Implementation of effect-based methods for water quality assessment

Final report
to the Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

Water is a strategic component to enhance the quality of life of South African citizens and the economy in general. Sustainable and quality water is thus a constitutional imperative, and the country shares the current global acknowledgement of the value and status of this vital resource.

Surface waters can contain a wide range of contaminants of concern (CECs), referred to as micropollutants. These include industrial compounds and agricultural compounds, such as pesticides, pharmaceuticals and personal/home care products, plasticizers, microplastics, and engineered nanoparticles, among others. The global community is acknowledging that there is a huge challenge to making water safe and the supply sustainable. South Africa (SA) is also currently facing the challenge of ensuring a sustained and safe supply of drinking water for its citizens. Mixtures of compounds are present in the aquatic ecosystem that can negatively affect human and environmental health, particularly the endocrine system, for example, reproduction, cancers, neurodevelopmental effects and obesity.

Despite the advances in increasing sensitivity in chemical analyses, it is challenging to measure and assess the combinatorial adverse health effects of these compounds (both known and unknown) to humans or aquatic organisms as they occur at low levels and in complex mixtures. Currently, the water quality assessment approaches are inadequate for evaluating the fitness for use of water containing such chemicals. It is also challenging to estimate the impacts of discharges containing such chemicals into water resources. Theoretically, it is possible for drinking or environmental water samples to comply with individual chemical guidelines or standard values, but could cause an adverse health effect due to the nature of these complex mixture components. These limitations have led to intense international research efforts to apply *in vivo* and *in vitro* bioassays (commonly used in the early pre-screening stages of drug development) for water quality assessment.

The members on this project team have been actively involved in a number of global projects for the development of bioassay toolboxes for water quality assessment, particularly the development of a toolbox to include *in vitro* and *in vivo* bioassays (WRC project 2020/2021-00165). In order for this toolbox to be successful, these selected assays need to be applied to different water sources in the form of case studies to determine their robustness and applicability.

South Africa (represented by the Water Research Commission) is currently investigating the feasibility of implementing EBM for water safety planning. The project aims to test the feasibility of the bioassays that can be found in the updated toolbox from the WRC project 2020/2021-00165 by applying them to different water sources and processes through different case studies. This will provide data on the most appropriate assays to apply to water quality monitoring, and to develop a decision-making tool for the water stakeholders as well as to affect policy in a positive direction by incorporating the bioassays in the relevant guidelines.

AIMS AND OBJECTIVES

The aims and objectives of the project were to use case studies to assess the success of the implementation of the *in vivo* and *in vitro* bioassays for water quality assessment.

1. To establish effect-based trigger values for highly specific modes of action and provide overall guidance on the interpretation of bioassays for drinking and environmental water quality assessment
2. To conduct case studies on the application of effect-based methods for evaluating the performance of treatment technologies (including conventional drinking water, water reuse, desalination and wastewater treatment plants) as part of water safety planning
3. To conduct case studies on the application of effect-based methods for characterising impacts to surface waters and determining potential impacts of wastewater discharges

4. To formulate recommendations for the application of EBMs for water quality monitoring within the framework of Integrated Water Quality Management and Water Safety Planning in South Africa
5. To develop factsheets and education material for regulators, water users and water quality practitioners
6. To develop a decision-making tool for the selection of appropriate bioanalytical tools for assessing environmental water quality and performance of water treatment processes

METHODS

A selection of *in vitro* and *in vivo* assays was applied to different water types: drinking water treatment plants (DWTPs), wastewater treatment plants (WWTP), groundwater (GW) and surface water (SW). The tests selected took into account the different trophic levels for the *in vivo* bioassays and the different adverse outcome pathways and modes of action of the *in vitro* assays.

In vivo tests:

Lethal or sub-lethal toxicity testing (as applied for this assessment) is applied by exposing biota to water sources to determine the potential risk of such waters to the biota/biological integrity of the receiving water bodies. A risk category (hazard class) is determined based on the percentage of mortalities (lethal) or inhibition (sub-lethal) of the exposed biota. It is important to note that the hazard class is based on the standardised battery of selected test biota and therefore represents the risk/hazard towards similar biota in the receiving aquatic environment. The toxicity hazard is therefore expressed in terms of the aquatic biotic integrity and does in no way represent toxicology towards humans or other mammals. A risk/hazard category is determined by using a hazard classification system whereby one can classify sites using the toxicity data of the non-diluted samples. The percentage effect (PE) of toxicity (mortalities, growth inhibition, luminescence inhibition, ingestion inhibition) is used to rank the sample into one of five classes based on the highest toxic response obtained in at least one of the tests applied. The results of the six *in vivo* assays (*A. fischeri, P. subcapitata, S.polyrhiza, T. platyurus, D. magna* and *P. reticulate*) indicated that the water samples from the selected case study sites fell between Class II-V hazard rankings.

In vitro assays:

The *in vitro* bioassays that were tested show activity, indicating that these assays are useful in determining biological effects in water and sediment samples.

A practical test battery of at least three or four bioassays representative of effects commonly detected in water extracts and aligned with relevant steps of adverse outcome pathways is suggested. Assays that show activation of AhR, activation of ER and oxidative stress response, as they are responsive to a range of water types, represent different stages of the cellular toxicity pathway. Non-potable water, like wastewater and reused water, can be tested with assays indicative of activation of AhR, activation of ER and oxidative stress response, as they are responsive to a range of water types, represent different stages of the cellular toxicity pathway. When considering drinking water treatment or water reuse for potable use, an assay indicative of either genotoxicity or mutagenicity added to activation of AhR, activation of ER, and oxidative stress response (ARE_c32 cells), can be used. While more bioassays may be included in a test battery and screening might be possible with fewer bioassays.

Due to the highly sensitive nature of the i*n vitro* bioassays, some can detect effects in clean water samples. This is where effect-based trigger values (EBTs) can be utilised to distinguish an acceptable response in a water sample from an unacceptable response. Various approaches can be applied, from simple translation from existing guideline values or acceptable daily intake (ADI) values to approaches that determine the *in vitro* effect at the guideline value concentration and consider mixture effects. Some approaches are only suitable for drinking water or assays where few potent chemicals dominate the effect, while other approaches can be

applied to any water type with guideline values and any assay. Guidance is available on what steps to take if the effect in a sample exceeds its EBT. It must be noted that work on EBT derivation is continuously evolving, and future work should focus on deriving EBTs for assays recommended for routine water quality monitoring. The results have shown the potential of EBM when applied to the different water sources.

CONCLUSIONS

Currently in South Africa, the capacity to do the bioassays lies in academic institutions, particularly for the *in vitro* assays, which are mainly research based. In order to implement EBMs in the laboratory, sophisticated infrastructure is required to cope with the high throughput assays on a larger scale. For example, in Europe, many of these laboratories are using robotics to perform the high throughput assays for EBM. In this project, a decision-making tool is proposed, but there is a necessity to refine the process, requiring more data and information. A comprehensive and strategic monitoring programme is recommended. Successful implementation of EBMs necessitates a collaborative effort involving multiple government departments, municipalities, water consumers, researchers and private sector water stakeholders.

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ACRONYMS & ABBREVIATIONS

AChE	Acetylcholinesterase
ADI	Acceptable daily intake
AhR	Aryl hydrocarbon receptor
AOPs	Adverse outcome pathways
AR	Androgen receptor
BEQ	Bioequivalent
BNR	Biological nutrient removal
CAT	Catalase
DBP	Disinfection by products
DEEEP	Direct estimation of ecological effect potential
DOC	Dissolved organic carbon
DTA	Direct toxicity assessment
DWAF	Department of Water Affairs and Forestry
DWS	Department of Water and Sanitation
DWTP	Drinking water treatment plant
EBM	Effect-based methods/ monitoring
EBT	Effect-based trigger values
ECPH	Environmental Chemical Pollution and Health
EDC	Endocrine disrupting chemicals
EQ	Equivalent concentration
EQS	Environmental quality standards
ER	Estrogen receptor
EtOH	Ethanol
EU	European Union
FET	Fish embryo test
GR	Glucocorticoid receptor
GW	Ground water
GWRC	Global Water Research Coalition
HLB	Hydrophilic lipophilic balance
IC	Inhibitory concentration
IWQM	Integrated water quality management
IWRM	Integrated water resources management
LLE	Liquid liquid extraction
LOD	Level of detection
LOQ	Level of quantification
MDA	Malondialdehyde
MOA	Mode of action
MeOH	Methanol
NTMP	National Toxicity Monitoring Program

NWA	National Water Act
NWU	North West University
PE	Percentage effect
RfD	Reference dose
ROS	Reactive oxygen species
SA	South Africa
SANS	South African National Standards
SOD	Superoxide dismutase
SPE	Solid-phase extraction
SW	Surface water
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TMP	1,1,3,3-Tetramethoxypropane
TWQR	Target water quality range
US EPA	United States Environmental Protection Agency
WET	Whole effluent toxicity
WFD	Water Framework Directive
WHO	World Health Organisation
WRC	Water Research Commission
WSA	Water safety Act
WSP	Water safety plan
WWTP	Wastewater treatment plant
YAS	Yeast Androgen Screen
YES	Yeast Estrogen Screen

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Water resources are globally under threat, mainly due to pollution, the impacts of climate change, as well as the mismanagement of freshwater ecosystems. The aquatic environment is polluted by a number of contaminants from diverse sources, providing reactive matrices for many biotic and abiotic chemical transformations, and consequently presents complex mixture exposure profiles (Schäfer et al., 2016, Malaj et al., 2014, Bradley et al., 2017). It is estimated that more than 100 000 chemicals and 4000 pharmaceuticals are in everyday commercial use (Schwarzenbach et al., 2006, Boxall et al., 2012). Many of these chemicals have been banned or listed for restricted use, particularly in Europe (Altenburger et al., 2019). At the same time, numerous alternative chemicals have replaced these chemicals, making the priority substance list outdated, with many of the chemicals no longer appearing on the list (De Baat et al., 2019, Neale et al., 2015). Drinking water quality is increasingly at risk due to the presence of unknown but also known chemicals and their mixtures in water ecosystems (as only a limited number are tested for in the current South African national drinking water quality standards, SANS 241). In order to improve understanding of these risks, it is better to establish biological effects first, not just the chemical analysis of individual priority substances. Combining effect-based methods with chemical analysis will give a holistic view of the level of pollution and the potential risks of both known and unknown chemicals, as well as their mixtures (Hamers et al., 2020, Leusch et al., 2014, De Baat et al., 2020). Currently in South Africa, only a few chemicals are listed in the water quality guidelines and regulatory standards.

These individual compounds, as well as their mixtures and transformation products have been reported to result in adverse human and environmental health effects, particularly the endocrine system. Most of the research until recently has focused on the effects of endocrine disrupting chemicals (EDCs) on the estrogenic system (Freitas, 2012). Therefore, identifying their point sources (e.g., wastewater treatment plants) and diffuse sources (e.g., agriculture) within the ecosystem, and attempting to find solutions may help to reduce their presence and impact on human and environmental health is increasingly becoming important (Neale et al., 2017). It is also important to note that while individual compounds might not elicit effects at these low concentrations, their combined presence may still be important, especially if they act on the same pathway (Silva et al., 2002).

The current water quality assessment and monitoring approaches are inadequate for evaluating the fitness for use of water containing such chemicals, as well as estimating the impacts of discharges containing such chemicals into water resources. This means that it is possible that drinking or environmental water samples, theoretically, comply with individual chemical guidelines or regulatory standard values, but could cause an adverse health effect due to the nature of these complex mixture components.

There is an increased need to assess the level of risks to human health under premium cost-effective and predictive monitoring frameworks to better ensure that there is no exposure to these early biological effects. The use of *in vitro* and *in vivo* screening assays that can provide biologically relevant information to establish a toxicity paradigm, referred to as adverse outcome pathways (AOPs), that not only determines endocrine disruptive activity, but also, for example, inflammation, mutagenicity, genotoxicity and oxidative stress has become a priority. Thus, future water quality monitoring or assessment should include more systematic efforts to identify contaminants relevant for compromised water quality and, importantly, to improve quantification of compounds that show increased biological activity (Altenburger et al., 2019, Berninger et al., 2019). This means testing for the combined effects of contaminant mixtures

as opposed to single compounds (Altenburger et al., 2019). Aligning this with AOPs, the use of effect-based monitoring approaches ranging from whole-organism exposure studies to the application of high throughput bioassays that can provide information on potential health effects associated with exposure to complex mixtures (Altenburger et al., 2019, Berninger et al., 2019).

While *in vivo* bioassays are already applied in water quality assessment, e.g., in surface and wastewater regulations (e.g., US EPA's Whole Effluent Toxicity (WET) testing requirements (https://www.epa.gov/cwa-methods/whole-effluent-toxicity-method) (Snyder and Leusch, 2018), the Direct Toxicity Assessment (DTA) in the Australia-New Zealand Guidelines for Fresh and Marine Water Quality (https://www.waterquality.gov.au/anz-guidelines) (Snyder and Leusch, 2018), and the Direct Estimation of the Ecological Effect potential (DEEEP) approach in South Africa (DWAF, 2003). An important aspect in the application of bioassays in water quality assessment is the development of response mechanisms for positive results. This would indicate at what level of response additional actions should be taken. One proposed method is the development of benchmark values, which have been termed Effects Based Trigger values (EBT) (Escher et al., 2018).

1.2 GLOBAL PROJECT ON THE APPLICATION OF EFFECT-BASED METHODS

The Global Water Research Coalition (GWRC) is an international water alliance that was established by twelve leading research organisations in 2002. The GWRC Members are: Canadian Water Association (Canada), KWR – Water B.V. (Netherlands), PUB – Public Utilities Board (Singapore), Stowa- Foundation for Applied Water Research (Netherlands), SUEZ - CIRSEE (France), TZW - Water Technology Center (Germany), UK Water Industry Research (UK), Veolia Research and Innovation (VERI) (France), Water Research Australia (Australia), **Water Research Commission (South Africa)**, The Water Research Foundation (USA), and the Water Services Association of Australia. The GWRC is a non-profit organisation that serves as a focal point for global collaboration for research planning and execution on water and wastewater-related issues. The Coalition focuses on water supply and wastewater issues and renewable water resources: the urban water cycle. They leverage funding and expertise among the participating research organisations, coordinate research strategies, secure additional funding not available to single country research foundations, and actively manage a centralised approach to global issues (https://globalwaterresearchcoalition.net/about/research-approach/; accessed 14 October 2024).

In 2019, the Global Water Research Coalition (GWRC) launched a project on the application of effect-based methods (EBMs) for water safety planning, with active participation from the Water Research Commission. This project aims to integrate and harmonise the application of EBMs into water quality monitoring frameworks, addressing complex chemical mixtures in water systems to enhance public health protection and aquatic ecosystem safety. The project was segmented into the following work packages (WPs):

- WP 1 – Bioassay Selection and Protocol Development: This WP involves identifying suitable bioassays for detecting various biological activities relevant to water quality, such as endocrine disruption, genotoxicity, and oxidative stress. The goal is to recommend a test battery of bioassays that provides comprehensive coverage for monitoring contaminants in different water matrices.
- WP 2 – Effect-Based Trigger (EBT) Values: Another WP focuses on establishing effect-based trigger values that correlate bioassay results to safe water quality levels for human and environmental health. These EBTs help in interpreting bioassay results and guiding decision-making.
- WP 3 – Decision-Making Tools: The project includes the creation of a tool to assist water quality professionals in selecting appropriate bioanalytical tools for specific water treatment contexts,

such as drinking water and wastewater treatment plants. This decision-making tool offers guidance on bioassay application based on the treatment type and intended monitoring objectives.

- WP 4 – Case Studies and Demonstration Sites: Through case studies from different regions, this WP evaluates the application of EBMs across various water treatment and reuse scenarios. The results help demonstrate the effectiveness of EBMs in real-world contexts, providing insights into regional adaptation needs.
- WP 5 – Guidance for Implementation in Water Safety Planning: This WP synthesises findings to formulate guidance on incorporating EBMs within water safety planning frameworks globally. It includes developing materials to support EBM adoption by regulators and water practitioners, promoting a shift towards integrated water quality management.

1.3 SOUTH AFRICAN PROJECT ON THE APPLICATION OF EFFECT-BASED METHODS

Similar to other countries all over the world, today the water industry in South Africa is faced with the challenge of ensuring a sustained and safe supply of drinking water. According to the Water Research Commission's (WRC) National Water Research, Development and Innovation Roadmap 2015- 2025, published in the WRC 2015 report, "water demand will outstrip supply by 17% in 2030". The effective and sustainable management of South Africa's limited resources is essential for community health, development, cohesion and economic growth (WRC, 2018). The use of alternative sources of water, such as wastewater, has been identified as one of the interventions for closing the supply and demand gap. Similarly, a variety of micropollutants, mixtures and transformation products have been detected in both freshwater and wastewaters in South Africa. Integrating chemical analysis and bioanalysis together with modelling for mixture toxicity has been recognized as the way forward in water quality assessment and monitoring (Neale et al., 2017). An updated and expanded version of the toolbox (WRC 2020/2021-00165) includes bioassay endpoints that are not only looking at endocrine disruptive endpoints, but also include assays to detect short-term toxicity, i.e. endpoints related to biological quality elements (fish, macroinvertebrates, phytoplankton and macrophytes). Secondly, it includes bioassays that are cell and organism based, which can detect chemical-biosystem interaction to serve as a proxy for long-term effects (i.e., chronic exposures).

South Africa (represented by the Water Research Commission) is investigating the feasibility of implementing EBM into the SANS 241 water quality guidelines and also the integrated water quality plan (IWQP). This project will be a first for SA to include the short-term *in vivo* bioassays, *in vitro* bioassays and chemical screening as part of EBM. The implementation of EBMs and inclusion in water safety plans for assessing the fitness-of-use of water for different uses, and for monitoring the impact of effluent discharges into water resources will be a novel water quality management approach in SA.

1.4 PROJECT AIMS

Project WRC2020/2021-00348 is the third component of a larger project that includes the toolbox of bioassays (WRC2020/2021-00165) and a chemical analysis component done by UNISA (WRC2020/2021-00347), commissioned and funded by the WRC in South Africa. It is aimed at advancing water quality management by demonstrating the application of effect-based methodologies in South Africa. Additionally, this project is aimed at developing tools and educational resources that support the assessment and monitoring of drinking and environmental water quality, improve water treatment processes, and guide regulatory and water safety planning efforts. The aims of the project are as follows:

1. To establish effect-based trigger values for highly specific modes of action and provide overall guidance on the interpretation of bioassays for drinking and environmental water quality assessment

2. To conduct case studies on the application of effect-based methods for evaluating the performance of treatment technologies (including conventional drinking water, water reuse, desalination and wastewater treatment plants) as part of water safety planning

3. To conduct case studies on the application of effect-based methods for characterizing impacts to surface waters and determining potential impacts of wastewater discharges

4. To formulate recommendations for the application of EBMs for water quality monitoring within the framework of Integrated Water Quality Management and Water Safety Planning in South Africa

5. To develop factsheets and education material for regulators, water users and water quality practitioners

6. To develop a decision-making tool for the selection of appropriate bioanalytical tools for assessing environmental water quality and performance of water treatment processes

CHAPTER 2: USE OF EFFECT BASED METHODS FOR WATER QUALITY ASSESSMENT IN SOUTH AFRICA

2.1 INTRODUCTION

Recently, there has been an increased focus on the use of effect-based methods (EBMs) for water quality assessment and monitoring. The use of EBMs offers a solution-oriented strategy, as they use both the responses of whole organisms (*in vivo*) or cellular bioassays (*in vitro*) to detect and quantify the effects of groups of chemicals on toxicological endpoints of concern. To this end, a minimum battery of bioassays has been recommended for inclusion in the Water Framework Directive (WFD) in Europe. The WFD, adopted since 2000 (EU, 2000) is an environmental legislation that attempts to ensure good water quality (Altenburger et al., 2019). Brack et al. (2017) states that a more coherent approach should be created to achieve a better-quality freshwater system in Europe, and this involves including short-term toxicity to algae, Daphnia and Zebra fish embryos, complemented with *in vitro* and short-term *in vivo* tests on mode-of-action specific effects as proxies for long-term toxicity. The likelihood of adverse impacts can be established with effect-based trigger values (EBTs), which differentiate good from poor water quality in close alignment with Environmental Quality Standards (EQS) for individual chemicals, while considering mixture toxicity. The use of EBMs is suggested in the WFD as one avenue to establish the likelihood of adverse effects due to chemical pollution in European water systems (Escher et al., 2018). Considering the progress made on bioassays MOAs, Escher et al. (2018) suggested that EBTs be determined for *in vitro* and *in vivo* bioassays performed on surface water extracts supporting the EQS of the European Union (EU) Water Framework Directive.

Similar to other countries all over the world, today the water industry in South Africa is faced with the challenge of ensuring a sustained and safe supply of drinking water. The effective and sustainable management of South Africa's limited resources is essential for community health, development, cohesion and economic growth (WRC., 2018). The use of alternative sources of water, such as wastewater, has been identified as one of the interventions for closing the supply and demand gap. Similarly, a variety of micropollutants, mixtures and transformation products have been detected in both freshwater and wastewaters in South Africa. Integrating chemical analysis and bioanalysis with modelling for mixture toxicity has been recognised as the way forward in water quality assessment and monitoring (Neale et al., 2017). The bioanalysis endpoints indicated are not looking at endocrine disruptive endpoints only, but also include assays to detect short-term toxicity, i.e., endpoints related to biological quality elements (fish, macroinvertebrates, phytoplankton and macrophytes). Secondly, it includes bioassays that are cell and organism based, which can detect chemical-biosystem interaction to serve as a proxy for long-term effects (i.e., chronic exposures).

South Africa (represented by the Water Research Commission), is currently investigating the feasibility of implementing EBM for water safety planning. This will be a first for SA to include short-term *in vivo* bioassays, as no data exists, and it will be meaningful to add this to the existing *in vitro* data. The implementation of EBMs and inclusion in water safety plans for assessing the fitness of use of water for different uses, and for monitoring the impact of effluent discharges into water resources will be a novel water quality management approach in SA.

2.2 BIOANALYTICAL TOOLS

2.2.1 Definition

The definition of bioanalytical tools is *in vivo* and *in vitro* (cell-based) bioassays based on their modes of action (i.e., endpoint) that are relevant to assess environmental and human effects (Escher et al., 2021). These include tests with unicellular and small organisms like *Daphnia*, whole cell and reporter gene assays, as well as enzyme and receptor binding assays (Escher et al., 2021). The European technical report can be found under the Water Framework Directive, describing the numerous bioassays, including biomarkers, that have been applied for water quality monitoring (Wernersson et al., 2015). The toolbox for EBM, WRC report 2020/2021-00165, defines the various *in vivo* and *in vitro* bioassays available in South Africa for effect-based monitoring.

Toxicity tests can be performed on different levels of organisation, for example, where disease in humans is associated with potential exposure to chemicals (i.e., epidemiological studies). Studies that include using human case studies or rodents in order to obtain toxicological information at the whole organism or organ level are *in vivo* studies. Several endpoints may be considered, including human toxicology and ecotoxicology, ranging across organisms, populations, ecosystems model ecosystems (Escher et al., 2021).

