1 |
| | 12.5% | 0 | 1 |
| | 6.25% | 5 | 0.025 |
| Honeymoon Creek | 100% | 5 | 0.025 |
| | 50% | 5 | 0.025 |
| | 25% | 0 | 1 |
| | 12.5% | 0 | 1 |
| | 6.25% | 0 | 1 |

| Site | Concentration | % Mortality | p<0.05 |
|---|---|---|---|
| Paul Kruger Bridge | 100% | 0 | 1 |
| | 50% | 0 | 1 |
| | 25% | 0 | 1 |
| | 12.5% | 0 | 1 |
| | 6.25% | 0 | 1 |
| BirdLife South Africa | 100% | 0 | 1 |
| | 50% | 0 | 1 |
| | 25% | 0 | 1 |
| | 12.5% | 0 | 1 |
| | 6.25% | 0 | 1 |
| WWTP | 100% | 55 | 0.025 |
| | 50% | 0 | 1 |
| | 25% | 5 | 0.025 |
| | 12.5% | 10 | 0.025 |
| | 6.25% | 0 | 1 |
| Martin’s Dam | 100% | 10 | 0.025 |
| | 50% | 0 | 1 |
| | 25% | 0 | 1 |
| | 12.5% | 0 | 1 |
| | 6.25% | 0 | 1 |