2.2.2 Cell-based bioassays

Bioassays that can target mechanisms of toxicity or particular endpoints are bioassays using native cells which include primary cells and immortal cell lines and bioassays using recombinant cell lines. Native (i.e., unmodified) cells have not been genetically modified and can be sourced directly from tissue samples and they have a limited life span *in vitro*. The term *in vitro* refers to keeping entities (parts) of an organism in an artificial environment while *in vivo* refers to a whole organism (Schirmer, 2006). Vertebrate cell lines arise from primary cultures while immortal cell lines are mutated cell lines that can proliferate indefinitely and are preferable due to their high reproducibility and improved animal ethics and cost. In mammals, only cancer and stem cells are immortal. More recently, methods have become available to immortalise cells but so far, they have not been widely used in practical applications for water quality assessment (Escher et al., 2021). Cell lines can be used to test the bioactivity of a given sample, native cells will respond to all bioactive substances and are suitable for the assessment of non-specific toxicity. Cell growth or viability (cytotoxicity) is usually the endpoint measured (Figure 2.1). Cytotoxicity assays can be more specific if cells are derived from particular tissues such as pulmonary epithelial cells or liver cells. Toxicity may vary between different cell types which can further indicate the mode of action (MOA) of the chemicals present in the sample. Certain cells may react to certain of groups of chemicals that together may have the same MOA. This could be for example a specific physiological response like direct inhibition of photosynthesis in algae or the proliferation of breast cancer cells in the presence of an estrogen (Escher et al., 2021).

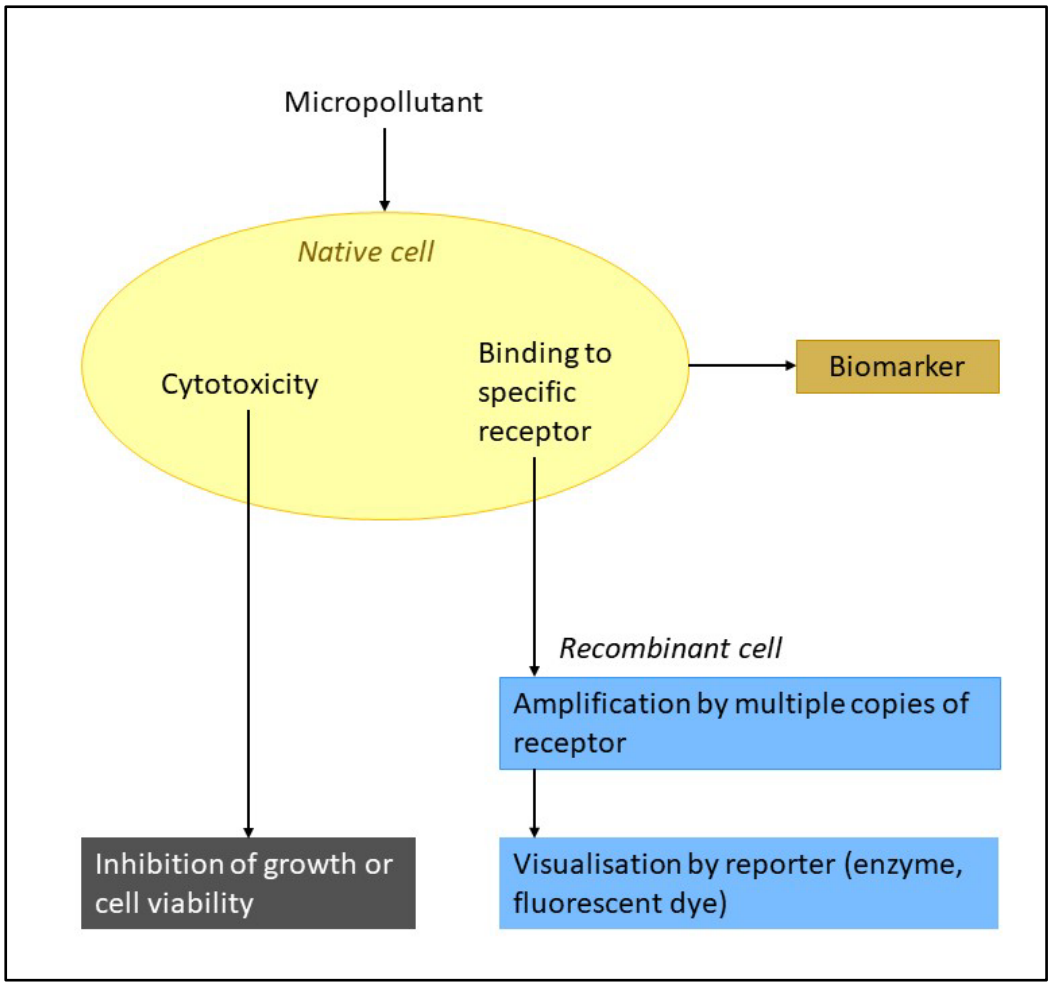

Figure 2-1: Design of cell-based bioassays. (Escher et al., 2021)

Genetically modified cells, like the recombinant cell lines, have been successfully applied in the last few years to detect and amplify toxic responses in the aquatic field (Figure 2.1). An example of this is cell lines that can bind to the AhR or detect hormone-mimicking activity, for example, estrogenic activity. A recombinant cell line usually indicates that there has been a genetic modification with the integration of a reporter plasmid into the cell, human or mammalian immortal cell line. A plasmid is a separate DNA molecule, circular in structure, and it carries a responsive element for the receptor of interest (e.g., ER_{α}), followed by a reporter gene that encodes a measurable feature such as an enzyme (e.g., β-galactosidase or luciferase) or a fluorescent protein. The amount of response quantified via the enzyme activity (colour change or light flash) is proportional to the amount of chemical bound to the receptor (Escher et al., 2021).

2.2.3 Modes of action

For adverse effects on humans or wildlife, several processes need to happen between exposure to the chemical and the effect on the cells. This needs to happen in two phases, namely toxicokinetic and toxicodynamic. The *toxicokinetic phase* describes all processes that link the external exposure (e.g., via drinking water, food and inhalation) to the biologically effective concentration within the cell. Processes include absorption, excretion, internal distribution and metabolism and elimination within the whole body and within cells. The *toxicodynamic phase* describes the actual toxicity pathways taking place inside the cell. This includes the initial molecular interaction of the chemical and its biological target (Figure 2.2). This can induce cellular defence mechanisms and other cellular responses that can

lead to observable toxic effects. In order for the bioassays to provide the information needed the selected assays must include both the relevant toxicokinetic steps and well-defined toxic mechanisms. Therefore, using whole-cell bioassays for the assessment of environmental samples like water is advocated, as the lipid bilayer of the cell membrane serves as a major barrier to chemical exposure. The molecular-based cell-free bioassays, like enzyme or receptor binding assays do not include the toxicokinetic process (Escher and Hermens, 2002, Escher et al., 2021).

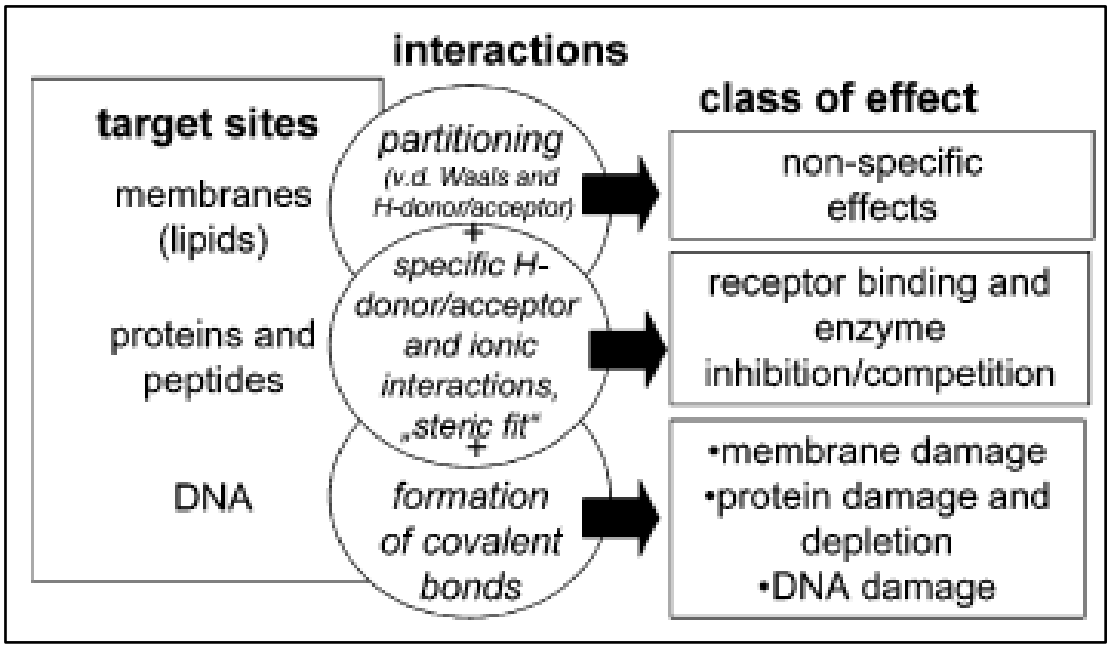

Figure 2-2: The underlying principle behind the classification of chemicals. (Source: Escher et al, 2002).

2.2.4 *In vivo* assays

Biomarkers are used to quantify sub-lethal effects. These are molecular characteristics that are objectively measured as indicators of a normal biological process or in response to harm (Escher et al., 2021). *In vivo* bioassays using whole-organism exposure tests are valuable for ecotoxicological assessment of specific chemicals, mixtures of chemicals, and applications for monitoring of water quality are generally limited to whole effluent testing and low-complexity assays, including those based on biomarker responses (Figure 2.3). Reproductive and developmental effects are rarely assessed (with the exception of the fish embryo test FET). Because these assays are usually done on whole water samples (directly or in diluted form), this is useful for mostly polluted water (Escher et al., 2021).

2.2.5 *In vitro* assays

In vitro assays are assays performed in tests done in a test tube or microtiter plates (e.g., 96 well or 348 well plates). These tests do not make use of animals; they are cell-based, including isolated tissue and enzyme extracts (Figure 2.3). The cells, mammalian, fish, yeast and bacterial in origin, can be obtained and grown without sacrificing animals. The advantage of molecular and cell-based assays is that they have a lower ethical impact compared to *in vivo* assays (Blaauboer and Andersen, 2007, Hartung, 2010). Some mammalian cells have to be isolated from tissue (primary cells) as they cannot be maintained indefinitely in culture for a length of time. Other cells, like mammalian cancer cells and fish cells, are immortal, and they can be cultured and reproduced indefinitely. *In vitro* assays require

less sample volume and are more practical for assessing environmental samples that have low levels of pollutants and need to be enriched (or concentrated) before toxicity testing. Sensitivity can also be improved through the use of genetically modified cell lines with an amplified response. Cell-based assays also lend themselves to automation and high-throughput screening, resulting in time- and cost-effectiveness (Escher et al., 2021).

In contrast to testing in *in vivo* assays, when assessing water quality using *in vitro* assays, the water sample is typically extracted and enriched before administration to *in vitro* bioassays. Because of the enrichment, a much wider range of sample matrices (e.g., from wastewater to drinking water) to be tested (Escher et al., 2021).

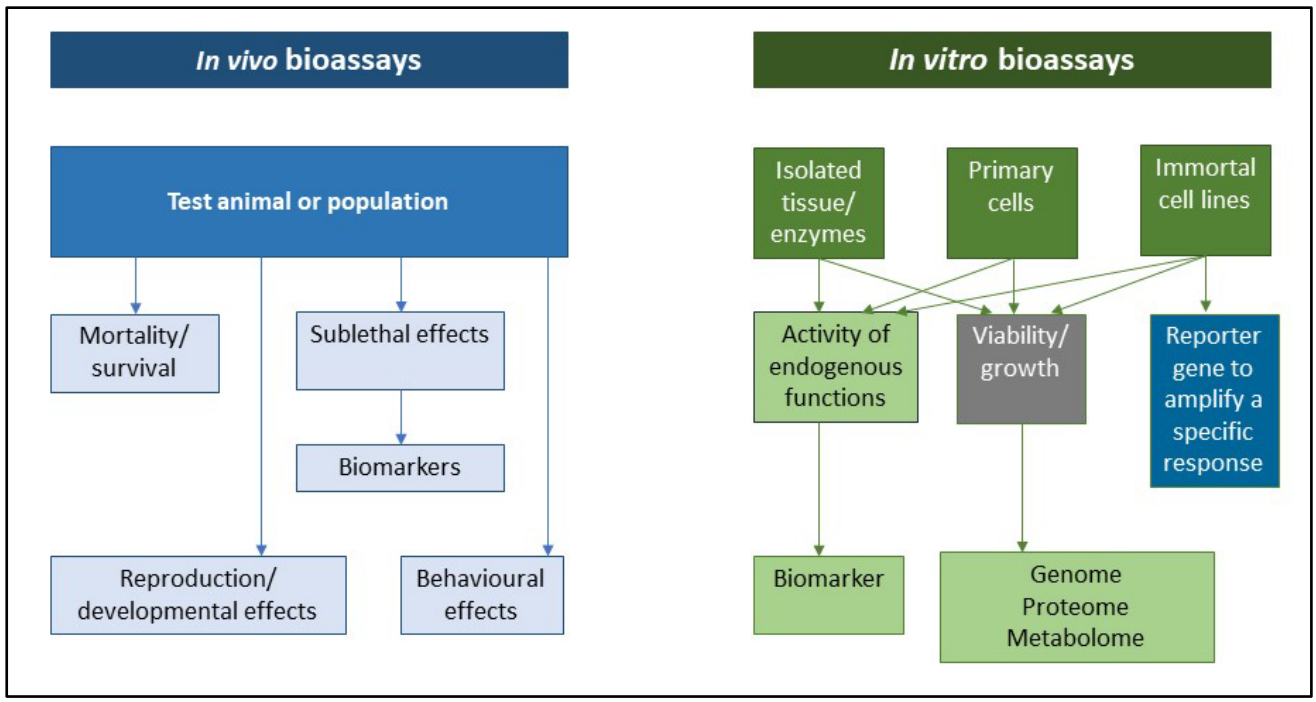

Figure 2-3: Principles of *in vivo* and *in vitro* bioassays used in water quality monitoring (Escher et al., 2021)

One advantage of bioassays is their ability to detect the cumulative toxicity of mixtures of both known and unknown chemicals in a sample (https://www.epa.gov/sites/default/files/2018-03/documents/bioassays-technical_brief_28mar18_final_0.pdf; accessed 9 Oct 2024). The bioassays measure the mixture toxicity or activity of a water sample. By measuring the mixture toxicity of a water sample, the bioassay approach includes a risk perspective as it explicitly accounts for the differences in toxicity across different chemicals and interactions among chemicals in a mixture. Chemical analysis can only quantify the concentration of known and/or targeted chemicals irrespective of toxicity (Leusch et al., 2018, Escher et al., 2021).

Bioassays can provide information on specific modes of action (MOA) and the general toxicity to cells (cytotoxicity). This is important, particularly when dealing with mixtures, because groups of chemicals with the same MOA can act together and result in concentration addition effects. By using a battery of bioassays that cover the various MOAs, it allows for the generation of mechanistic information relevant to predicting the adverse health outcomes (Escher et al., 2021, GWRC, 2020c). The bioassays can be run in parallel and give a comprehensive bioanalytical result for the toxicity of the bioactive substances found in the water sample.

2.2.6 Effect-based trigger values (EBT)

2.2.6.1 The importance of effect-based trigger values (EBTs)

Many *in vitro* bioassays are highly sensitive and can detect effects at very low levels. Samples that test positive in bioassays do not necessarily imply that the water quality is unacceptable. For this reason, EBTs were proposed. Effect-based trigger values allow the evaluation of the significance of bioassay results and thus are critical for the wider acceptance of *in vitro* bioassays and well plate-based *in vivo* assays by regulators and the water industry (GWRC, 2020c). Comparing measured effects in water samples against EBTs can help bioassay users to differentiate between acceptable and unacceptable responses. Effect-based trigger values are also needed before EBM can be implemented into Water Safety Plans (WSP) (GWRC, 2020c).

2.2.6.2 The derivation of EBTs

There are many different approaches to calculating EBTs, including deriving it from an acceptable daily intake (ADI) or an established guideline value (GV). When deriving EBTs from an ADI, toxicokinetic parameters may be incorporated, and when GVs are used, relative potencies and mixture factors may be incorporated. The aforementioned approaches are depicted in Figure 2.4 (GWRC, 2020c).

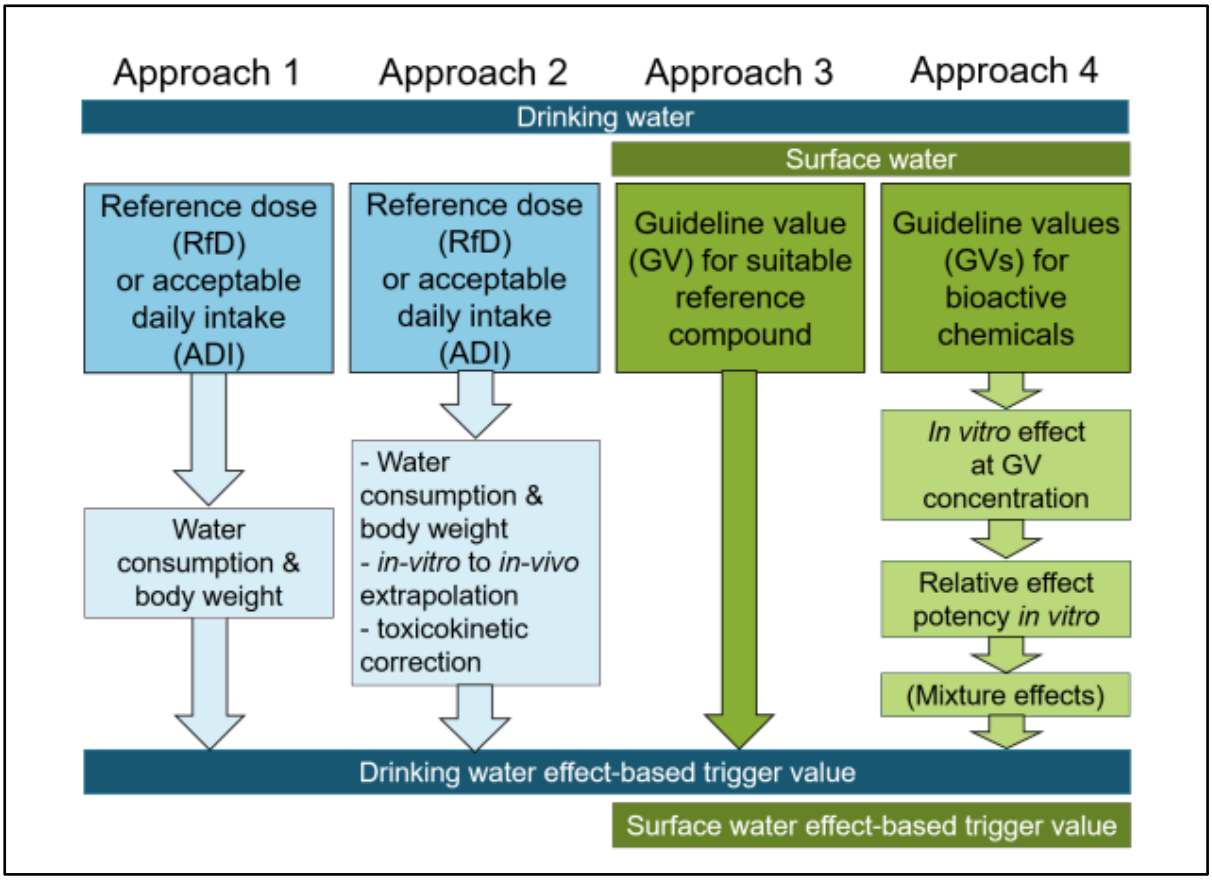

Figure 2-4: Derivation of effect-based trigger values (EBT) for drinking water and surface water by different approaches

1) from an acceptable daily intake (ADI); (2) from an ADI incorporating toxicokinetic parameters; (3) from an established guideline value (GV); and (4) from an established GV incorporating relative potencies and mixture factors. (GWRC, 2020c)

Other approaches to calculate EBM values include using multiple lines of evidence, including converting from *in vivo* toxicity data and field investigations (van der Oost et al., 2017) and comparison of *in vitro* and *in vivo* responses to determine maximum sensitivity and specificity cut-offs (Brion et al., 2019). A more detailed description and discussion of the various approaches can be found in the GWRC 2020, WP 3.4 report (GWRC, 2020c).

Effect-based trigger values are most commonly expressed as bioanalytical equivalent concentrations (BEQ) and relate the activity/effect of a water sample to the activity/effect of a reference compound used in the bioassay. The specific assay, reference compound and water source will affect the EBT value. Differences in sensitivity and potency between assays will result in different EBTs for different assays measuring the same endpoint. It is therefore important to use an EBT derived for a specific assay (e.g., yeast estrogen screen or T47D-KBluc) rather than an endpoint (e.g., estrogenic activity). The reference compound used for a particular endpoint may also differ in different studies. It is therefore important to make sure that the EBT for the relevant reference compound is used. Different EBTs may apply to different water sources, e.g., for drinking versus surface water. The reason for this is that the health targets for the different sources and uses are different, e.g., humans consuming drinking water versus aquatic animals living in surface water. The EBTs for surface water are generally lower compared to drinking water (as adverse effects are seen at very low concentrations in vulnerable aquatic organisms), and therefore, EBTs for surface water should be protective for drinking water as well (GWRC, 2020c).

2.2.6.3 Currently available effect-based trigger values

The majority of EBTs are derived from receptor-mediated effects, with estrogenic activity the most common endpoint. There are fewer EBTs available for the induction of xenobiotic metabolism, adaptive stress responses and apical effects (GWRC, 2020c). Table 2.1 gives a summary of the proposed EBT values for both human health and ecological health that are currently available in the literature (GWRC, 2020c).

Table 2-1: Summary of currently available EBT values for both human health and ecological health expressed as bioanalytical equivalent concentrations (BEQ)

Endpoint	Assay name	Human EBT (Drinking and recycled water for indirect potable reuse)	Ecological EBT (Surface water)
Xenobiotic metabolism			
AhR activity	AhR-cis FACTORIAL	18 µg/L Carbaryl EQ [1]	
	PAH CALUX		150 ng/L B[a]P EQ [2] 6.2 ng/L B[a]P EQ [3] 62.1 ng/L B[a]P EQ [4]
	DR CALUX		0.05 ng/L TCDD EQ [2]
	H4L1.1c4 AhR assay		6.4 ng/L B[a]P EQ [3] 4.3 ng/L B[a]P EQ [5]
PPARγ activity	PPARγ CALUX		10 ng/L Rosiglitazone EQ [2]
	PPARγ-GeneBLAzer		36 ng/L Rosiglitazone EQ [3] 19 ng/L Rosiglitazone EQ [5]
PXR activity	PXR-cisFACTORIAL	59 µg/L Metolachlor EQ [1]	
	PXR CALUX		3.0 µg/L Nicardipine EQ [2] 272 µg/L DEHP EQ [3] corresponding to 54 µg/L Nicardipine EQ 5.4 µg/L Nicardipine EQ [4]
	HG5LN-hPXR		16 µg/L DEHP EQ [3]
Receptor-mediated effects			
Estrogenic activity	[#]	0.7 ng/L EEQ [6]	0.4 ng/L EEQ [9]
	ERα CALUX	0.2 ng/L EEQ [1] 3.8 ng/L EEQ [7] 0.25 ng/L EEQ [8]	0.5 ng/L EEQ [2] 0.10 ng/L EEQ [3] 0.28 ng/L EEQ [10] 0.2 – 0.4 ng/L EEQ[#] [11]

	ERα GeneBLAzer	1.8 ng/L EEQ [1]	0.34 ng/L EEQ [3] 0.24 ng/L EEQ [10]
	E-SCREEN	0.9 ng/L EEQ [1]	0.1 – 0.3 ng/L EEQ# [11]
	YES	12 ng/L EEQ [1]	0.2 – 0.4 ng/L EEQ# [11]
	HeLa-9903	0.6 ng/L EEQ [1]	1.0 ng/L EEQ [3] 0.18 ng/L EEQ [10]
	MELN		0.37 ng/L EEQ[3] 0.56 ng/L EEQ [10] 0.2 – 0.3 ng/L EEQ# [11]
	MVLN		0.1 – 0.3 ng/L EEQ# [11]
	ERα-Luc-BG1		0.62 ng/L EEQ [3]
	A-YES		0.56 ng/L EEQ [3]
	3d YES		0.88 ng/L EEQ [3]
	ISO-LYES (Sumpter)		0.97 ng/L EEQ [3]
	ISO-LYES (McDonnell)		1.1 ng/L EEQ [3]
	pYES		0.5 ng/L EEQ [10]
	EASZY (Cyp19a1b-GFP)		2.2 ng/L EEQ [3]
	REACTIV (unspiked)		0.80 ng/L EEQ [3]
Androgenic activity	AR CALUX	11 ng/L DHT EQ [7] 4.5 ng/L DHT EQ [8]	
	AR GeneBLAzer	14 ng/L Testosterone EQ [1]	
Anti-androgenic activity	Anti-AR CALUX	4.8 µg/L Flutamide EQ [8]	25 µg/L Flutamide EQ [2] 14 µg/L Flutamide EQ [3]
	Anti-AR GeneBLAzer		3.3 µg/L Flutamide EQ [3]
	Anti-MDA-kb2		3.5 µg/L Flutamide EQ [3]
	Anti-AR RADAR (spiked)		3.6 µg/L Flutamide EQ [3]
Glucocorticoid activity	GR CALUX	150 ng/L Dexamethasone EQ [1] 21 ng/L Dexamethasone EQ [7]	100 ng/L Dexamethasone EQ [2]
Progestagenic activity	PR CALUX	724 ng/L Levonorgestrel EQ* [7]	
Anti-progestagenic activity	Anti-PR CALUX		1967 ng/L Endosulfan EQ [3]

Thyroid activity	TTR RLBA		0.06 µg/L Thyroxine EQ [3]
Thyroid activity	TTR FITC-T4		0.49 µg/L Thyroxine EQ [3]
Thyroid activity	XETA (unspiked)		0.62 ng/L Triiodothyronine EQ [3]
Anti-thyroid activity	Anti-TR-LUC-GH3		0.60 µg/L Bisphenol A EQ [3]
Photosynthesis inhibition	Combined algae assay (2 h–PSII)	0.6 µg/L Diuron EQ [1]	0.07 µg/L Diuron EQ [3]
Acetylcholinesterase inhibition	AChE assay	26 µg/L Parathion EQ [1]	
Adaptive stress response			
Oxidative stress response	AREc32	284 µg/L Dichlorvos EQ† [12]	156 µg/L Dichlorvos EQ [3] 140 µg/L Dichlorvos EQ [5]
Oxidative stress response	Nrf2 CALUX		10 µg/L Curcumin EQ [2] 26 µg/L Dichlorvos EQ [3]
Oxidative stress response	ARE GeneBLAzer		392 µg/L Dichlorvos EQ [3]
Apical effects in well plate-based in vivo assays			
Bacterial toxicity	Microtox	4100-4392 µg/L Baseline TEQ‡ [13]	1264 µg/L Baseline TEQ [3]
Algal growth	72 h algal growth inhibition		0.12 µg/L Diuron EQ [3]
Algal growth	24 h synchronous algae reproduction		0.11 µg/L Diuron EQ [3]
Algal growth	Combined algae assay (24hr growth)		0.13 µg/L Diuron EQ [3]
Immobilization	48 h Daphnia immobilization test		15 ng/L Chlorpyrifos EQ [3]
Mortality	Fish embryo toxicity (48 h)		276 µg/L Bisphenol A EQ [3]
Mortality	Fish embryo toxicity (96 - 120 h)		183 µg/L Bisphenol A EQ [3]

Note:

i) EBTs in units of BEQ are not currently available for assays indicative of reactive toxicity, so these assays are not included in the table.

ii) The green rows indicate the bioassays available in South Africa, further details are available in the WRC 2020/2021-00165 Toolbox report.

2.2.7 What to do if the bioassay is positive

The other main application of bioanalytical tools is to benchmark water quality. This can be done by comparison between sites and time of sampling. Effect-based trigger (EBTs) values are available that can differentiate between acceptable and poor water quality. Although EBTs have not yet been implemented in regulation, they are widely used for research purposes and various methods have been developed for their derivation. Most of the EBTs for drinking water have been developed by read across from drinking water guideline values, in some cases using toxicokinetic corrections. EBTs for surface water are also mainly read-across methods from guideline values and environmental quality standards but several methods specifically account for mixture effects.

2.3 TOWARDS EFFECT-BASED MONITORING IN WATER SAFETY PLANNING IN SOUTH AFRICA

As leaders in water research, a team of researchers from the various GWRC members put together a project titled “Effect-Based Monitoring in Water Safety Planning”. The project aimed to develop a Chemical Safety framework to assess innovative effect-based methods (EBMs) or monitoring tools to capture adverse toxic pathways missing from current substance-based targeting. The key challenge to this project is to use these innovative methods to assess water quality profiles potentially triggered by residual organic micro-pollutants at different parts of the Drinking Water Treatment Process (DWTP), from resource to tap, through the whole water cycle. Including wastewater treatment plants (WWTPs), conventional and alternative water treatment schemes and water reuse. Water resources can be impacted by different types of chemical pollution and specific treatment interaction, possibly generating other water quality profiles by degrading some compounds into by-products or metabolites. One of the main objectives of the project is to combine substance/chemical-based to EBM tools to capture any adverse toxic pathways missing from current substance-based target analysis. Therefore, many of the objectives of the GWRC project will benefit SA in making sure that they include these various endpoints recommended by the WHO and EU in a context that will be suitable to the SA scenario. This also meets several of the strategic objectives set out in the WRC CP18 (https://www.wrc.org.za/wp-content/uploads/WRC_Corporate-Plan_final.pdf).

2.3.1 Water quality legislative context in South Africa

The **National Water Act (NWA)** (Act 36 of 1998), is one of two important water laws in South Africa and provides the legal framework for the effective and sustainable management of our water resources. Act 36 of 98 was published in 1998 with the aim of fundamentally reforming the past laws relating to water resources, as these were discriminatory and inappropriate for the South African conditions. The NWA recognises that in South Africa, water is a scarce and precious resource that belongs to all the people, and to achieve the sustainable use of water for the benefit of all South Africans (De la Harpe and Ramsden, 2017). The second law is the **Water Services Act (WSA)** (Act 108 of 1997). The difference between the two acts can be seen in Figure 2.5.

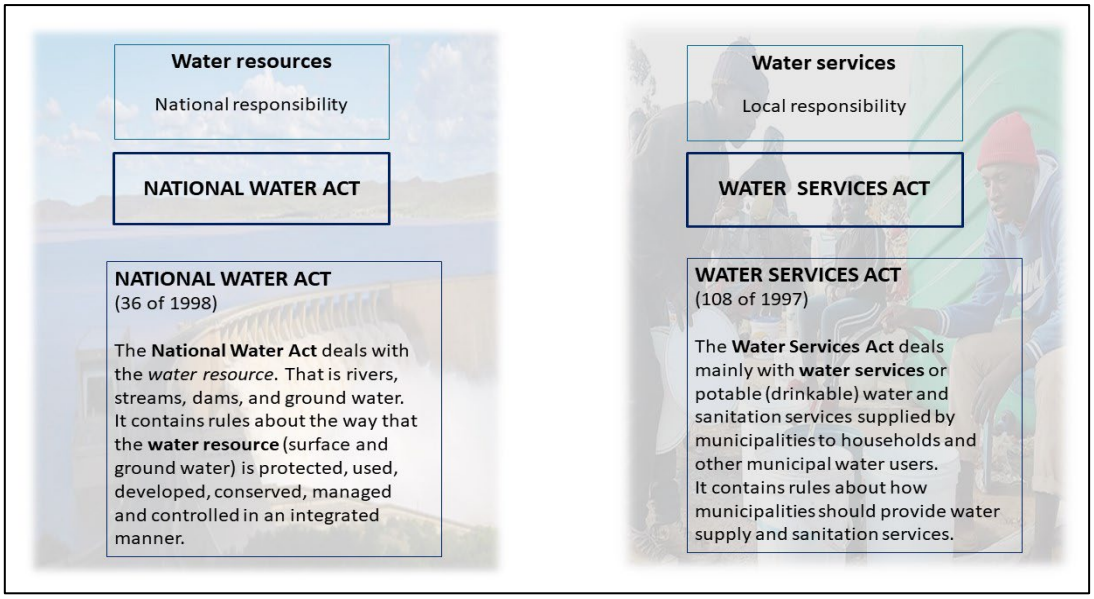

Figure 2-5: Summary highlighting the differences between the National Water Act (NWA) and the Water Services Act (WSA)

The WSA contains rules about how municipalities should provide potable water and sanitation services to households and other municipal water users(De la Harpe and Ramsden, 2017). Under the NWA, the national government is responsible for the establishment of the National Water Resource Strategy, and one of the many items to be addressed was to set targets for water quality for different water resources (De la Harpe and Ramsden, 2017). This was done in the form of the Water Quality Guidelines (volumes 1-8) (http://www.waternet.co.za/policy/g_wq.html, accessed 19 Oct 2024); however, only the drinking water guidelines have been updated since publication in 1996. Seven volumes contain water quality criteria—referred to as the Target Water Quality Range (TWQR)—together with other useful information. The eighth volume summarises the TWQR for each of the other volumes of different water uses: domestic, industrial, irrigation, livestock watering, aquaculture and aquatic ecosystem (Table 2.2) (Kruger et al., 2022).

The domestic water guideline has been fine-tuned into the South African National Standard 241 for drinking water (SANS 241, 2015) and is the mandate of the WSA. The TWQR of the SANS 241 document is also included in Table 2.2. It differs from that of domestic use and was mainly derived from the World Health Organization's Guidelines for drinking-water quality (SANS, 2015). The SANS 241 is currently under revision again (Kruger et al., 2022).The Directorate Resource Quality Information Services (RQIS) in the National Department of Water and Sanitation (DWS) administer several monitoring programmes on a national level: (i) chemical, (ii) microbial, (iii) eutrophication, (iv) toxicity, and (v) radioactivity monitoring programmes along with (vi) ecosystem monitoring programme (DWS, 2019a) to ensure good environmental water quality from which safe drinking water is prepared. Therefore, the collection of data and the interpretation of information on water and sanitation are critical for effective water and sanitation management (Kruger et al., 2022).

The NWA mandated the establishment of national monitoring systems to reduce and prevent degradation of water resources and to assess their quality. South Africa's water resources comprise inland surface water, water courses (rivers, springs, natural channels, wetlands, lakes and dams into which and from which water flows), estuaries and aquifers. A crucial implication of the Act is that an ecological effect-based approach needs to be applied to water resource management (DWAF, 2003), thus supporting regular toxicity testing of water resources as well as complex industrial wastewaters (effluents) which are released into water resources.

The National Toxicity Monitoring Programme (NTMP) suggests monitoring the concentrations of toxicants in the rivers and dams of South Africa, but with limited biological endpoints, toxicity tests included. The latest reference regarding the work done by RQIS on the NTMP itself that could be found on the RQIS website (https://www.dws.gov.za/iwqs/water_quality/ntmp/index.aspx) is a 'Draft phase 3: Pilot implementation and testing of design 2008–09' in which a case study was reported: Several sites were selected in the polluted Jukskei River in the Gauteng province. The aim was to establish the optimal sampling frequencies for various selected compounds, which included several organochlorine pesticides, some alkyl phenols, a few phthalates, and toxaphene. A final version of this has not yet been published on the site. Some toxicity tests using *Danio rerio*, *Daphnia pulex*, *Poecilia reticulata*, *Selenastrum capricornutum* and engineered *Aliivibrio fischeri* enzyme inhibition tests were also included (DWS, 2018). A shortlist of peer-reviewed research papers (Rimayi et al., 2015, Rimayi et al., 2016, Rimayi et al., 2017, Rimayi et al., 2018a, Rimayi et al., 2018b, Rimayi et al., 2018c). A small number of sites for selected targeted compounds is also listed (DWS 2018). Rimayi co-authored more recent papers in the same line (Batayi et al., 2020, Rimayi and Chimuka, 2019, Rimayi et al., 2019) but these have not been referenced on the RQIS website. The Blue Drop and the Green Drop Certification Programmes were introduced in 2008 and implemented in 2009 by DWS (Burgess, 2016). It will be important going forward that, should the NTMP gain traction that EBM is included.

Table 2-2: Guidelines stipulated by the Department of Water Affairs and Forestry (DWAF) in 1996 and the South African National Standards (SANS) 241 in 2015

Constituent in µg/L	DWAF V1 Domestic use	DWAF V2 Recreational use	DWAF V3 Industrial use	DWAF V4 Agricultural use: Irrigation	DWAF V5 Agricultural use: Livestock watering	DWAF V6 Agricultural use: Aquaculture	DWAF V7 Aquatic Ecosystems	SANS 241	General effluent standard	
									General limit	Special limit
Alkalinity ($CaCO_3$)			i) ≤ 5×10^4 ii) ≤ 1.2×10^5 iii) ≤ 3×10^5 iv) ≤ 10^6			2×10^4–10^5				
Aluminium	≤ 150			≤ 5×10^3	≤ 5×10^3	< 30	5–10	≤ 300		
Ammonia	≤ 1×10^3					≤ 25	≤ 7×10^{-9}	≤1.5×10^3	6×10^3	2×10^3
Antimony								≤ 20		
Arsenic	≤ 1×10^{-8}			≤ 100	≤ 10^3	≤ 50	10	≤ 10	20	0
Atrazine	≤ 2×10^{-9}					≤ 1.8×10^{-8}	10			
Barium								≤ 700		
Beryllium				≤ 100						
Boron				≤ 500	≤ 5×10^3			≤ 2.4×10^3	10^3	500
Cadmium	≤ 5			≤ 10	≤ 10		≤ 0.4	≤ 3	5	1
Calcium	≤ 3.2×10^4				≤ 10^6					
Chemical oxygen demand			i) ≤ 10^4 ii) ≤ 1.5×10^4 iii) ≤ 3×10^4 iv) ≤ 7.5×10^4						7.5×10^4	3×10^4
Chloride			i) ≤ 2×10^4 ii) ≤ 4.5×10^4 iii) ≤ 10^5 iv) ≤ 10^5	≤ 10^5	≤ 1.5×10^6	< 6×10^5		≤ 3×10^5		
Chlorine	≤ 10^5						0.2	≤ 5×10^3	250	0
Chromium (VI)				≤ 100	≤ 10^3	< 0–2×10^{-8}	7		50	20
Cobalt				≤ 50	≤ 10^3		≤ 1.4			
Copper	≤ 10^3			≤ 200	≤	< 5		≤ 2×10^3	10	2
Cyanide						< 20	1	≤ 200	20	10
Dissolved organic carbon	≤ 5×10^3									
Dissolved oxygen						6–9	80–120% of saturation			

Endosulfan							0.01			
Fluoride	≤ 10^3			≤ 2×10^3	≤ 2×10^3		750	≤ 2.5×10^5	10^3	10^3
Iron	≤ 100		i) ≤ 100 ii) ≤ 200 iii) ≤ 300 iv) ≤ 10^4	≤ 5×10^3	≤ 10^4	< 10	< 10% of background dissolved iron concentration	≤ 2×10^3	300	300
Lead	≤ 10^{-8}			≤ 200	≤ 100	< 10	≤ 1.2	≤ 10	10	6
Lithium				≤ 2.5×10^3						
Magnesium	≤ 30 000				≤ 5×10^5					
Manganese	≤ 50		i) ≤ 50 ii) ≤ 100 iii) ≤ 200 iv) ≤ 1×10^4	≤ 20	≤ 10^4	< 100	180	≤ 400	100	100
Mercury	≤ 10^{-9}				≤ 10^3	< 10^3	0.04×10^{-9}	≤ 6	5	1
Molybdenum				≤ 10	≤ 10					
Monochloramine								≤ 3×10^3		
Nickel				≤ 200	≤ 10^3			≤ 70		
Nitrate	≤ 6×10^3				≤ 10^5	< 3×10^5		≤ 1.1×10^4	1.5×10^4	1.5×10^3
Nitrite						< 50		≤ 900		
Nitrogen (inorganic)				≤ 5×10^3			v) < 500 vi) 500–2.5×10^3 vii) 2.5×10^3–10^4 viii) >10^4			
pH	6–9	6.5–8.5	i) 7–8 ii) 6.5–8 iii) 6.5–8 iv) 5–10	6.5–8.4		6.5–9	5–10	5–9.7	5.5–9.5	5.5–7.5
Phenol	≤ 10^{-9}					< 10^3		≤ 10		
Phosphorus (inorganic)						100	v) < 5×10^{-9} vi) 5×10^{-9}–25×10^{-9} vii) 25×10^{-9} – 250×10^{-9} viii) >250×10^{-9}		10^4	10^3 (median); 2.5×10^3 (max)
Potassium	≤ 5×10^4									
Selenium	≤ 2×10^{-8}			≤ 20	≤ 5×10^{-8}	< 300	2	≤ 40	20	20
Silica			i) ≤ 5×10^3 ii) ≤ 10^4 iii) ≤ 2×10^4 iv) ≤ 1.5×10^5							

Sodium adsorption ratio				2						
Sodium	≤ 10^5			≤ 7×10^4	≤ 2×10^6			≤ 2×10^5		
Sulphate	≤ 2×10^5		i) ≤ 3×10^4 ii) ≤ 8×10^4 iii) ≤ 2×10^5 iv) ≤ 5×10^5		≤ 10^6			≤ 5×10^5		
Sulphide						< 1				
Suspended solids			i) ≤ 3×10^3 ii) ≤ 5×10^3 iii) ≤ 5×10^3 iv) ≤ 2.5×10^4	≤ 5×10^4					2.5×10^4	10^4
Total chromium								≤ 50		
Total dissolved solids	≤ 4.5×10^5		i) ≤ 10^5 ii) ≤ 10^5 iii) ≤ 4.5×10^5 iv) ≤ 1.6×10^6	≤ 256	≤ 10^6	< 2×10^3				
Total Hardness			i) ≤ 5×10^4 ii) ≤ 10^5 iii) ≤ 2.5×10^5 iv) ≤ 10^6			20–100				
Total organic carbon								≤10		
Trihalomethanes	≤ 10^{-7}							≤ 1		
Uranium	≤ 70			≤ 10				≤ 10		
Vanadium	≤ 100			≤ 100	≤ 10^3					
Zinc	≤ 3×10^3			≤ 10^3	≤ 2×10^4		2	≤ 5×10^3	100	40
References	DWAF, 1996a	DWAF, 1996b	DWAF, 1996c	DWAF, 1996d	DWAF, 1996e	DWAF, 1996f	DWAF, 1996g	SANS 241, 2015	Government Gazette, 2013	

i: Industrial processes that needs high quality water; ii: Water of intermediate to high quality is required for these processes; iii: Domestic water quality with baseline minimum standards; iv: Industrial processes which can use water of any quality; v: Under oligotrophic conditions; vi: Mesotrophic conditions; vii: Eutrophic conditions; viii: Hypertrophic conditions

These programmes were incentive based and aimed to improve drinking water quality (Blue Drop) and management of wastewater treatment plants (WWTPs) (Green Drop), which are the responsibilities of municipalities. One of the requirements of the Blue Drop programme is that the SANS 241 guidelines should be met. And one of the requirements for the Green Drop programme is that, at a minimum, the general effluent standard (Table 2.2 'General effluent standard') should be met in the case of an unlicensed WWTP (Government Gazette, 2013). A licensed WWTP would receive its own, customised requirements upon receiving its licence. The customisation is based on the size of the WWTP and the receiving river. In his 2020 State of the Nation Address, South African president Cyril Ramaphosa said the government is working to revive the Blue and Green Drop certification programme, which was disbanded in 2014 (Bega, 2021), but the last Blue Drop and Green Drop report found on the Department of Water and Sanitation's webpage is from 2012. Public interest organisations such as AfriForum took over the monitoring and tested 118 WWTPs and the drinking water quality of 220 towns (AfriForum, 2020). Their report shows that 90 sewage systems and 5 towns did not comply with the limits of the general effluent standard (Table 2.1) ((AfriForum, 2020)(integrated water management plan SA)).

2.3.2 Integrated Water Quality Management (IWQM) in South Africa

The DWS's responsibility lies not only in guaranteeing equitable distribution of water to all South Africans, but also protecting the water resource for future generations (DWS., 2017). This means managing the water quality of the resource and controlling the inputs from the sources of pollution are very important. Water quality management (WQM) is a balance between protecting the resource and the need for development and growth in South Africa. A successful Integrated Water Quality Management (IWQM) plan calls for an inclusive approach of all key role players in the water sector (government, private sector and civil society) (DWS., 2017). In 2015, the DWS began the "Water Quality Management Policies and Strategies for South Africa project", which was proposed to run from October 2015 to September 2017. This project included five phases; i) Inception phase; ii) Assessment phase; iii) Policy phase; iv) Strategy phase; v) Practice phase (DWS., May 2016). The key elements of this policy can be seen in Figure 2.6 (DWS., July 2016).

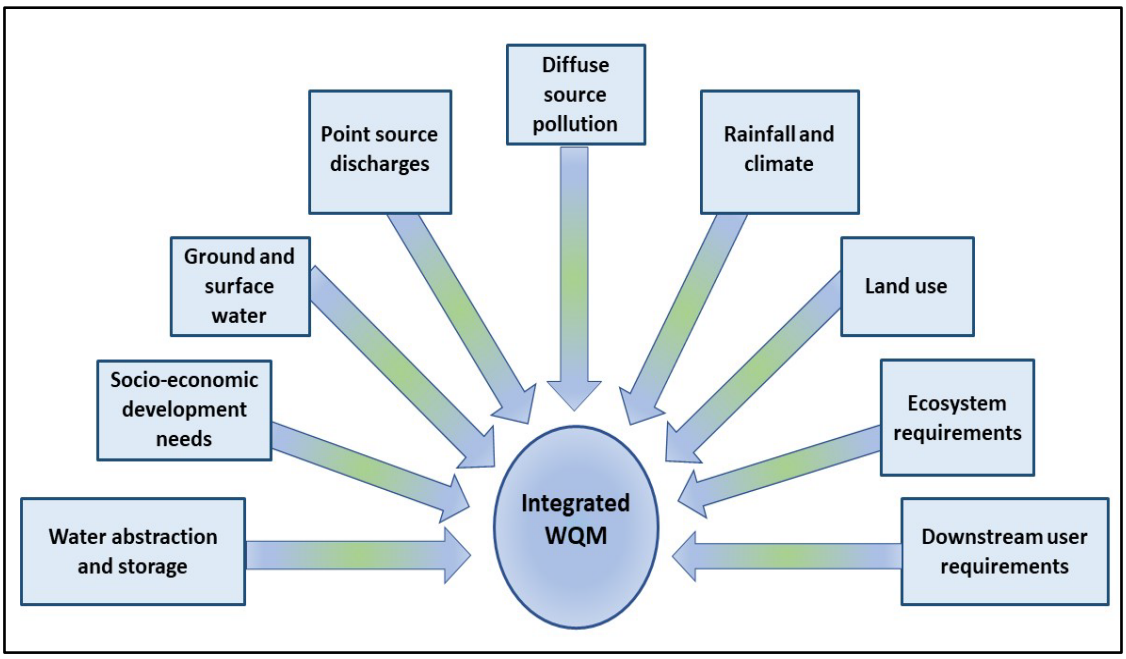

Figure 2-6: The key elements if the Integrated Water Quality Management process (DWS, July 2016)

There are already concerns with regards to the significant issues of eutrophication, salinization, acid mine drainage and other widespread water quality challenges. The IWQM Policy and Strategy is a response to the many water quality challenges that South Africa is facing. Currently, this is a government-wide task led by the DWS. The process of WQM Vision to the Strategic issues is presented in Figure 2.7 (DWS., December 2016).

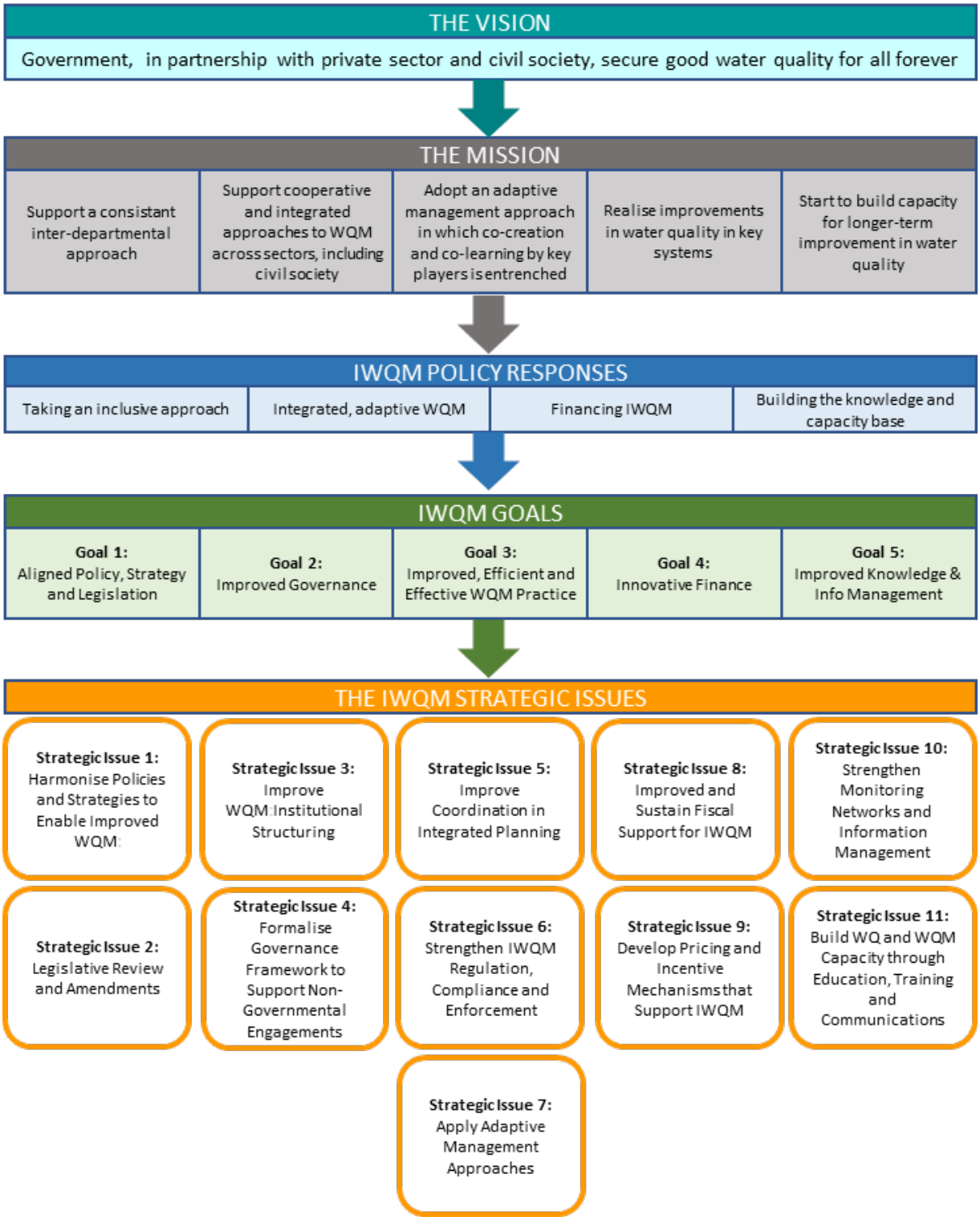

Figure 2-7: A representation of the Water Quality Management Vision and Strategic Issues for South Africa. (DWS., December 2016)

2.3.3 How does this impact water safety planning in South Africa?

According to Schreiner (2013) and Takacs (2016) the South African National Water Act (NWA) (Act 36 of 1998) was lauded by the international water community as one of the most progressive pieces of water legislation, particularly in the translation of the concept of integrated water resources management (IWRM) into legislation. Core to the NWA of 1998 is the principle that water is a scarce natural resource that belongs to all of the people of South Africa, and thus it should be used beneficially and in the public interest. The act was also more appropriate for a water-scarce country such as South Africa than water-rich Europe. The Act has also been used as an example when countries like China and Zambia wanted to revise their water legislation (Schreiner, 2013). Unfortunately, the implementation of the act has only been moderately successful and, in some cases, non-existent. Specifically, monitoring the quality of water resources regularly has declined to a point where it seems to be non-existent, apart from a number of smaller research studies (Kruger et al., 2022). Water quality monitoring is lacking due to corruption, lack of expertise, ineffective management of sewage and finances and, as a result, there are gaps in the monitoring data (DWS 2019b). These gaps cause incomplete and erroneous assessments, which prevent decision-making (Kruger et al, 2022).

Many of the objectives of the GWRC project will benefit SA in making sure that they include these various endpoints recommended by the WHO and EU. Currently, SA is in the process of updating the SANS 241 drinking water quality regulations. The updated regulations will include testing for contaminants of emerging concern and endocrine activity bioassays. However, many of these assays are unavailable or unaffordable in private settings. There are academic institutions in South Africa that do have some of these bioassays or those with similar endpoints (*in vitro* and tier 1 *in vivo*) that can measure mode of action (MOA). This also meets several of the strategic objectives set out in the WRC CP18.

Although the ultimate goal is to integrate EBMs into a water safety plan in South Africa, this was not in the scope of this study. There is still a lot of research and work needed before this can be achieved.

CHAPTER 3: DECISION-MAKING TOOLS FOR SAMPLE COLLECTION AND ANALYSIS USING BIOASSAYS

3.1 INTRODUCTION

Over the last few decades, *in vitro* bioassays have been widely applied for water quality screening, and there is a large body of literature to support this (Escher et al., 2014, Escher et al., 2021, Altenburger et al., 2019, Snyder and Leusch, 2018). As a result, many of the advantages and limitations are recognised, and over the last few years, they have shown their usefulness in developing an effect-based assessment in conventional water quality monitoring (Snyder and Leusch, 2018). Environmental water is a complex sample made up of a mixture of chemicals, and therefore a single bioassay cannot capture all the effects that may be induced (e.g., estrogenic, anti-androgenic and thyroid activity). There are a large number of assays with different MOAs on the AOP, and therefore it is impractical to try to test the sample for all endpoints. It is preferable to select a practical battery of assays, three to four that are representative of effects commonly found in water samples.

Assay selection will also depend on the context of the sampling campaign (i.e., what you are wanting to test for; e.g., DWTP product or treatment efficacy) and the type of water (e.g., influent, effluent or the different steps of processing) required. For example, water type (e.g., WWTP). The GWRC (2020a) WP 3.2 in the case of wastewater or reuse of non-potable water recommends assays that would represent the different stages of toxicity pathway, for example, xenobiotic metabolism, receptor-mediated effects and adaptive stress responses. Note that some of these assays have not yet been established in SA, but are included in the toolbox as various team member groups have decided to establish them in the laboratory.

The most commonly applied assays are those that are indicative of activation of aryl hydrocarbon receptor (AhR), activation of the estrogen receptor (ER) and oxidative stress response, respectively. Proposed EBTs are available for these endpoints (Brand et al., 2013, Escher et al., 2013, Escher et al., 2015, van der Oost et al., 2017, Escher et al., 2018). In addition, these three endpoints are responsive in various water sources or types. These recommendations are also suggested by Brack et al (2019) for rivers that may well be receiving effluent input, but at the same time are used as a source for DWTPs (a scenario often seen in SA). By using the same test battery, it allows effects of the site to be monitored over time and the effects can be compared. If additional endpoints need to be investigated for endocrine disruption, then the glucocorticoid receptor (GR) or anti-androgenic activity assays can be added. Unfortunately, EBT values for these assays are not currently available in the scientific literature

3.2 GUIDANCE ON SAMPLE COLLECTION AND PRE-TREATMENT

3.2.1 Sample collection

When planning a sampling event there are certain steps or processes to bear in mind to have a successful sample collection.

3.2.1.1 Representativeness of the samples

*E*xposure may be assessed for a number of different reasons which may influence the choice of sampling locations or sampling matrices. In other words, where to take the sample at the site of choice, which will also depend on the type of water you are testing (GWRC, 2020a, Altenburger et al., 2019).

3.2.1.2 Timing and frequency of sampling

Collection of samples should address exposure of greatest concern (e.g., long-term chronic exposure vs. intermittent short-term exposure), patterns of contamination (e.g., continuous discharge vs. one-time accidental contamination), and address the endpoints of greatest concern.

Note on passive sampling

Passive samplers are not commonly used as less than 10% of reviewed studies in the GWRC 2020, WP3.2 and most studies used them in surface water protocols applied passive sampling (Creusot et al., 2014, van der Oost et al., 2017, Toušová et al., 2019, GWRC, 2020a). Passive samplers are useful as they can be placed on site in the water for a longer period of time in the water environment over a longer period of time and allows chemical and bioassay analysis of very low concentrations of chemicals. A limitation may be that the composition of the chemical mixture taken up into the sampler may differ from the chemical mixture in the water as different chemicals will have different uptake rates into the passive sampler. For more information on passive sampling please refer to the WRC project no. C2020-2021 -00347.

3.2.1.3 Selection of matrices

The choice of matrix is determined by factors such as relevance to route of exposure, ease and practicability of sampling, which analytes are to be assessed, and in some cases, ethical considerations. In this case our matrix is water.

3.2.1.4 Statistics

Appropriate statistical methods need to ensure the relevance of sampling (e.g., pooled vs. individual samples, numbers of samples from different locations/species)

3.2.2 Sample pre-treatment, preservation, filtration and storage

Special methods may need to be used to avoid contamination with other chemicals with potential EDC activity, which are commonly found in sampling and laboratory equipment (e.g., plasticizers).

Internationally and currently, there is no standard for water preparation, which is a critical step in the process of EBM analysis, particularly as it can affect the sensitivity and accuracy of the bioassays. This is further discussed in the review by Robitaille et al. (2022). The GWRC developed a flow chart for water preparation; however, this has been adapted for the SA scenario (Figure 3.1). The pre-treatment of samples for other EBMs can be found in the draft toolbox from the WRC project C2020/2021-00165. It is important to remember that after collecting the samples they should remain on ice until they are delivered to the testing laboratory. Ideally, any pre-treatment step like adjusting the pH (usually to 3)

should be done as soon as possible after sample collection. One of the main reasons for sample acidification is to prevent bacterial growth, which could degrade contaminants over time. This can be done by adjusting the pH prior to SPE to 3 using HCl or H_2SO_4. For some studies, methanol, formaldehyde, or copper sulphate (Schilirò et al., 2009, Schilirò et al., 2012, Li et al., 2011, Conley et al., 2017a, Kibambe et al., 2020, Spina et al., 2020) were used for preservation of the sample (Robitaille et al., 2022).

Acidification could impact the recovery of some chemicals. Other studies have found that the recovery of some compounds, such as progestins and mifespristone is decreased (Šauer et al., 2018), while Stalter et al. (2016a) showed that acidification could improve the recovery of DBS for various SPE sorbents for chemical and cytotoxicity assays. Samples containing disinfection by-products (DBP), particularly chlorinated samples, should be treated with sodium thiosulphate. While chlorine will not be extracted by SPE, quenching is important to prevent the formation of additional DBPs and to prevent the chlorine from potentially reacting with the SPE sorbent. Many studies use sodium thiosulphate to quench the residual chlorine (Macova et al., 2010a, Escher et al., 2014, Neale et al., 2020), some use ascorbic acid instead (Conley et al., 2017b, GWRC, 2020a). Because EDCs fall into different categories of compounds, acidification could have various effects on the recovery of the SPE. It is important that this should be further investigated (Robitaille et al., 2022).

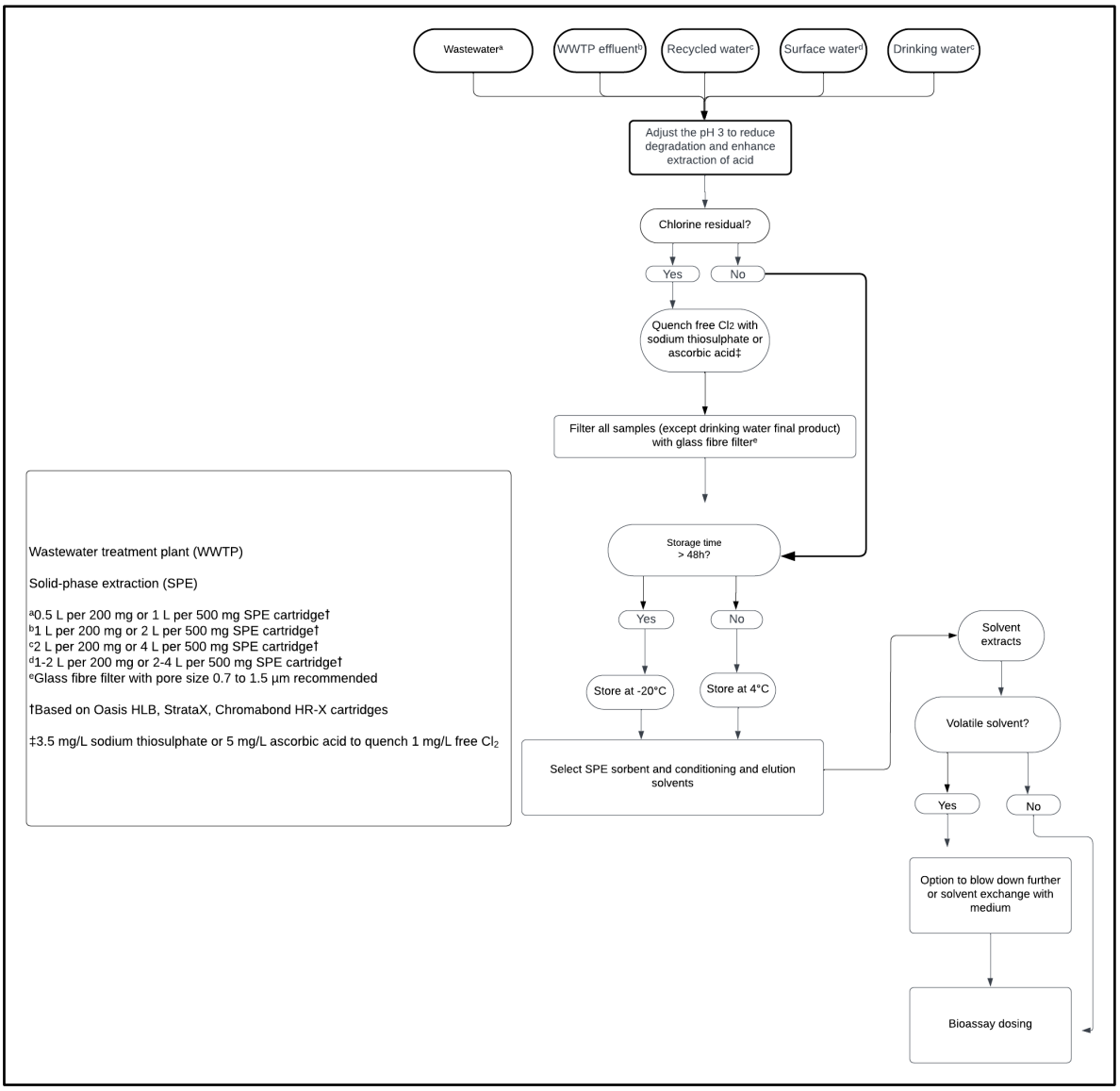

Figure 3-1: Decision-making flow chart for sample pre-treatment and processing (adapted for South Africa from Neale et al.2020)

Depending on the source of water, the sample is not only acidified but may also be treated to remove large particulates that may cause an obstruction in the SPE cartridge. These particulates can be removed by filtration with glass-fibre filters 0.7-2µm if turbidity is greater than 5 Nephelometric turbidity units (NTU) or additional filter discs with a 0.22–5 µm pore size Figure 3.1 (GWRC, 2020b) (as discussed in the relevant methods in the toolbox from WRC project C2020/2021-00165). If any pre-treatment has taken place, it is important to run the sample through the SPE cartridge as soon as possible. Once this step is complete, the dry cartridge can be stored in the fridge for 24 hours and then placed for longer storage at -20˚C. The review by Robitaille et al. (2022) gives further details and a summary on the preparation, pre-treatment and storage of water samples for the different bioassays.

3.2.3 Sample concentration/extraction and elution methods

3.2.3.1 Solid phase extraction SPE

This is the most commonly used method for EDC enrichment and can be in a cartridge or disc form. It allows for the concentration of hydrophobic to neutral contaminants in the water sample. At the same time, it removes salts, inorganics metals. Most of the SPE methods allow for the concentration of hydrophobic to neutral contaminants present in water samples, as well as allowing the removal of salts, inorganics, metals and most of the dissolved organic carbon (DOC), which may cause interferences with bioassays (Poole, 2003, Robitaille et al., 2022). More importantly, the SPE results in a lower limit of detection for quantification of EDCs and can be used for multiple bioassays. There are five steps involved in basic SPE for water samples:

I. Water must be passed on a pre-conditioned (with selected solvents) column
II. The column is washed and dried
III. The contaminants are eluted with chosen solvents
IV. The eluate can be concentrated even more by evaporation of the solvents
V. The concentrated eluate is reconstituted into a small volume with a solvent of choice.

Although the basic steps are found in most articles, methods and solvents may vary depending on the study and initial volume of the sample, pre-treatment, choice of sorbent, choice of solvent and the temperature of evaporation (Robitaille et al., 2022). The concentrated extract can be run in bioassays. According to Ademollo et al. (2012) SPE has a number of advantages, including good recovery of a wide range of contaminants and the ability to be automated.

3.2.3.2 Liquid-liquid extraction (LLE)

Liquid-liquid extraction use is extremely limited most likely due to the high solvent use and time-consuming nature. It is not a method that is recommended for sample enrichment of water samples (GWRC, 2020b).

3.2.3.3 Extraction limitations

Any extraction procedure using solvent extraction, passive sampling and conventional SPE sample processing involves a blow-down step. Many solvents, volatile chemicals and some DBPs, like trihalomethanes, will be lost during the evaporation process and therefore are lost in the final extract. There is also the potential for the loss of volatile chemicals when using mammalian cell-based assays (incubated at 37°C for often 16 to 24 h). According to Stalter et al., (2013), some bioassays can be adapted to run without a headspace to prevent the loss of volatile chemicals. It seems that volatile DBPs appear to only have a minor contribution to the overall effects (Stalter et al., 2016b), which suggests that we can capture the majority of DBP-associated toxicity with simpler common SPE methods (GWRC, 2020b).

3.2.4 Sample elution

At the end of the process of extracting the sample which has sorbed on the SPE sorbent, the cartridge can be dried in a vacuum or under nitrogen stream for up to two hours. At this point, the dried cartridge can be sealed with parafilm and kept dark by wrapping it in aluminium foil and stored at -20°C until elution (Tang et al., 2014). This is advantageous, particularly if the sample cannot be immediately tested

in the bioassays or has to be sent to other laboratories for testing as opposed to sending litres of unenriched water. Further details for elution and specific solvents can be found in the GWRC 2020, WP 3.3 report, and can also be found in the review by Robitaille et al., (2022).

3.2.5 Guidance on sampling for sample analysis using *in vitro* and *in vivo* bioassays

The GWRC (2020b) "Effect-based monitoring in water safety planning" WP 3.3 has suggested sampling strategies, which depend on the purpose, objective and sample context. As summarised in Figure 3.2:

I. If the sampling purpose is to evaluate the product of a drinking water treatment plant (DWTP), the objective would be to ensure that the water is safe to drink, and the final water will be compared to an EBT. Therefore, the only sample required would be the product water and an appropriate blank and control sample.

II. If the treatment process efficiency needs to be tested, then the source water and product water (including the blank and control) will be required. This may include samples from every step of the treatment process (e.g. advanced oxidation or disinfection).

III. Wastewater would need composite samples to capture diurnal variation; many studies collected 24h composite influent and effluent samples (Nelson et al., 2011, Petrie et al., 2017, Körner et al., 2001, Bicchi et al., 2009, Macova et al., 2010b, Reungoat et al., 2010, Jálová et al., 2013, Bain et al., 2014, Roberts et al., 2015).

IV. Drinking or recycled water should not change in quality over time, then grab sampling is a suitable mode of collection.

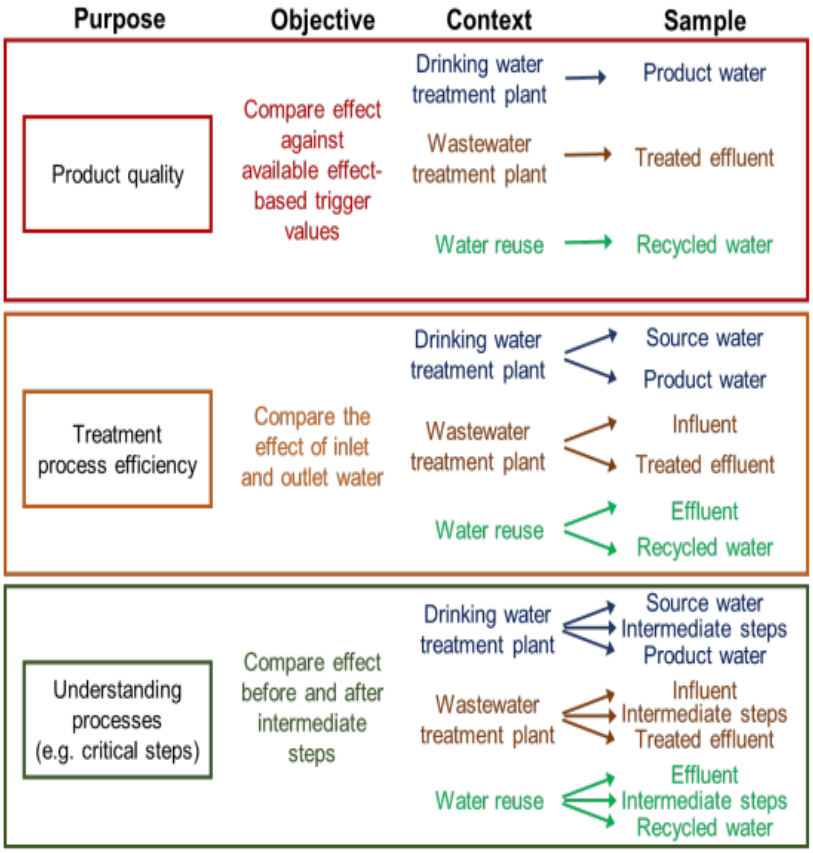

Figure 3-2: Examples of different sampling campaign purposes for drinking water, wastewater and water reuse with the required samples for each purpose indicated. (GWRC 2020. WP3.3)

V. Glass bottles, preferably amber, should be carefully prepared by washing with an appropriate solvent or burnt-out (500°C for 2 h) amber glass bottles with caps that do not contain polymer liners. (If amber bottles are unavailable, cover the clear bottles with tin foil to keep the contents in the dark. Once the samples have been collected, the bottles should be stored on ice (or in a portable cooler at 4˚C) and in the dark. The samples should reach the laboratory as soon as possible for further processing and assessment.

VI. It is important to include field and process blanks in any sampling strategy. Denison et al. (2020) also recommended matrix-spiked samples for recovery experiments.

VII. Sample volume - Sample volume should also be noted, as this will depend on numerous factors:
   a. The expected level of chemical contamination and the need for concentration, for example, if you are investigating a WWTP, taking a sample from the influent will require a lower volume compared to that of drinking water, which will require a larger volume (Table 3.1).
   b. The type and size of the SPE cartridge.

Table 3-1: Example of sample volumes based on 200 mg HLB SPE cartridge

Matrix	Sample volume	Final extract volume	Enrichment factor
Wastewater influent	**0.5 L**	**0.5 mL**	**1000**
Wastewater effluent or surface water	**1 L**	**0.5 mL**	**2000**
Drinking water or recycled water	**2 L**	**0.5 mL**	**4000**
**** Majority of studies in South Africa for all types of water**	**1 L**	**1 mL**	**1000**

** Samples are always taken in duplicate (2 x 1 L) to ensure there is a backup sample in case of breakage.

Further information on SPE for EBM is found in detail in the WRC 2020/2021-00165. The number of assays that can be run from a single extract will depend on the number of repeats planned and how much extract is dosed, but typically only small extract volumes (microlitres) are required for 96 and 384-well plate assays, meaning that a number of different assays can often be run.

According to Neale et al. (2020) a practical test battery/suite of assays should consist of three to four assays representative of effects commonly detected in water samples; other effects may be missed (GWRC, 2020b) . Neale et al. (2017) stresses that the exact type of bioassay is not essential, but the battery of selected assays should be diverse and indicative of crucial steps in the toxicity pathway relevant to the micropollutants occurring in the source water being investigated. Therefore, assay selection will also largely depend on the water context (type), e.g. wastewater, desalinated water, drinking water, etc. The purpose of testing is also important in utilising the decision tool guideline. This will also vary depending on what you want to test. For example, when testing the water quality of ground or surface water or if you are looking at the efficacy of treatment processes (Neale et al, 2017). The test batteries will be aligned for this purpose. See Tables 3.2, 3.3 and 3.4 and Figures 3.3 and 3.4. Details are also available on the Factsheets in WRC report 2020/2021-00165.

Note: Guidance on the specific bioassays, quality control, technical guidance on bioassay data evaluation and bioequivalents are available on the Factsheets in WRC report 2020/2021-00165.

3.3 DECISION-MAKING TOOL FOR THE SELECTION OF BIOASSAYS

3.3.1 Bioassay selection based on the assays available in South Africa

There are a number of different bioassays measuring different endpoints that are recommended by the GWRC for effect-based monitoring of water quality. However, not all are available in South Africa. Based on a literature search and also through discussions with other institutions and the private sector, the project team has developed a list of potentially suitable bioassays to use to develop a water safety framework (in line with the GWRC recommendations) for South Africa. These assays are part of a battery of tests to test the biological effects in water extracts and are aligned with relevant steps of AOP (Figure 3.3) as recommended by the GWRC for water safety.

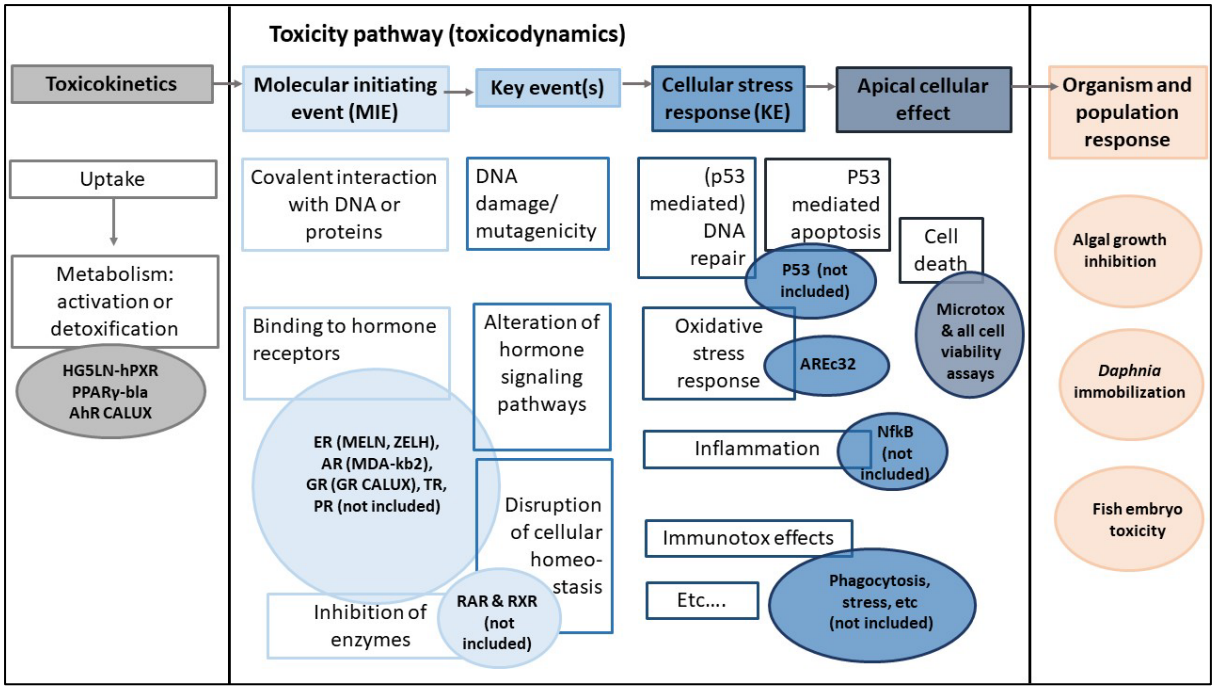

Figure 3-3: Design of the panel of bioassays/biological endpoints (ovals) recommended for water quality assessment, and where they are situated along the adverse outcome pathway (boxes)

The bioassays selected for this part of the project are to test the effective application of EBM methods that can be found in detail in the toolbox of bioassays from WRC 2020/2021-00165 or the summary factsheets.

3.3.1.1 In vivo bioassays

These tests can be used to measure apical effects. They are whole organism bioassays, some are accredited with international standards organisations (e.g. OECD, ISO, EEC, USEPA, ASTM); 30-minute *Allivibrio fischeri* bioluminescent test (bacteria), 72-hour *Selenastrum capricornutum* growth inhibition test (algae), 48-hour *Daphnia magna* acute toxicity test (invertebrate, crustacean) and two-day *Brachionus calyciflorus* chronic toxicity test (rotifer). The Integrated Water Use Authorisation Bioassay Toolkit (Pearson. et al., 2015) includes three of the proposed bioassays. The endpoints of these bioassays range from growth inhibition or stimulation, mortality and behaviour.

***In vivo* bioassays – Acute toxicity**
Bioluminescent test:

- *Aliivibrio fischeri* (bacteria)

Growth inhibition test:

- *Pseudokirchneriella subcapitata* (micro algae)
- *Spirodela polyrhiza* (duckweed plant)

Mortality:

- *Daphnia magna* (invertebrate)
- *Thamnocephalus platyurus* (invertebrate; fairy shrimp)
- *Poecilia reticulata* (vertebrate; guppy)

When doing toxicity tests, particularly on whole effluent tests, it is important that it is representative and different trophic levels should be tested (Figure 3.4)(Escher et al., 2021).

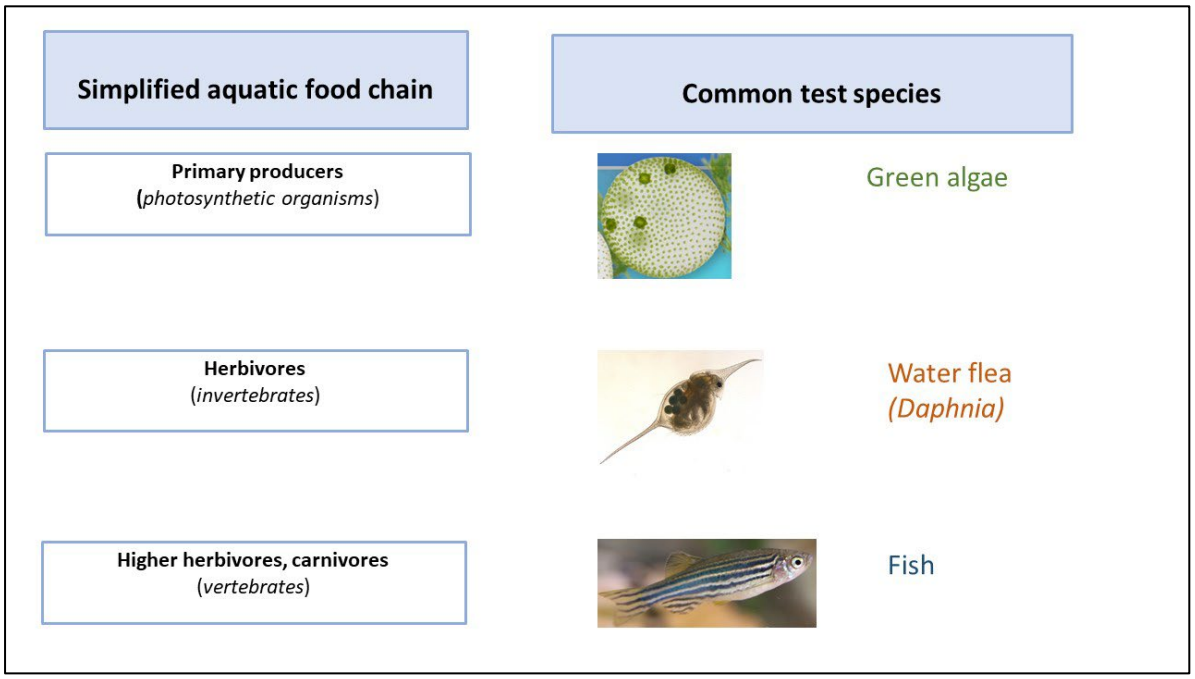

Figure 3-4: Test organisms representing different trophic levels in aquatic toxicology

NOTE:
Factsheets for the *in vivo* assays can be found in the Annexure section of the WRC 2020/2021-00165, bioanalytical toolbox report.

3.3.1.2 In vitro bioassays

The following *in vitro* bioassays are included to assess the best for the application (to test DW, WW, ground or surface water).

***In vitro* bioassays – Proxies for long-term effects, i.e., Indicators**
EDCs:

- T47D-KBluc reporter gene assay (estrogenic activity)
- MDA-KB2 reporter gene assay (androgenic and glucocorticoid activity)
- GH3.Tre.Luc reporter gene assay (thyroid activity)
- AhR reporter gene assay (commercially known as the DR-CALUX)
- Yeast Estrogen screen (YES)
- Yeast androgen screen (YAS)

Cytotoxicity:

- MTT or the very expensive eXelligence (real-time cell analyser) on any of the cell lines.
- Cell lines not genetically modified can be used here: HuTu (human intestinal cells (adenocarcinoma))

Oxidative stress:

- Reactive oxygen species (ROS)
- Catalase activity (CAT)
- Superoxide dismutase activity (SOD)
- Other oxidative stress assays can be added such as protein carbonyl formation
- Lipid peroxidation indicated by malondialdehyde (MDA) content
- The AREc32cell line is an indicator of oxidative stress this assay was established in the Environmental Chemical Pollution and Health (ECPH) Research Unit UP- GWRC collaboration with UFZ)

NOTE:
Factsheets for the *in vitro* assays can be found in the Annexure section of the WRC 2020/2021-00165, bioanalytical toolbox report.

3.3.2 Decision-making tools for bioassay selection, sample collection and processing

Based on the GWRC Effect-based Monitoring in Water Safety Planning project decision-making tool, these are the following recommendations. It is critical that the appropriate bioassays for water quality monitoring are selected based on what is available in South Africa. An important point to consider is why and what you sampling for. Therefore, choosing the correct sampling strategy, suitable sample collection, for example, time of the year (seasonal or according to rainfall), how often (daily, weekly, monthly, etc), particularly when monitoring a system. Correct sample collection is also important, and this can include volume of sample, site selection and pre-treatment options. Processing strategies are important, for example, if the sample strategy is to test the drinking water process, it is important to take note of, for example, the presence of chlorine in the post-treatment water and final water, as it may require treatment, can be seen in Figure 3.1.

A reliable, good assay to include in the battery of assays is activation of the AhR for most water sources. For DWTP or water reuse for potable use, an assay indicative of either genotoxicity or mutagenicity should be used in addition to activation of AhR, ER and oxidative stress response (e.g., AREc32 assay). Adding an oxidative stress response assay can detect increased effects after drinking water disinfection, which is important. The GWRC 2020 WP 3.2 report has suggested a decision-making tool that includes three test batteries. Table 3.2 gives the recommended endpoints in the different test batteries for drinking water, specifically as suggested by the GWRC. The battery of tests for environmental water quality monitoring recommended for South Africa (by this project team) is shown in Table 3.3. Table 3.4 recommends the battery required for a specific sampling campaign.

Table 3-2: The recommended endpoints within the relevant test battery for drinking water quality monitoring from the GWRC

Test battery	Recommended bioassays			
Battery 1	Low sensitivity ER*	Oxidative stress	AhR	
Battery 2	High sensitivity ER**	Oxidative stress	AhR	
Battery 3	High sensitivity ER**	Oxidative stress	AhR	Mutagen or genotoxicity (e.g. Ames test)

* Low sensitivity ER includes the yeast reporter gene assay; ** High sensitivity ER needs a mammalian reporter gene assay (Taken from the GWRC 2020, WP 3.2 report)

Table 3-3: The recommended endpoints within the relevant test battery for environmental water quality monitoring available in South Africa

Test battery	Recommended bioassays			
Battery 1	Low sensitivity ER*	Oxidative stress	AhR	***In vivo* tests:** *Allivibrio fischeri, Pseudokirchneriella subcapitata (algae), Daphnia magna, Poecillia reticulata* **Optional:** *Spirodela polyrhiza, Thamnocephalus platyurus*
Battery 2	High sensitivity ER**	Oxidative stress	AhR	***In vivo* tests:** *Allivibrio fischeri, Pseudokirchneriella subcapitata (algae), Daphnia magna, Poecillia reticulata* **Optional:** *Spirodela polyrhiza, Thamnocephalus platyurus*
Battery 3*	High sensitivity ER**	Oxidative stress	AhR	Mutagen or genotoxicity (eg Ames test)

*Battery 3 in this table refers to environmental water that is directly used for drinking water

Table 3-4: Test battery selection depending on sampling campaign context (water type) and purpose

Type of water sample required	Purpose		
	Assess product quality	*Assess treatment efficacy*	*Understand treatment processes (eg CCP)*
Wastewater treatment	Battery 1	Battery 1	Battery 2
Water reuse (non-potable)	Battery 2	Battery 2	Battery 2
Drinking water (Including potable)	Battery 3	Battery 3	Battery 3
Groundwater	Battery 3	Not applicable	Not applicable
Surface water	Battery 2	Not applicable	Not applicable

* If the surface water is used for drinking water, mutagen or genotoxicity assays should also be included (Adapted from GWRC 2020, WP 3.2 for SA conditions)

Table 3.2 to 3.4 can be used together by water laboratories or stakeholders to decide on a way forward with sample testing strategies discussed later in this report. In the case of the bioassays in Table 3.2 and 3.3 the word “Sensitivity” stands for assay responsiveness. This means that the detection limit of the assay must be sufficiently low to be able to detect effects in very clean water samples (e.g., DWTP effluent). For this to be successful, either the sample needs to be highly concentrated or the bioassays that respond already to low concentrations of chemicals are used.

Example 1: Sampling campaign purpose is to assess WWTP product quality alone then it is only necessary to use a battery of low sensitivity assays, such as yeast reporter gene assays, could be applied as these assays are typically sufficiently sensitive to detect effects in treated effluent i.e., Test battery 1.

Example 2: Sampling campaign is to understand critical processes in WWTPs or in a water reuse context use Test battery 2. (Why - this is because yeast reporter gene assays are unlikely to be sensitive enough to detect effects after advanced treatment processes. But the majority of mammalian reporter gene assays are similarly sensitive – so these can be applied).

Example 3: If the sampling campaign is investigating drinking water. Test battery 3 must be used. Therefore, this should also include a genotoxicity or mutagenicity assay, such as Ames or umuC, which will be particularly important if disinfected, e.g., chlorinated, water is being evaluated.

NOTE:

In all test batteries, the specific effects measured should be accompanied by cytotoxicity assessment. This is because cytotoxicity may cause false negative results (e.g., masking the effect) or false positive results (e.g., “cytotoxicity burst” phenomena (Judson et al., 2016). If a reporter gene assay is used that cannot be duplexed with a quantitative cytotoxicity assay that reports effect concentrations for cytotoxicity (e.g., inhibitory concentration IC10), then it is imperative to include an assay with an apical endpoint (Robitaille et al., 2022).

3.4 INTEGRATING EBM INTO A WATER SAFETY PLAN (WSP)

3.4.1 EBM for water quality monitoring

Applying EBM (*in vivo* and *in vitro* assays) for water quality monitoring, the following is important;

I. Select the correct sampling strategy i.e. the purpose of the investigation
II. Use suitable pre-treatment and processing methods
III. Extraction methods (often referred to as enrichment techniques)
IV. Select the appropriate assays for the purpose (drinking water quality, the treatment processes or wastewater treatment plant (WWTP) quality)

While bioassays are sensitive screening tools to detect complex mixtures of organic micropollutants in water samples, micropollutants are often present at low concentrations (e.g., nanogram per litre) in drinking water and clean source waters (Glassmeyer et al., 2017, Tröger et al., 2018), so water samples may need to be enriched up to 100 times in the assay before an effect can be detected. *In vitro* assays typically target complex mixtures of organic micropollutants but not inorganics and metals, which can be comprehensively analysed using chemical methods. Therefore, extraction methods also serve to separate the organic micropollutants from the matrix, inorganics and metals in a water sample. The

literature reviewed in WP3.2 “Medium-to-high throughput bioanalytical tools and decision-making tool for selection of bioassays” indicated that three methods were used to enrich and isolate organic micropollutants from whole water samples: solid-phase extraction (SPE), passive sampling and liquid-liquid extraction (LLE) (GWRC, 2020a). It is important to note that these methods are continuously being developed or improved.

3.4.2 Developing a framework for integrating effect-based monitoring into water safety planning

This section provides a basic background on how to develop a framework for how effect-based monitoring can be integrated into a WSP. The framework is a generic guide to using the EBM to get information required to ensure safe water and good water quality. This includes test frequencies and how to set alert level triggers and what to do if the effect in a sample exceeds its EBT (Figure 3.4). Guidance is given on what to do if the effect in a sample exceeds EBT.

There are four different monitoring categories within the WSP framework. System assessment monitoring, operational monitoring, verification monitoring, and validation monitoring. Effect-based monitoring can be applied within the four categories and from catchment to customer. The four monitoring categories, the frequency of sampling, and how often to apply effect-based monitoring within the campaign, and where to sample are important for water safety (Figure 3.5).

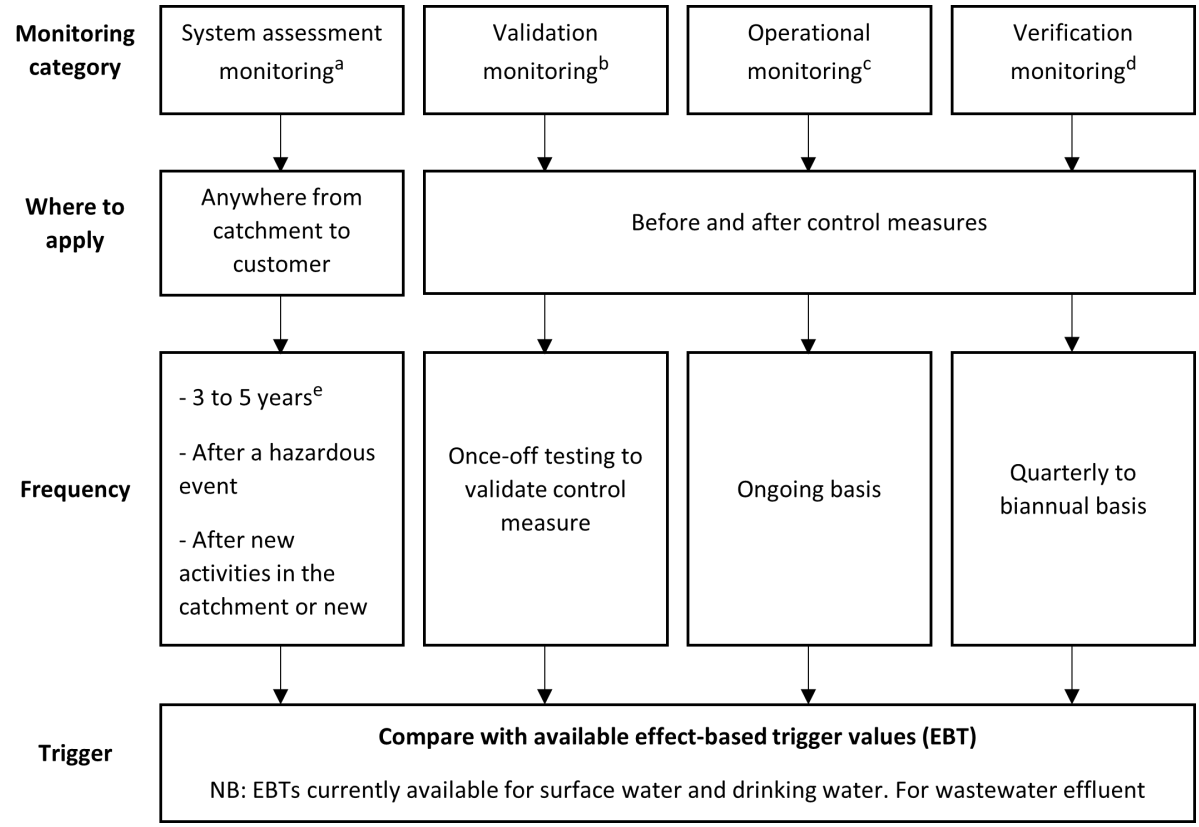

Figure 3-5: Overview of how effect-based monitoring can be applied within WSPs, with a focus on different monitoring categories, monitoring location and monitoring frequency

[a]monitoring to characterise water quality and help inform risk assessments and define treatment requirements; [b]monitoring to provide evidence that control measures are effective; [c]monitoring of control measures on a continual

basis to confirm they are working in a timely manner; [d]monitoring used to verify routine operations; [e]more frequent system assessment monitoring is required for smaller reservoirs or reservoirs with more variable water quality; [f]a dilution factor of 10 was selected as a conservative estimate of dilution of wastewater effluent into a receiving waterbody. However, if the operator knows that the dilution factor is larger or smaller, the correct dilution factor can be used instead

Effect-based methods can account for the effects of complex mixtures of chemicals and a wide range of chemicals in water. Including those present below the analytical detection limits. They provide fundamental advantages for risk assessment and risk management, especially when applied as a complement to targeted chemical analysis. In many countries, EBM has not been integrated into WSPs as yet and particularly not in South Africa. In order for it to be implemented, certain things need to be in place, and these include the development of guidance documents, frameworks and standard operating procedures. This is required for both bioassay operators (laboratory technicians and scientists) and the WSP team (water stakeholders) to support the uptake of EBM into WSPs (Neale et al., 2022). This project is one of the first in South Africa currently contributing to developing a plan to integrate these assays for water quality and safety assessment to protect our water sources.

For all types of monitoring, the observed effect should be compared to an EBT. Effect-based trigger values are available for drinking water and surface water for a number of endpoints, but are not readily available for wastewater effluent. The effect in wastewater effluent can be compared to surface water EBT after dilution. If the dilution factor is not known, a dilution factor of 10 can be used as a conservative estimate of wastewater dilution into a receiving water body. Available EBTs are provided on a factsheet from WRC 2020/2021-00165.

The response in a bioassay, expressed as BEQ_{bio}, is compared to the EBT-BEQ if available. If the BEQ_{bio} is lower than the EBT-BEQ then no further action is required. If the measured BEQ_{bio} value exceeds the EBT-BEQ, the first step is to check the bioassay quality control (QC) and collect another water sample from the same site and re-test (this can pose a challenge in South Africa as many laboratories are not close to the sample sites, therefore a second “back-up” sample is always collected). This is comparable to what is currently done for chemical analytes. If the BEQ_{bio} of the second sample is below the EBT-BEQ, then no further action is required. If the second test confirms the initial positive result and both samples report a BEQ_{bio} > EBT-BEQ, then further action is needed. The magnitude of the response should depend on the magnitude of the exceedance and regulatory advice (Figure 3.6).
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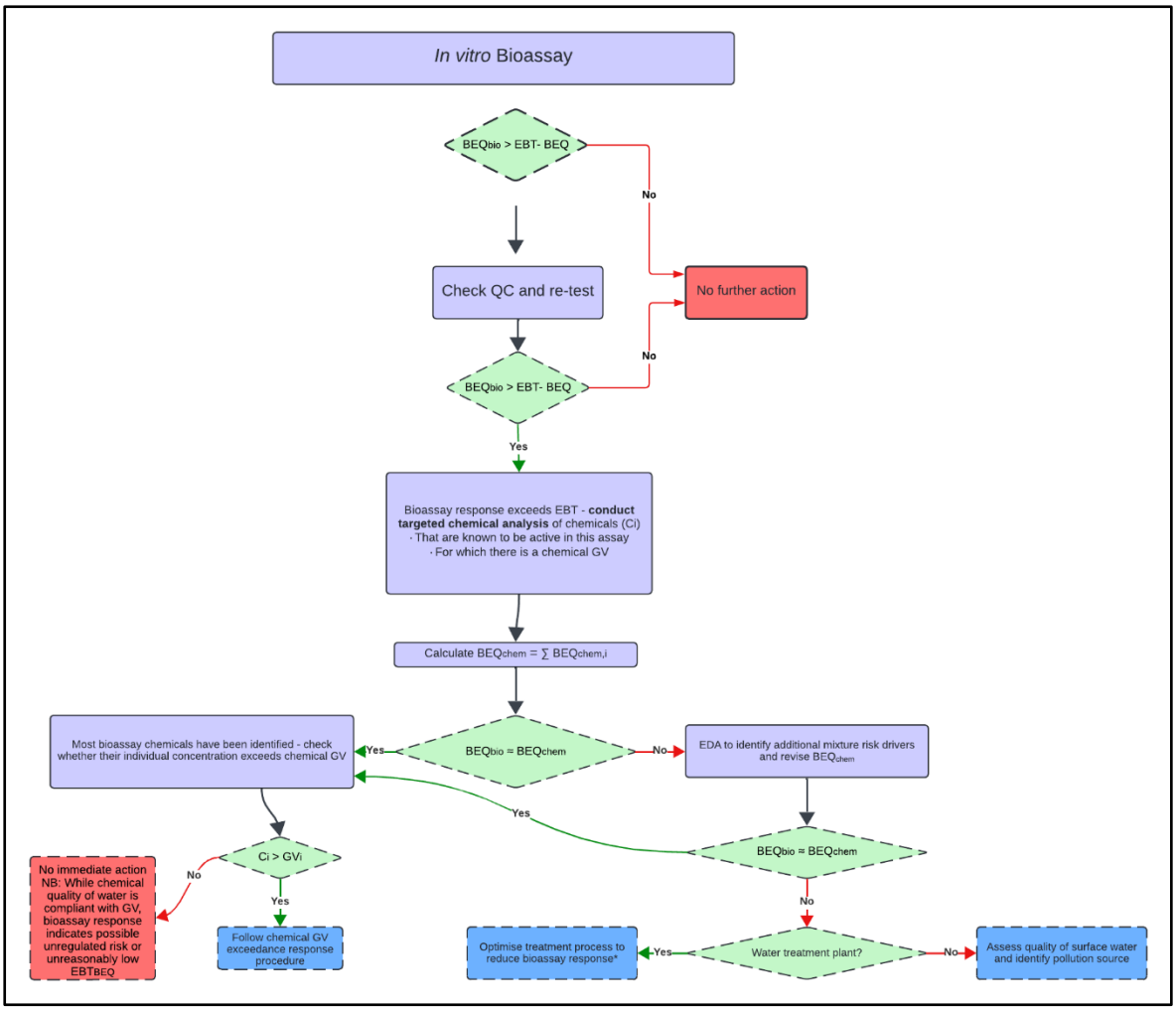


Figure 3-6: Flow diagram to represent the process of assessing the outcome of the bioassay (BEQ_{bio}) versus the EBT of a water sample. (Adapted from Escher et al, 2021).

CHAPTER 4: CASE STUDIES

4.1 INTRODUCTION

Before any definitive decisions can be made about which bioassay should be included for effect-based monitoring of water quality and incorporated into any water safety plan, it is advisable to “test run” these assays on the various water sources and test their suitability. This can be done through case studies focusing on drinking water, groundwater, surface water and wastewater. Doing this will help verify treatment efficacy and validate control measures. The selected *in vivo* and *in vitro* bioassays were selected based on the toolbox of bioassays from WRC 2020/2021-00165.

4.2 CASE STUDY SITE CRITERIA

The South African water industry is facing a challenge of water scarcity and pollution, both of which threaten the sustained, safe supply of drinking water to the population. Water resources pollution, mainly caused by factors such as agricultural runoff, discharge of poorly treated industrial effluents, lack of wastewater management in settlements, and discharge of inadequately treated municipal wastewater, has compromised the quality of water resources in many areas in South Africa. Water scarcity, exacerbated by climate change, population growth, and over-extraction, puts additional pressure on the already limited water resources. Due to water scarcity, a diverse water sources (such as surface water, groundwater, seawater and wastewater), often contaminated with natural and anthropogenic compounds, are used for drinking water production. These compounds may adversely affect the ecosystems in which they are found.

To investigate the application of effect-based methods for evaluating the performance of treatment technologies (such as conventional drinking water, water reuse, desalination and wastewater treatment plants), as well as for characterizing the impacts of wastewater discharges into water resources, the following considerations were used for case study selection;

- A drinking water treatment plant that uses conventional water treatment technologies for drinking water production
- A drinking water treatment plant that abstracts raw water from an impacted water source to represent an unplanned and indirect potable water reuse technology
- A drinking water treatment plant that uses treated wastewater as feed water for drinking water production (planned potable water reuse technology)
- A drinking water treatment plant that uses seawater for drinking water production (desalination technology)
- A wastewater treatment plant that treats domestic wastewater for discharge into a water resource
- A surface water body that receives discharges from point and/or non-point sources

The case study locations selected were spread across various provinces, and each had at least one or more water treatment plants, except for Limpopo Province, where surface water is used by the local communities for drinking and household use. The following was considered for analysis of the water samples collected;

- At least one *in vitro* or small-scale *in vivo* whole-organism (e.g., algae, daphnids, fish embryos) bioassay used
- At least two different types of water tested in a plant (e.g., untreated and treated)

- Water samples prepared using solid-phase extraction (SPE)
- Bioassay results expressed quantitatively (i.e., effect concentration or equivalent concentration rather than just +/-
- Quality control parameters for bioassays reported (i.e., positive control (=potent reference compound); negative controls (= clean water run through the whole enrichment process with SPE; solvent control)
- More than one bioassay used (preferably a test battery with assays that target different modes of action)
- Chemical analysis of organic micropollutants conducted alongside bioanalysis (in the same water samples/extracts)

4.3 DESCRIPTION OF CASE STUDY SITES

4.3.1 Mpumalanga province

A town in Mpumalanga province was identified as a suitable study site for this project. It is an example where wastewater is first treated before being released into ponds, where some nitrogen is lost through denitrification and some by drainage to the groundwater, from where it discharges into a stream feeding an adjacent wetland. The northern part of the wetland is connected to a river via a dam, which serves as the raw water source for the drinking water treatment plant. The town's wastewater treatment plant is located northeast of the wetland. The effluent from the plant enters the wetland after the dam that serves as the source for the DWTP. This active wetland serves as a home to a number of bird species and other animals that may be at risk of exposure to various pollutants like EDCs. The location of sites selected for sample collection at the Mpumalanga town is shown in Figure 4.1, and they are as follows:

i. Dam 1 (raw water source for drinking water treatment plant MP-DWTP2a)
ii. Wastewater treatment plant (MP-WWTP3b) effluent (settling dams)
iii. Upstream wetland (MP-SW2)
iv. Middle of wetland (MP-SW3)
v. Downstream wetland (MP-SW4)
vi. Natural spring (MP-GW2)
vii. Dam 2 (dam downstream of the wetland – MP-SW1)
viii. Borehole water (MP-GW1)
ix. Tap water (from DWTP – MP-DWTP2b)
x. Bottled water from a local natural spring (MP-GW4)

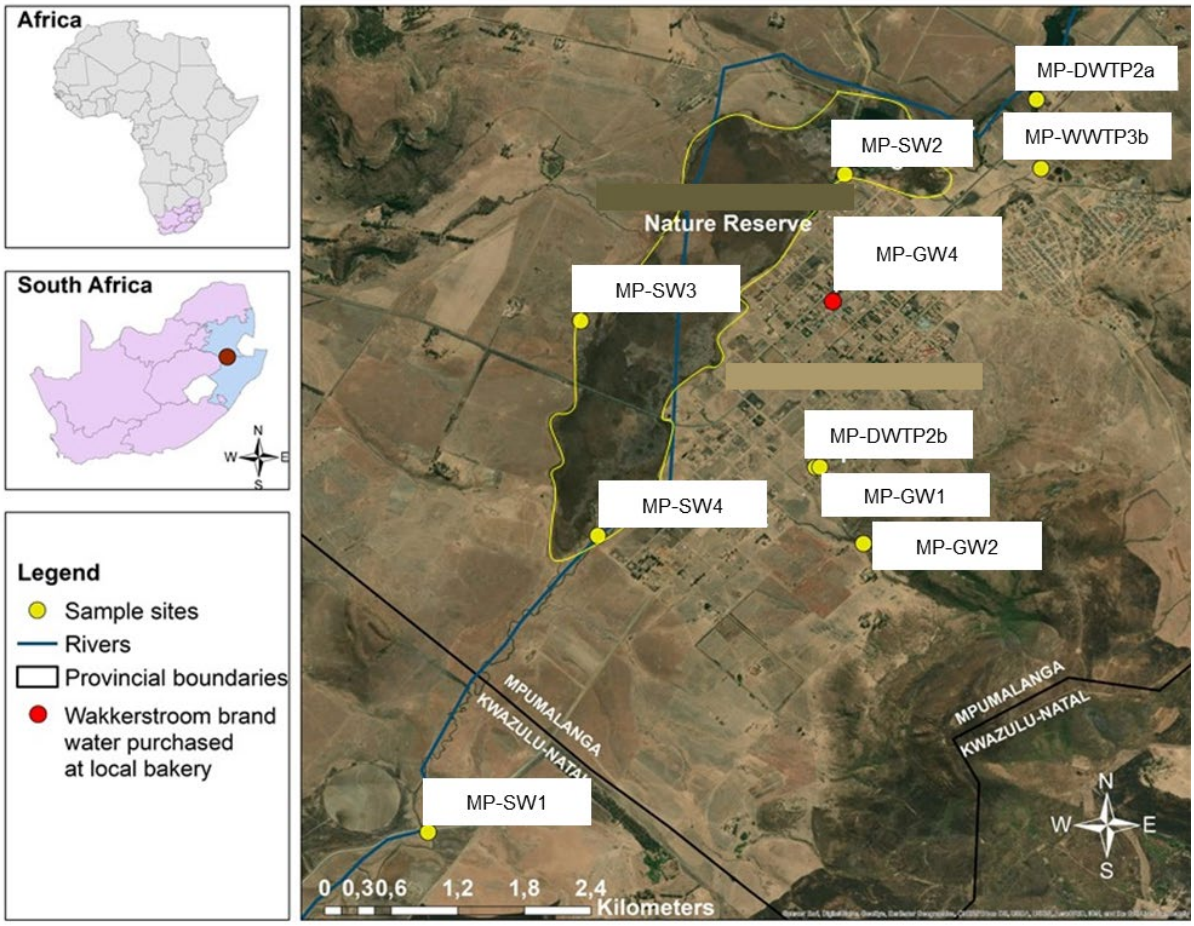


Figure 4-1: Map of sampling sites in Mpumalanga

4.3.2 North West Province

A water utility that abstracts water from Department of Water and Sanitation (DWS) dams and provides bulk water and secondary services directly to communities, mines, industry and municipalities in the North West province was selected for this study. Raw water is channelled to four drinking water treatment plants where it is treated. The municipalities that are serviced by this utility draw the water supplied through reservoirs to consumers for household use. An indirect potable water reuse plant (NW-DWTP3) was selected for this project.

This plant has been in existence for more than 46 years and has been regularly upgraded as new technologies were available, and to keep up with the demand for water. At the same time, there has been a deterioration in raw water quality. The plant has grown from 18ML/d 270ML/d in late 2016; all four plants can be seen in Figure 4.2 taken from Ncobo's MSc dissertation (2019). Raw and final treated water were collected from this plant for the scope of this project.

Figure 4-2: NW-DWTP3 Google Maps, 11 August 2018 (Ncobo, 2019)

4.3.3 Gauteng Province

4.3.3.1 Conventional drinking water treatment plant (GP-DWTP1)

Gauteng has one of the world's largest urban nature reserves, situated south of the City of Tshwane (also known as Pretoria) but still within the city limits. On its border is a drinking water treatment plant that supplies 17 – 19% of Pretoria's drinking water (Figure 4.3). The stream into the reserve receives effluent from wastewater treatment plants, industries and informal settlements in the catchment areas. There are two dams, interconnected with a wetland and channel. Although previous studies have shown estrogenic activity in the water sources within the reserve (Bornman et al., 2007), this plant is still considered a conventional DWTP as it primarily uses standard treatment processes, including coagulation, flocculation, sedimentation, filtration, and disinfection, to produce drinking water. Raw, final treated and backwash water were collected from this plant for the scope of this study.

Figure 4-3 GP-DWTP1 (Google maps, 27 Oct 2024)

4.3.3.2 Conventional drinking water treatment plant (GP-DWTP6)

GP-DWTP6 (Figure 4.4) is the largest bulk water utility in Africa and is one of the largest in the world. This water utility provides bulk potable water to more than 11 million people in Gauteng, parts of Mpumalanga, the Free State and North West – an area that stretches over 18 000 km^2. The Utility has thirteen tertiary pumping stations and 60 strategically located service reservoirs and secondary booster stations; as well as a multi-billion Rand regional pipeline network of approximately 3500km. Raw and final treated water were collected from this plant for the scope of this study.

Figure 4-4 GP-DWTP6 (Google maps, 15 Nov 2024)

4.3.3.3 Wastewater treatment plant (GP-WWTP1)

A wastewater treatment plant (WWTP) situated in downtown Tshwane (Pretoria) (GP-WWTP1) was selected for this study. The plant was constructed between 1913 and 1920 on the southern banks of the Apies River in Pretoria (Figure 4.5). The plant is situated adjacent to the central business district and is one of 15 wastewater treatment plants in operation. The WWTP employs technologies, such as biological nutrient removal (BNR) activated sludge, and biological filters for liquid processing. On the sludge side, it uses DAF thickening, anaerobic digestion and solar drying beds. Influent (GP-WWTP1a) and effluent (GP-WWTP1b) samples were collected from the WWTP. Upstream (GP-SW12) and downstream (GP-SW13) water samples were also collected from the Apies River.

Figure 4-5 GP-WWTP1, Pretoria (Google maps, 27 October 2024)

4.3.3.4 Wastewater treatment plant (GP-WWTP 2)

GP-WWTP2 is one of the largest wastewater treatment works found in Johannesburg, Gauteng. It is located on the Jukskei River, some 30 km north of the Johannesburg central business district and serves most of the city of Johannesburg's sewer basins north of the Witwatersrand (Figure 4.6). The influent (GP-WWTP2a) and effluent (GP-WWTP2b), as well as upstream (GP-SW10) and downstream (GP-SW11) water samples from the Jukskei River, were collected for this site.

Figure 4-6 GP-WWTP2 situated near Johannesburg (Google maps, 27 Oct 2024)

4.3.4 Western Cape Province

A seawater desalination plant (WC-DWTP4) that treats water for drinking purposes through reverse osmosis at a rate of 2 megalitres of water a day was selected. This plant does not run all year round, but functions during peak periods over the summer months. The plant draws beach sand filtered borehole seawater through submersible pumps sinking 12 m deep adjacent to the beach (Figure 4.7). The waste product of the reverse osmosis process, brine, is pumped through an outlet pipe 250m out to sea, where the tides and wave action on the rocks ensure that the brine and sea water are well mixed to protect the environment from the effects of brine. The hydraulic retention time of the treatment process is 1 hour. Grab water samples were collected from the intake tap (WC-DWTP4a) before undergoing a series of treatment processes. The product water samples (final water WC-DWTP4b) were collected from a dedicated sampling tap after completion of the purification process. Samples were also collected from a borehole (WC-GW3) at the facility.

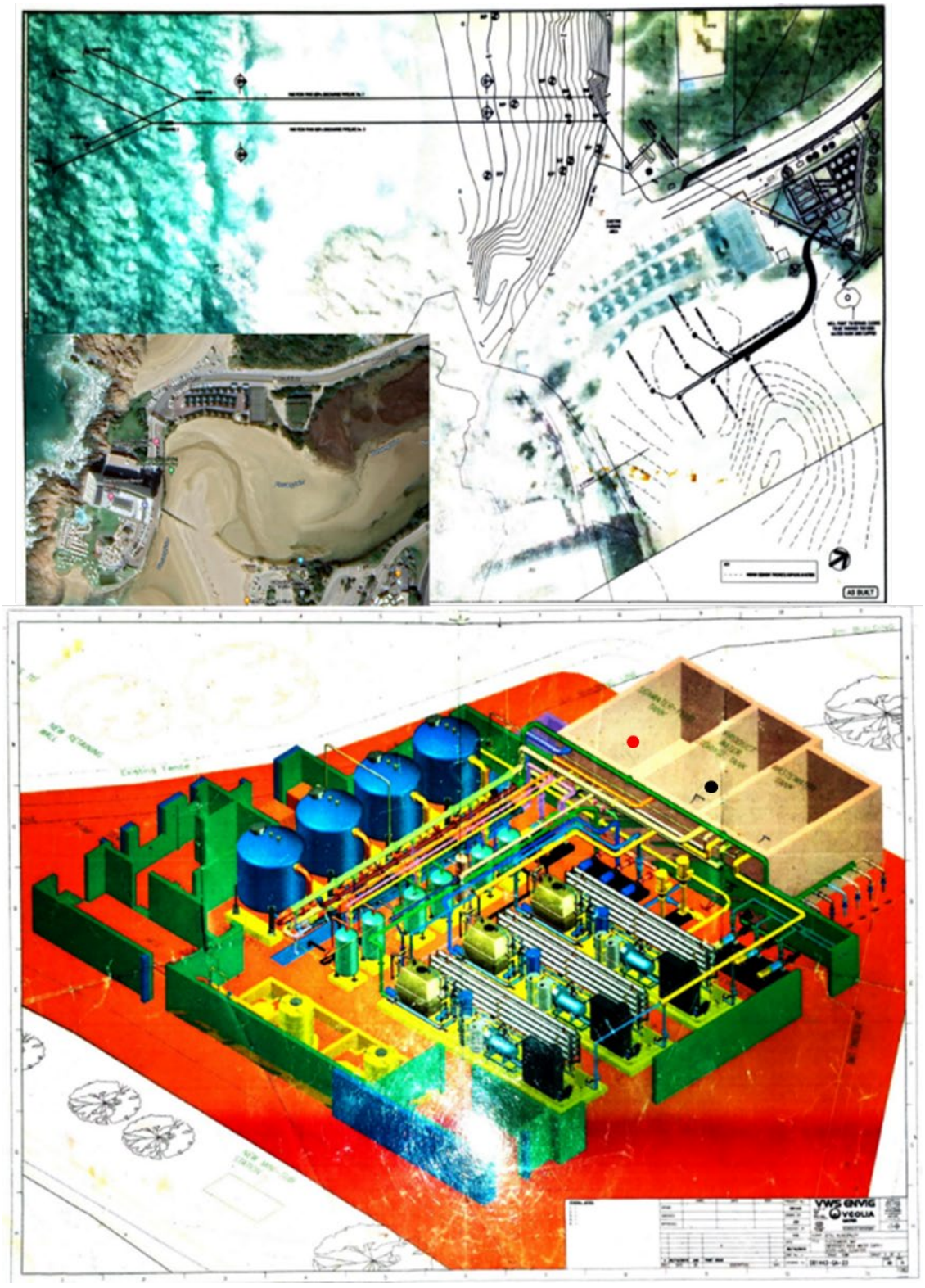


Figure 4-7 Desalination plant layout showing various stages of the treatment train. The seawater feed water tank (raw water) sampling point (1) and the product water (final water) storage tank sampling point (2). (Source: WRC Project final report 2020/2021-00347)

4.3.5 Limpopo Province

The Vhembe District Municipality is situated in Limpopo in the north-eastern corner of South Africa, bordering Zimbabwe and Mozambique. This is a malaria-endemic area where insecticides are used for malaria vector control. Many of the communities in this area rely on surface water for their needs. This water may be contaminated with pollutants like insecticides (DDT, DDE and pyrethroids), and pharmaceuticals (anti-retrovirals, TB drugs and malaria prophylaxis).

Two dams were selected in the area, namely Albasini dam, which lies outside the malaria spray programme area and the Nandoni dam that falls inside the sprayed area. The Luvuvhu and Doornspruit rivers flow into Albasini dam. Figure 4.8 shows the locations where samples were collected, and they are as follows:

i. Doornspruit River (Albasini dam inlet 1, L-SW5)
ii. Luvuvhu River (Albasini dam inlet 2, L-SW6)
iii. Albasini dam outlet (L-SW7)
iv. Nandoni dam inlet (L-SW8)
v. Nandoni dam outlet (L-SW9)

Figure 4-8 Map of sampling sites for Vhembe district, Limpopo Province, South Africa
Sample site

4.3.6 KwaZulu-Natal province

A direct water reuse plant (KZN-DWTP5) is a demonstration facility that receives treated effluent directly from wastewater treatment works and employs advanced technologies to treat it to potable drinking water standards. The treatment train processes include advanced oxidation, biologically activated

filters, and ultrafiltration membranes, which collectively provide multiple barriers against contaminants of emerging concern, such as nanomaterials, pharmaceuticals, and endocrine disruptors (Figure 4.9). Grab water samples from both the treated effluent from wastewater treatment works, which serves as the raw water for the DWTP, and final treated water from the demonstration DWTP were taken.

Figure 4-9 KZN-DWTP5 direct potable water reuse plant in KwaZulu Natal (Google maps, 15 Nov 2024)

4.4 SAMPLE COLLECTION

Samples were collected during the period 08/2021-08/2023. Table 4.1 shows the location of the samples (province), sample type and samples collected. The sample sites have been categorised for this progress report into DWTPs, WWTPs, groundwater (includes boreholes and spring water), and surface water (includes rivers, dams and wetlands). Each of the samples were screened using bioassays and analysed for micropollutants.

Table 4-1: Sites categorised according to the water sample sources

Province	Water sample type/treatment technology	Site ID	Collected sample(s) ID
Drinking Water Treatment Plant (DWTP)			
Gauteng (GP)	Conventional drinking water treatment plant	GP-DWTP1	GP-DWTP1a (raw) GP-DWTP1b (final treated)
Mpumalanga (MP)	Conventional drinking water treatment plant	MP-DWTP2	MP-DWTP2a (raw) MP-DWTP2b (final treated)
North West (NW)	Indirect potable water reuse plant	NW-DWTP3	NW-DWTP3a (raw) NW-DWTP3b (final treated)
Western Cape (WC)	Seawater desalination plant	WC-DWTP4	WC-DWTP4a (feed/raw) WC-DWTP4b (final treated)
KwaZulu Natal (KZN)	Direct potable water reuse plant	KZN-DWTP5	KZN-DWTP5a (raw) KZN-DWTP5b (final treated)
Gauteng (GP)	Conventional drinking water treatment plant	GP-DWTP6	GP-DWTP6a (raw) GP-DWTP6b (final treated)
Wastewater treatment plants			
Gauteng	Biological nutrient removal system	GP-WWTP1	GP-WWTP1a (influent) GP-WWTP1b (effluent)
Gauteng	Biological nutrient removal system	GP-WWTP2	GP-WWTP2a (influent) GP-WWTP2b (effluent)
Mpumalanga	Settling pond system	MP-WWTP3	MP-WWTP3a (influent) MP-WWTP3b (effluent)
Borehole, spring water, groundwater (GW)			
Mpumalanga	Borehole water	MP-GW1	MP-GW1
Mpumalanga	Natural spring	MP-GW2	MP-GW2
Western Cape	Borehole	WC-GW3	WC-GW3
Mpumalanga	Bottled water from a local natural spring	MP-GW4	MP-GW4
Surface water (rivers, dams and wetlands) (SW)			

Mpumalanga	Dam	MP-SW1	MP-SW1
Mpumalanga	Upstream wetland	MP-SW2	MP-SW2
Mpumalanga	Middle wetland	MP-SW3	MP-SW3
Mpumalanga	Downstream wetland	MP-SW4	MP-SW4
Limpopo	River (dam 1 inlet 1)	L-SW5	L-SW5
Limpopo	River (dam 1 inlet 2)	L-SW6	L-SW6
Limpopo	Dam (dam 1 outlet)	L-SW7	L-SW7
Limpopo	Dam (dam 2 inlet)	L-SW8	L-SW8
Limpopo	Dam (dam 2 outlet)	L-SW9	L-SW9
Gauteng	River – upstream GP-WWTP2	GP-SW10	GP-SW10
Gauteng	River – downstream GP-WWTP2	GP-SW11	GP-SW11
Gauteng	River – upstream GP-WWTP1	GP-SW12	GP-SW12
Gauteng	River – downstream GP-WWTP1	GP-SW13	GP-SW13

4.5 SAMPLES PRE-TREATMENT

The analysis of water samples using effect-based methods (bioassays) and analytical techniques enables the detection of a broad range of contaminants, allowing researchers to assess not only the presence of specific pollutants but also their potential biological effects on ecosystems and human health. Samples will usually require some form of pre-treatment before they can be analysed in the bioassays.

4.5.1 Sample preparation and extraction procedure for *in vitro* bioassays

Water samples selected for analysis were collected from the identified sample sites within the study areas in specially prepared (rinsed with HPLC grade methanol) 1L glass Schott bottles, in triplicate. The pH of the water was adjusted to 3 using concentrated hydrochloric acid, and then filtered using a 0.22-micron and glass wool filters. All samples were stored at 4ºC in the dark until extraction.

Samples were extracted at UP using a solid phase extraction method (SPE). The Oasis HLB (hydrophilic lipophilic balance) glass cartridges (5 cc/200 mg) were placed on the SPE vacuum manifold and pre-conditioned with 5mL EDC free water (Ultrapure water, MilliQ system fitted with an EDS filter) followed by 5mL HPLC grade methanol (MeOH) and followed by 5 mL EDC free water. One litre of the sample was loaded onto the SPE cartridge, taking care not to let the cartridge run dry and never

exceeding a flow rate of 10 mL/min. After the entire 1 L sample has passed through the column, the cartridges were dried under vacuum. The cartridges at this point were carefully wrapped in aluminium foil and stored at -20°C.

The cartridges were disseminated to the NWU for further testing in their suite of bioassays and a second cartridge was kept by UP to test in their bioassays. The cartridges were eluted using 5 mL MEOH. The solvent was evaporated under a gentle stream of nitrogen (37°C) and reconstituted in 1 mL HPLC grade ethanol (EtOH). The reconstituted samples were stored in amber glass bottles at -20°C and then applied to the bioassays.

4.5.2 Sample preparation and extraction procedure for chemical analysis

Grab water samples were collected and transported on ice to the laboratory, where they were processed within 48 hours. Samples were extracted using OasisTM HLB and dual stack MCX and MAX SPE cartridges.

4.5.2.1 OasisTM HLB

Extraction was performed using preconditioned OasisTM HLB SPE cartridges. Cartridges used for extraction were sequentially conditioned with 5 mL of methanol and 5 mL of ultrapure water at a flow rate of 5 mL/min. Thereafter, 500 mL of the water sample was loaded into the cartridge at a flow rate of 5 mL/min. The cartridge was then washed with 5 mL of 5% methanol in water, followed by vacuum drying with the help of vacuum suction for 5 min. Elution was performed with 2 x 5 mL of methanol at 2.5 mL/min. Methanol extracts were concentrated to dryness under a stream of nitrogen and reconstituted to a final volume of 1 mL in 50% methanol: ultra-pure water and stored at - 20°C until analysis.

4.5.2.2 Dual stack of MCX and MAX

The extraction process utilized a tandem cartridge configuration with Waters Oasis MAX and MCX SPE cartridges. This configuration facilitated a 3-tiered extraction mechanism that employed reversed-phase, anion exchange, and cation exchange methods. The extraction protocol was designed to ensure the retention of acidic, basic, and neutral compounds. The Oasis MCX cartridge was connected below the MAX cartridge, and both cartridges were conditioned by passing 5 mL of methanol and 5 mL of water. A vacuum was used to load a 1L water sample at 10 mL/min onto the dual stack using a bottle to SPE adapter. After the loading step was complete, the cartridge stack was disassembled, and each cartridge underwent specific wash and elution steps

The Oasis MAX cartridge was washed with 5 mL of ammonium hydroxide in water. The elution was performed in two steps: first with 5 mL of methanol (neutral) and second with 5 mL of methanol containing 5% formic acid (acidic compounds). Both elution fractions were collected in a 20 mL glass tube. The Oasis MCX cartridge was washed with 5% formic acid and eluted with 5 mL of methanol containing 5% ammonium hydroxide (basic). The MCX and MAX elution fractions were pooled and evaporated to dryness at 60°C under a gentle stream of nitrogen. The dried eluate was reconstituted with 1000 µL of 10 mM ammonium formate.

4.6 SAMPLES SCREENING USING *IN VIVO* BIOASSAYS

4.6.1 Analysis of physico-chemical parameters

Between 28/02/2022 and 01/03/2022, thirteen water samples were delivered to the BioTox Lab office for analysis. The pH levels of the samples ranged from 7.1 (MP-SW3 wetland middle) to 8.4 (MP-GW1 borehole). The pHs of all the samples were within the acceptable range (pH 6-9) in which pH can be excluded as a driving factor for toxicity (USEPA, 1996). The conductivities of the samples ranged from 8.8 mS/m (BW1 local bottled water Wakkerstroom brand) to 54 mS/m (Water Treatment Plant). A dissolved oxygen (DO) concentration above 4 mg/L is required for aquatic organisms (USEPA, 1996) to survive. The DO levels for the 13 samples submitted ranged from 7.9 mg/L (Unknown) to 9.7 mg/L (Norma). The physical-chemical parameter results are summarised in Table 4.2.

Table 4-2: Summary of the physical-chemical parameter results

Sample name	pH	Conductivity (mS/m)	Dissolved oxygen concentration (mg/L)
Drinking water treatment plants (DWTP)			
DWTP raw water			
GP-DWTP1a	8.01	17.4	9.43
DWTP final treated water			
GP-DWTP1b	7.83	10.9	9.21
Wastewater Treatment Works			
Wastewater Treatment Works influent			
ND	-	-	-
Wastewater Treatment Works effluent			
MP-WWTP3	8.12		9.01
Borehole/Spring water/Ground water			
MP-GW1, (borehole)	8.36	43.2	9.09
MP-GW2 (natural spring)	8.20	28.8	8.89
MP-GW4 (bottled water from local natural spring)	7.49	8.8	7.94
Surface water (rivers, dams and wetland)			

MP-SW1 (dam)	8.00	17.2	9.34
MP-SW2 (upstream wetland)	7.59	11.7	9.47
MP-SW3 (middle of wetland)	7.11	20.4	7.64
MP-SW4 (downstream wetland)	7.75	17.4	9.65
L-SW5 (river)	8.14	19.5	9.56
L-SW6 (river)	8.10	15.4	8.82
L-SW7 (dam)	8.07	18.1	9.90
L-SW8 (dam)	8.01	14.2	8.86
L-SW9 (dam)	8.15	14.7	8.92

4.6.2 Selection of *in vivo* bioassays

Table 3.4 shows the list of samples analysed using *in vivo* methods. For the scope of this study all the available methods were applied to all types of water, but the decision-making tool for the selection of *in vivo* bioassays can be found in Table 4.3, and is also further discussed in the relevant result sections for each water type as well as in the fact sheets in the appendixes. The samples were exposed to screening tests on 5 trophic levels as follows: *Aliivibrio fischeri* (bacteria), *Pseudokirchneriella subcapitata* (micro-algae), *Spirodela polyrhiza* (plant), *Daphnia magna* (invertebrate), *Thamnocephalus platyurus* (invertebrate) and *Poecilia reticulata* (vertebrate).

Table 4-3 A description of the different water samples and *in vivo* screening tests applied

Sample name	**Screening test**					
	Aliivibrio fischeri	*Pseudokirchneriella subcapitata*	*Spirodela polyrhiza*	*Daphnia magna*	*Thamnocephalus platyurus*	*Poecilia reticulata*
Drinking water treatment plants (DWTP)						

DWTP raw water						
GP-DWTP1a						
MP-DWTP2a	X	X	X	X	X	X
DWTP final treated water						
GP-DWTP1b						
MP-DWTP2b	X	X	X	X	X	X
Wastewater Treatment Plants (WWTP)						
WWTP influent						
GP-WWTP1a						
GP-WWTP2a						
MP-WWTP3a						
WWTP effluent						
GP-WWTP1b						
GP-WWTP2b						
MP-WWTP3b	X	X	X	X	X	X
Borehole/Spring water/Ground water (GW)						
MP-GW1	X	X	X	X	X	X
MP-GW2	X	X	X	X	X	X
MP-GW4	X	X	X	X	X	X
Surface water (rivers, dams and wetland) (SW)						
MP-SW1	X	X	X	X	X	X
MP-SW2	X	X	X	X	X	X
MP-SW3	X	X	X	X	X	X
MP-SW4	X	X	X	X	X	X
L-SW5	X	X	X	X	X	X
L-SW6	X	X	X	X	X	X
L-SW7	X	X	X	X	X	X

L-SW8	X	X	X	X	X	X
L-SW9	X	X	X	X	X	X

Prior to analysis using *in vivo* bioassay, physical and chemical properties of the samples were established as required by the standard toxicity methods, and are included in this report as additional data to the toxicity testing data. The toxicity tests were done at the BioTox laboratory in Pretoria in environmentally controlled rooms using standard techniques. Standard, internationally accepted methods (ISO/SANS) and materials were applied in order to conduct lethal and sub-lethal toxicity testing.

4.6.3 *Aliivibrio fischeri* bioluminescent test

Table 4-4 *Aliivibrio fischeri* bioluminescent test method summary

Standard method:	ISO/SANS 11348-3: 2013
Deviation from the method:	None
Test endpoint:	% growth inhibition or stimulation, relative to control
Exposure period:	15 and 30 minutes
Test chamber type:	Polystyrene cuvettes for luminometer
Test sample volume:	500 µL
Number of replicates per sample:	2
Test temperature (14-16°C):	14.8°C
Test organism species name and source:	Lyophilized *Aliivibrio fischeri* luminescent bacteria (NRRL B-11177)
Luminescent measurement:	Luminoscan TL, Hygiena Monitoring System
Test organism species name	*Aliivibrio fischeri*
Aliivibrio fischeri batch number(s):	VF 200505/2024-03
Reagent batch number(s):	RD 200505/2024-03
Sample diluent batch number(s):	SD 200505/2023-08
Statistical methods used:	Microsoft Excel® and regression analysis
Date(s) of performance of the test(s):	2022/04/07 2022/04/12
Validity criteria (CF 0.6-1.8):	0.90; 0.80

4.6.4 *Pseudokirchneriella subcapitata* growth inhibition test

Table 4-5 *Pseudokirchneriella subcapitata* growth inhibition test method summary

Standard method:	ISO/SANS 8692: 2015
Deviation from the method:	None
Test endpoint:	% growth inhibition or stimulation, relative to control
Exposure period:	72 hours
Test chamber type:	10cm path length long cells
Test sample volume:	25 mL
Number of replicates per sample:	3
Test temperature (21-25°C):	23.7 – 24.7°C

Test organism species name and source:	*Pseudokirchneriella subcapitata, Selenastrum capricornutum*, Printz algae beads (CCAP 278/4 Cambridge, UK)			
Synonym:	*Pseudokirchneriella subcapitata, Selenastrum capricornutum, Raphidocelis subcapitata;*			
Optical density measurement:	Jenway 6300 Spectrophotometer			
Algal beads batch number(s):	SC 181121			
Matrix dissolving batch number(s):	MD 190721			
Nutrient batch number(s):	A: SC170521	B: SC170521	C: SC170521	D: 170521
Statistical methods used:	Microsoft Excel® spreadsheet formulated by supplier (MicroBioTests Inc., Belgium) – RegTox and Regression analysis			
Date(s) of performance of the test(s):	2022.03.29			
Validity (Regtox sheet: cell density factor ≥67):	Yes			

4.6.5 *Spirodela polyrhiza* growth inhibition test

Table 4-6 *Spirodela polyrhiza* growth inhibition test method summary

Standard method:	ISO SANS 20227: 2017
Deviation from the method:	None
Test endpoint:	% growth inhibition or stimulation, relative to control
Exposure period:	72 hours
Test chamber type:	Polystyrene plates (9x13 cm) with 48 wells (1 mL)
Test sample volume:	1 mL
Number of replicates per sample:	8
Test temperature (24-26°C):	25°C
Test organism species name and source:	*Spirodela polyrhiza* – Turions obtained from MicrobioTests test kit
Area measurement:	Image J from photograph taken of test plate
Spirodela batch number(s):	SPP 091121
Steinberg medium batch number(s):	SM 200919
Statistical methods used:	Microsoft Excel® spreadsheet formulated by supplier (MicroBioTests Inc., Belgium) – RegTox and Regression analysis
Date(s) of performance of the test(s):	2022.03.28
Validity (mean growth of first fronds in cups of control column after 3 days incubation at 25°C and under 6000lux illumination ≥10mm^2):	47.16mm^2

4.6.6 *Daphnia magna* acute toxicity test

Table 4-7 *Daphnia magna* acute toxicity test method summary

Standard method:	ISO/SANS 6341: 2015
Deviation from the method:	None
Test endpoint:	% mortality

Exposure period:	24 and 48 hours
Test chamber type:	Polycarbonate test plates (6 rinsing wells and 24 testing wells)
Test sample volume:	25 mL
Number of replicates per sample:	4
Number of test organisms per chamber:	5
Test temperature (20-22°C):	21°C
Test organism species name, age & source:	*Daphnia magna* – ephippia obtained from MicroBiotests, <24h old
Feeding frequency during testing:	None
Ephippia batch number(s):	DM171121
ISO media batch number(s):	ISO0191121
Statistical methods used:	Microsoft Excel®
Date(s) of performance of the test(s):	2022/03/28
Validity criteria (control mortality≤10%):	5%

4.6.7 *Thamnocephalus platyurus* acute toxicity

Table 4-8 *Thamnocephalus platyurus* acute toxicity test method summary

Standard method:	ISO/SANS 14380: 2011
Deviation from the method:	None
Test endpoint:	% mortality and presence/absence of coloured particles
Exposure period:	15 to 30 minutes
Test chamber type:	10 mL conical base polystyrene tubes with plugs
Test sample volume:	5 mL
Number of replicates per sample:	2
Number of test organisms per chamber:	0.5 mL larval suspension
Test temperature (24-26°C):	25°C
Test organism species name, age & source:	*Thamnocephalus platyurus* – freshly hatched
Cyst batch number(s):	TP251120
Statistical methods used:	Microsoft Excel®
Date(s) of performance of the test(s):	2022/04/17
Validity criteria (≥50% ingestion in controls):	79.07%

4.6.8 *Poecilia reticulata* acute toxicity

Table 4-9 *Poecilia reticulata* acute toxicity test method summary

Standard method:	ISO/SANS 7346-1: 2013
Deviation from the method:	None
Test endpoint:	% mortality
Exposure period:	96 hours
Test chamber type:	250 mL disposable polystyrene cups
Test sample volume:	200 mL
Number of replicates per sample:	2
Number of test organisms per chamber:	6

Test temperature (20-22°C):	19 - 23°C
Test organism species name, age & source:	*Poecilia reticulata* – 7-21 days old. Obtained from external stock
Feeding frequency during testing:	None
ISO media batch number(s):	ISO191121
Statistical methods used:	Microsoft Excel®
Date(s) of performance of the test(s):	2022/04/07
Validity criteria (control mortality≤10%):	8.33%

4.6.9 Hazard Classification System

Lethal or sub-lethal toxicity testing (as applied for this assessment) is applied by exposing biota to water sources to determine the potential risk of such waters to the biota/biological integrity of the receiving water bodies. A risk category (hazard class) is determined based on the percentage of mortalities (lethal) or inhibition (sub-lethal) of the exposed biota. It is important to note that the hazard class is based on the standardised battery of selected test biota and therefore represents the risk/hazard towards similar biota in the receiving aquatic environment. The toxicity hazard is therefore in terms of the aquatic biotic integrity and does in no way represent toxicology towards humans or other mammals.

A risk/hazard category is determined by using a hazard classification system developed by Persoone et al. (2003) whereby one can classify sites using the toxicity data of the non-diluted samples. The percentage effect (PE) of toxicity (mortalities, growth inhibition, luminescence inhibition, ingestion inhibition) is used to rank the sample into one of five classes (Table 4.10) based on the highest toxic response obtained in at least one of the tests applied.

Table 4-10 Hazard classification system for natural waters/screening samples (Persoone et al., 2003)

Class	Symbol	Hazard rating	PE	Percentage effect
I	☺	No lethal/sub-lethal hazard	≤10/20%	None of the tests show a toxic effect (i.e., an effect value that is significantly higher than that noted in the controls)
II	☹	Slight lethal/sub-lethal hazard	10/20%≤PE<50%	A statistically significant (P<0.05) PE is reached in at least one test, but the effect level is below 50%
III	☠	Lethal/sub-lethal hazard	50%≤PE<100%	The 50% effect level is reached or exceeded in at least one test, but the effect level is below 100%
IV	☠☠	High lethal/sub-lethal hazard	PE 100% in at least one test	The 100% effect is reached or exceeded in at least one test
V	☠☠☠	Very high lethal/ sub-lethal hazard	PE 100% in all tests	The 100% effect is exceeded in all the tests

Each sample is furthermore weighted (Table 4.11) according to its relative toxicity level (out of 100%). Higher values indicate that more of the individual tests indicated toxicity within a specific class.

Table 4-11 Weight score allocation for each test type (Persoone et al., 2003)

Test score	Category
0	No significant toxicity effect
1	Significant toxicity effect < PE50
2	Toxicity effect >PE50 but <PE100
3	The PE100 is reached

Class weight score calculated as follows:
Class weight score = (∑ all test scores)/n) where n is the number of tests performed
Class weight score % = (class score) / (maximum class weight score) x 100

4.7 SAMPLES SCREENING USING *IN VITRO* ASSAYS

4.7.1 Selection of *in vitro* bioassays

For the scope of this study all the available methods were applied to all types of water, but the decision-making tool for the selection of *in vitro* bioassays can be found in Table 3.2-3.4, and is also further discussed in the relevant result sections for each water type as well as in the fact sheets in the appendixes. Bioassays for xenobiotic metabolism and endocrine disruption (estrogenic, androgenic, and thyroid activity) and oxidative stress have been included. The project team has attempted to stay in line with the recommended tests for battery 3 and has included the following bioassays.

Xenobiotic metabolism:
- H4IIE-luc reporter gene assay

Estrogenic activity:
- YES assay
- T47D-KBluc assay (estrogenic and anti-estrogenic activity)

Androgenic activity:
- YAS assay
- MDA-Kb2 assay (androgenic and anti-androgenic activity)

Thyroid activity:
- GH3.TRE-*Luc* assay (thyroid and anti-thyroid activity)

Oxidative stress
- AREc32 assay is an *in vitro* assay to assess the oxidative stress response and cytotoxicity of chemicals in water samples

The water extracts were analysed according to this list of bioassays, according to the methods in the updated toolbox from WRC 2020/2021-00165.

4.7.2 Interpretation of results

The *in vitro* bioassay results are expressed as bioanalytical equivalent concentrations (BEQs). The BEQs are interpolated from a positive control standard curve (agonist or antagonist) that is assay specific. The BEQ value is corrected for the appropriate dilution factor for each sample. Detailed

calculations can be found in the latest Toolbox for EBM in South Africa (WRC project no.2020/2021-00165).

The BEQ values are compared to EBT values to differentiate between acceptable and poor water quality. The current available EBT values for surface and drinking water can be found in Table 2.1. Groundwater is compared to the EBT values for surface water. For wastewater, a 10x dilution is applied to the BEQ values to account for the dilution effect of wastewater effluents into receiving water bodies, before it can be compared to surface water EBT values. If the BEQ is lower than the EBT-BEQ, then no further action is required. If the measured BEQ value exceeds the EBT-BEQ, the first step is to check the bioassay quality control (QC) and collect another water sample from the same site and re-test. If the BEQ of the second sample is below the EBT-BEQ, then no further action is required. If the second test confirms the initial positive result and both samples report a BEQ > EBT-BEQ, then further action is needed. The magnitude of the response should depend on the magnitude of the exceedance and regulatory advice (refer to Figure 3.6).

4.8 CHEMICAL ANALYSIS

Non-targeted and targeted chemical analyses were done using the following analytical methods:

4.8.1 Optimised UPLC/Q-TOF-MS analysis

The separation of the analytes was carried out using a Dionex Ultimate 3000 UHPLC system (Dionex Softron GmbH, Dornierstr. 4, Germany) equipped with a reversed-phase C18 analytical column of 100 mm × 2.1 mm and 1.7 µm particle size (Acquity UPLC® BEH, Waters, Ireland). Column temperature was maintained at 35 ºC. The injected sample volume was 5 µL. Mobile phases A and B were water and methanol with 0.1% formic acid, respectively. The optimized chromatographic method was programmed as follows: the initial mobile phase composition (2% B) constant for 1 min, followed by a linear gradient from 2% B to 100 % B for 9 mins, kept 100% B for 2 mins and then dropped back to 2% B 12.1mins and kept constant at 2%B for 2 mins. The flow rate used was 0.3 mL/min, and the total run time was 14 mins. This UHPLC system was connected to an ultrahigh resolution quadrupole time-of-flight mass spectrometer Impact II Bruker (Bruker Daltonics GmbH Fahrenheitstr. 4, Bremen, Germany) equipped with electrospray ionisation, operating in positive ion mode. LC/MS accurate mass spectra were recorded across the range 50–1600 m/z. The data recorded was processed with Bruker Compass DataAnalysis 4.3 software. Accurate mass measurements of each peak from the extracted ion chromatograms were obtained by means of a sodium formate calibrant solution delivered by a KdScientific external pump. The instrument was operated in full-scan mode, except in those cases where automated MS–MS was necessary to discriminate isobars/isomers, as well as for identification of selected compounds and degradation products, as explained in the results.

4.8.2 Optimised LC – HRAMS Q Exactive system

The Q Exactive mass spectrometer was run in both positive and negative ionization mode. The electrospray ionization was set at 2.5 kV (for negative) and 3.5 kV (for positive) with an auxiliary gas set at 5 arbitrary units, the sheath gas set at 36 arbitrary units, and the capillary temperature was set at 320°C. The scan parameters for the mass spectrometer included a run time of 13 min duration time and 6 s chromatogram peak width in DDA mode. MS1 used the Orbitrap mass analyser with a resolution of 70,000, a maximum injection time (MIT) of 300 ms, one scan, an RF lens (%) of 50, and a scan range

from 65 to 750 m/z. The Automatic Gain Control (AGC) target was set to 3e6. MS2 data were acquired using a resolution of 17,500, MIT of 80 ms, AGC target of 1e5 and a scan range from 65 to 750 m/z. The top 1 abundant precursor within an isolation window of 1.0 m/z was chosen for MS/MS analysis. A minimum intensity threshold of 1.0e5 and dynamic exclusion of 6 s were used during the data-dependent scanning. For precursor fragmentation, high energy collision dissociation (HCD) normalized three-step collision energy was set to 10, 30, and 60. For the PRM method, the scan parameters for the mass spectrometer included a run time of 13 min duration time and 6 s chromatogram peak width in PRM mode. MS2 data were acquired using a resolution of 17,500, MIT of 160 ms, AGC target of 2e5 and an inclusion list of all the precursor ions of our target compounds. The loop and maximum number of precursors to be multiplexed in a scan event were set at 1 within an isolation window of 4.0 m/z. For precursor fragmentation, high energy collision dissociation (HCD) normalized collision energy for specific precursor was listed in the inclusion list and ranged from 10 to 40eV.

4.8.3 GC TOF MS analysis

DLLME n-hexane extracts as well as methanol extracts of Chemcatcher, were analysed by gas chromatography (Agilent Technologies, Inc., Wilmington, Delaware, USA) coupled with a LECO HT time of flight mass spectrometer (LECO Corporation, St. Joseph, MI, USA) and a Gerstel Multi-Purpose Sampler MPS 2 from Gerstel GmbH (Mülheim an der Ruhr, Germany).) on-column injection system in a splitless injection mode. The GC oven was equipped with a Restek Rxi®-5Sil MS, 30 m, 0.25 mm ID, 0.25 µm and the helium carrier gas was maintained at a constant flow of 1 mL per minute. The injection temperature was set at 300ºC, and the oven temperature was programmed as follows: 40ºC held for 2 minutes; ramped from 40ºC - 240ºC at 30ºCmin-1, then 240ºC - 320ºC at 10ºC per minute. The mass spectrometry conditions were set as follows: Transfer line temperature: 300°C; Ionization: Electron ionization at -70 eV; source temperature: 280°C; stored mass range: 50-500 um; solvent delay: 240 seconds; acquisition rate: 8 spectra/second; detector voltage: -1654 V. Also, the system was equipped with ChromaTOF data acquisition software and the NIST library for performing the integration of chromatograms and compound quantification. External calibration standards were used for quantification.

4.8.4 NexION 350D ICP MS analysis

The elemental analysis of water samples was performed by Perkin Elmer NexION 350 ICP-MS system. The typical instrumental parameters were set as follows: cell gas: argon; nebulizer: glass concentric; spray chamber: glass cyclonic, sample uptake rate: 0.3 mLmin-1; RF power:1600 W, Triple cone interface: nickel/aluminium, sweeps per reading: 10, replicates: 3-10, dwell time: 50 – 150 ms, lens: Quadrupole Ion Deflector, scanning mode: STD or KED (He) or DRC (NH3).

CHAPTER 5: USE OF EFFECT-BASED METHODS FOR DRINKING WATER QUALITY ASSESSMENT – CASE STUDIES

5.1 INTRODUCTION

Drinking water catchment areas may contain organic micropollutants, which are taken up into the DWTP and DBPs can be formed during the various treatment processes (e.g., chlorination). The case studies represented here aimed to evaluate the efficacy of treatment processes at DWTPs in five provinces. A battery of bioassays was used to test the efficacy of the method and also the DWTP. These tests included a battery of *in vivo* bioassays and *in vitro* assays focusing on hormone receptor-mediated effects to evaluate micropollutant removal (i.e., evaluate treatment efficacy), and the reactive toxicity and adaptive stress responses were to assess DBP formation. Samples were also subjected to non-targeted and targeted chemical analysis to complement the *in vivo* and *in vitro* bioassays. For all DWTPs, samples were collected from the source water and after chlorination.

For drinking water, battery 3 bioassays are recommended (refer to the decision-making tool in Table 3.2 and 3.4), consisting of high sensitivity *in vitro* bioassays, oxidative stress, AhR and mutagen or genotoxicity assays. The high sensitivity bioassays are mammalian-based assays, as yeast-based assays are not sensitive enough for drinking water. The *in vivo* assays are also not indicated for drinking water, but for the scope of this study, the yeast-based assays and *in vivo* assays were included.

5.2 APPLICATION OF *IN VIVO* BIOASSAYS FOR DRINKING WATER QUALITY ASSESSMENT

The DWTP *in vivo* bioassay results are summarised in Table 5.1. For sample MP-DWTP2a, the highest effect (53% growth inhibition) was noted following the duckweed (*S. polyrhiza*) test. The micro-algae (*R. subcapitata*) effect (26% growth inhibition) also slightly exceeded the minimum acceptable effect level of ≤20% for this test. This sample was allocated a Hazard Class III (weight score of 30).

Table 5-1: Summary of *in vivo* bioassay results for DWTP samples

Sample name	*A. fischeri*	*R. subcapitata*	*S. polyrhiza*	*D. magna*	*T. platyurus*	*P. reticulata*	Hazard Class and weight score
Raw Water							
MP-DWTP2a	-4	-26	-53	-5	NP	0	Class III 30
GP-DWTP6a	-11	-3	-54	0	-1	0	Class III 17
Final Treated Water							
MP-DWTP2b	-16	-26	-57	0	-86	-25	Class III 50
GP-DWTP6b	-11	-6	-86	0	-14	0	Class III 17

NP – test not performed	Slight effect		Significant effect

For sample MP-DWTP2b (the final treated water from this site), the highest effect (86% ingestion inhibition) was noted following the macro-invertebrate (*T. platyurus)* bioassay, and tests of which the results exceeded the minimum acceptable effect levels include the micro-algae (*R. subcapitata*) with 26% growth inhibition, duckweed (*S. polyrhiza*) with 57% growth inhibition and the vertebrate (*P. reticulata*) tests with 25% mortality. This sample was also allocated a Hazard Class III, but with a weighting of 50, indicating an increase in the effect noted from the raw water.

For sample GP-DWTP6a (raw water), the highest effect (54% growth inhibition) noted was also following the duckweed (*S. polyrhiza*) test, with no other test exceeding the minimum acceptable effect levels. This sample was allocated a Hazard Class III (weight score of 17).

For sample GP-DWTP6b (final treated water from this site), the highest effect (86% growth inhibition) noted was also following the duckweed (*S. polyrhiza*) bioassay, with no other test showing any significant effect; however, the effect levels were slightly higher in two tests. This site was also allocated a Hazard Class III (weight score of 17).

These two sites (MP-DWTP2a/MP-DWTP2b and GP-DWTP6a/GP-DWTP6b) are in two different locations, one being more rural than the other.

All four samples were allocated a Hazard Class III (where the percentage effect is reached or exceeded in at least one test, but the effect level is below 100%), indicating an acute hazard to the environment. Based on the weight scores, the samples are ranked from highest to lowest toxicity as follows: MP-DWTP2b, then MP-DWTP2a followed by GP-DWTP6a/GP-DWTP6b.

The results obtained from MP-DWTP2 final treated water showed more significant effects than those of the raw water. This may potentially be because of the chemicals used for disinfection. The level of effects noted for GP-DWTP6, between raw and final water, remaining relatively stable (same Hazard Class and weight score may be because this treatment plant utilizes better processes or chemicals for the disinfection process, not having such a significant effect on the aquatic organisms as those used at site MP-DWTP2.

Three of the four drinking water samples (raw and treated) indicated that the duckweed test (*S. polyrhiza*) shows the highest effects, and for the fourth sample, this was seen following the *T. platyurus* bioassay. The results obtained from these aquatic plant tests, in the case of the duckweed test, indicate that these types of samples will mostly have a negative impact on the aquatic plants in the environment. As indicated by the results obtained at site MP-DWTP2, the final treated water will impact all aquatic life represented by these trophic levels (macro-algae, aquatic plants, invertebrates and vertebrates) as these results clearly indicate effects above the acceptable levels of 20% recommended.

5.3 APPLICATION OF *IN VITRO* BIOASSAYS FOR DRINKING WATER QUALITY ASSESSMENT

5.3.1 Xenobiotic metabolism

The H4IIE-luc reporter gene assay was used to evaluate xenobiotic metabolism and its effects on biological systems, particularly through their interaction with the aryl hydrocarbon receptor (AhR). In drinking water quality assessment, this assay is applied due to its sensitivity in detecting micropollutants (and their mixtures) with AhR activity, which includes a wide range of organic pollutants that may pose health risks if present in drinking water. The results of the DWTP samples using the H4IIE-luc reporter gene assay are shown in Table 5-2. None of the samples that were tested were above the LOD of the assay. No cytotoxicity was detected in the H4IIE-luc cells.

Table 5-2: H4IIE-luc reporter gene assay results for DWTP samples

Sample site	TCDD-EQ (ng/L)	
	Sample collection 1	Sample collection 2
DWTP 1a	Not analysed	Not analysed
GP-DWTP1b	Not analysed	Not analysed
GP-DWTP1c	Not analysed	Not analysed
MP-DWTP2a	Not analysed	Not analysed
MP-DWTP2b	Not analysed	Not analysed
NW-DWTP3a	<LOD	<LOD
NW-DWTP3b	<LOD	<LOD
WC-DWTP4a	<LOD	No sample
WC-DWTP4b	<LOD	No sample
KZN-DWTP5a	No sample	<LOD
KZN-DWTP5b	No sample	<LOD
GP-DWTP6a	<LOD	No sample
GP-DWTP6b	<LOD	No sample

<LOD: Below the limit of detection of the assay.

5.3.2 Hormone receptor/Endocrine disruption

5.3.2.1 Yeast estrogen screen (YES)

The results of the DWTP samples using the YES assay are tabulated in Table 5-3. The activity of the samples was measured against the positive control, 17β-estradiol, in order to determine the bioequivalent concentrations from the dose response curves. Estrogenic activity could be quantified in six of the raw water samples and in four of the final treated water samples. In general, estrogenic activity was higher in raw water samples compared to treated water samples, indicating that the DWTPs were working effectively to remove contaminants. The exception was NW-DWTP3, where estrogenic activity was higher in the treated water samples for both sample collection events. NW-DWTP3 is an indirect reuse drinking water treatment plant.

Table 5-3 Estrogenic activity of DWTP samples using the yeast estrogen screen

Sample site	EEQ (ng/L)	
	Sample collection 1	Sample collection 2
DWTP 1a	<LOD	0.23 ± 0.021
GP-DWTP1b	<LOQ	<LOQ
GP-DWTP1c	<LOQ	<LOD
MP-DWTP2a	<LOD	<LOD
MP-DWTP2b	<LOQ	<LOD
NW-DWTP3a	0.090 ± 0.006	0.52 ± 0.056 *
NW-DWTP3b	0.50 ± 0.032	1.6 ± 0.20
WC-DWTP4a	0.18 ± 0.037	No sample
WC-DWTP4b	<LOD	No sample
KZN-DWTP5a	No sample	0.28 ± 0.069 *
KZN-DWTP5b	No sample	0.22 ± 0.042
GP-DWTP6a	0.36 ± 0.027	No sample
GP-DWTP6b	0.20 ± 0.072	No sample

<LOD: Below the limit of detection of the assay. <LOQ: below the level of quantification. * cytotoxicity observed at 50x concentration

5.3.2.2 T47D-KBluc reporter gene assay

The DWTP samples were assessed for estrogenic and anti-estrogenic activity using the T47D-KBluc reporter gene assay. The results are tabulated in Table 5.4. In the T47D-KBluc assay, 19 of the samples could be quantified for estrogenic activity, compared to only 10 samples that could be quantified with the YES assay. The results suggest that the T47D-KBluc assay might be more sensitive for testing DWTP samples. None of the samples showed anti-estrogenic activity.

Table 5-4 Estrogenic activity of DWTP samples using the T47D-KBluc reporter gene assay

Sample site	EEQ (ng/L)	
	Sample collection 1	Sample collection 2
GP-DWTP1a	1.4 ± 0.3	0.88 ± 0.07
GP-DWTP1b	0.39 ± 0.07	1.2 ± 0.05
GP-DWTP1c	1.7 ± 0.27	0.069 ± 0.027
MP-DWTP2a	0.20 ± 0.04	0.15 ± 0.04
MP-DWTP2b	0.35 ± 0.01	0.004 ± 0.001
NW-DWTP3a	0.79 ± 0.18	<LOQ
NW-DWTP3b	0.98 ± 0.24	0.99 ± 0.62
WC-DWTP4a	0.098 ± 0.015	No sample
WC-DWTP4b	0.064 ± 0.002	No sample
KZN-DWTP5a	No sample	0.22 ± 0.02
KZN-DWTP5b	No sample	1.2 ± 0.3
GP-DWTP6a	1.9 ± 0.63	No sample
GP-DWTP6b	1.0 ± 0.03	No sample

<LOQ: below the level of quantification.

5.3.3 Androgenic activity

To assess androgenic activity in drinking water, two assays have been recommended; the Yeast androgen screen (YAS) and MDA-kb2 reporter gene assay. The DWTP samples were not subjected to the YAS assay. No androgenic, anti-androgenic or glucocorticoid activity were detected in any of the DWTP samples using the MDA-kb2 reporter gene assay.

5.3.4 Thyroid activity

No thyroid activity was detected in the DWTP samples using the GH3.TRE-Luc assay.

5.3.5 Oxidative stress

The AREc32 bioassay was used to assess oxidative stress potential in the collected drinking water samples. The assay is particularly valuable for evaluating the presence of oxidative stress-inducing contaminants in drinking water as they can have adverse health effects. The oxidative stress results are summarised in Table 5.5. Samples GP-DWTP1a and GP-DWTP1c for the second sample collection were cytotoxic at REF of >200, which is past the limit of concentrations range that can be evaluated. Dichlorvos-EQs values could be calculated for all the other samples.

Table 5-5 Oxidative stress response results of DWTP samples using the AREc32 reporter gene assay

Sample site	Sample collection 1 (Dry Season)			Sample collection 2 (Wet Season)		
	IC_{10} ± SE (REF)	$EC_{IR1.5}$ ± SE (REF)	Dichlorvos -EQ (µg/L)	IC_{10} ± SE (REF)	$EC_{IR1.5}$ ± SE (REF)	Dichlorvos -EQ (µg/L)
GP-DWTP1a	42.6 ± 3.3	6.7 ± 0.3	253.9	>200	>200	-
GP-DWTP1b	64.3 ± 20.7	5 ± 0.2	342.4	60.9 ± 13.9	5.1 ± 0.2	333.4
GP-DWTP1c	48.1 ± 3.4	6.5 ± 0.4	263.1	>200	>200	-
MP-DWTP2a	>200	20.9 ± 1	81.2	63.3 ± 9.1	12.9 ± 0.5	131.7
MP-DWTP2b		135,5 ± 9	12.5		60.8 ± 2.5	28.0

REF = relative enrichment factor of a water sample in the bioassay (a REF of 1 means that the concentrations of the extracted chemicals are the same as in the original water sample); IC_{10} = 10% inhibition concentration; effect concentration ($EC_{IR1.5}$), and SE = Standard error.

5.4 CHEMICAL ANALYSIS

In order to identify contaminants in the raw and final product water that might contribute to the effects seen in the bioassays, samples were subjected to non-targeted and targeted chemical analysis. The results are summarised in Table 5.6. Target chemicals could only be quantified in NW-DWTP3a.

Table 5-6 Non-targeted and targeted chemical analysis results of DWTP samples

Sample code	Sample collection no	Non-targeted analysis (number of compounds detected)	Targeted analysis (list of compounds and concentrations in ng/L)
GP-DWTP1a	1	NA	NA
GP-DWTP1a	2	NA	NA
GP-DWTP1b	1	NA	NA
GP-DWTP1b	2	NA	NA
GP-DWTP1c	1	NA	NA
GP-DWTP1c	2	NA	NA
MP-DWTP2a	1	49	NA
MP-DWTP2a	2	50	NA
MP-DWTP2b	1	49	NA
MP-DWTP2b	2	49	NA

NW-DWTP3a	1	446	4-Aminobiphenyl (0.5), Acetaminophen (3), Atenolol (13.7), Atrazine (26.9), Benzophenone (12.4), Bisphenol A (7.0), Bufexamac (3.2), Caffeine (5.9), Carbamazepine (310.1), Danofloxacin (27.8), Digoxin (17.4), Efavirenz (262.8), Enrofloxacin (3.0), Estrone (16.5), Flumequine (1.1), Imidacloprid (46.1), Isoniazid (4.5), Ketoprofen (9.5), Levoflaxacin (605.9), Lidocaine (17.2), Lopinavir (492.6), Medroxyprogesterone (15.6), Metoprolol (310.2), Phenacetin (2.7), Pindolol (50.8), Primidone (19.5), Roxithromycin (0.4), Sulfadimethoxine (0.8), Sulfadoxine (78.7), Sulfamethoxazole (27.4), Sulfisoxazol (1.4), Terbuthylazine (57.2), Terbutryn (3.9), Thiabendazole (7.9), Thiacloprid (3.0), Tramadol (268.9), Trimethoprim (5.1), Valsartan (26.3)
NW-DWTP3a	2		
NW-DWTP3b	1	354	<LOQ
NWDWTP3b	2		
WC-DWTP4a	1	26	<LOQ
WC-DWTP4b	1	11	<LOQ
KZN-DWTP5a	1	446	<LOQ
KZNDWTP5b	1	315	<LOQ
GP-DWTP6a	1	94	<LOQ
GP-DWTP6b	1	119	<LOQ

NA Not analysed; <LOQ Below the level of quantification

5.5 APPLYING EBT VALUES TO BIOASSAY RESULTS FOR DRINKING WATER QUALITY MONITORING

The percentage of DWTP samples below the LOQ, above the LOQ and above the EBT in the various bioassays are graphically displayed in Figure 5.1. All DWTP samples were below the LOD in the H4IIE-luc, MDA-kb2 (androgenic) and GH3.TRE-luc (thyroid) assays. In the assays for estrogenic activity, 10 samples could be quantified in the YES assay and 19 could be quantified in the T47D-KBluc assay. In the YES assay, one sample was above the EBT value for drinking water and 10 samples were above the EBT value in the T47D-KBluc assay. This discrepancy could be explained by the fact that the YES cells only contain the estrogen receptor alpha, whereas the T47D-KBluc cells contain both estrogen receptor alpha and beta, making it a more sensitive assay. The target chemical analysis could only quantify target chemicals in the raw water from NW-DWTP3a, which is an indirect reuse plant. At this site, the estrogenic activity was also above the EBT value. One of the target chemicals that was quantified in this sample that could contribute to the estrogenic activity was estrone, at a concentration of 16.5 ng/L. In the AREc32 assay for oxidative stress, three of the five samples that could be quantified were above the EBT value for drinking water. The hazard classification system that is used for the *in vivo* bioassays indicated a Class III hazard rating for all four the DWTP samples that were tested. Besides NW-DWTP3a, no target chemicals were above the LOQ for any of the other DWTP samples that showed activity in the bioassays, indicating the value of bioassays to complement chemical analysis.

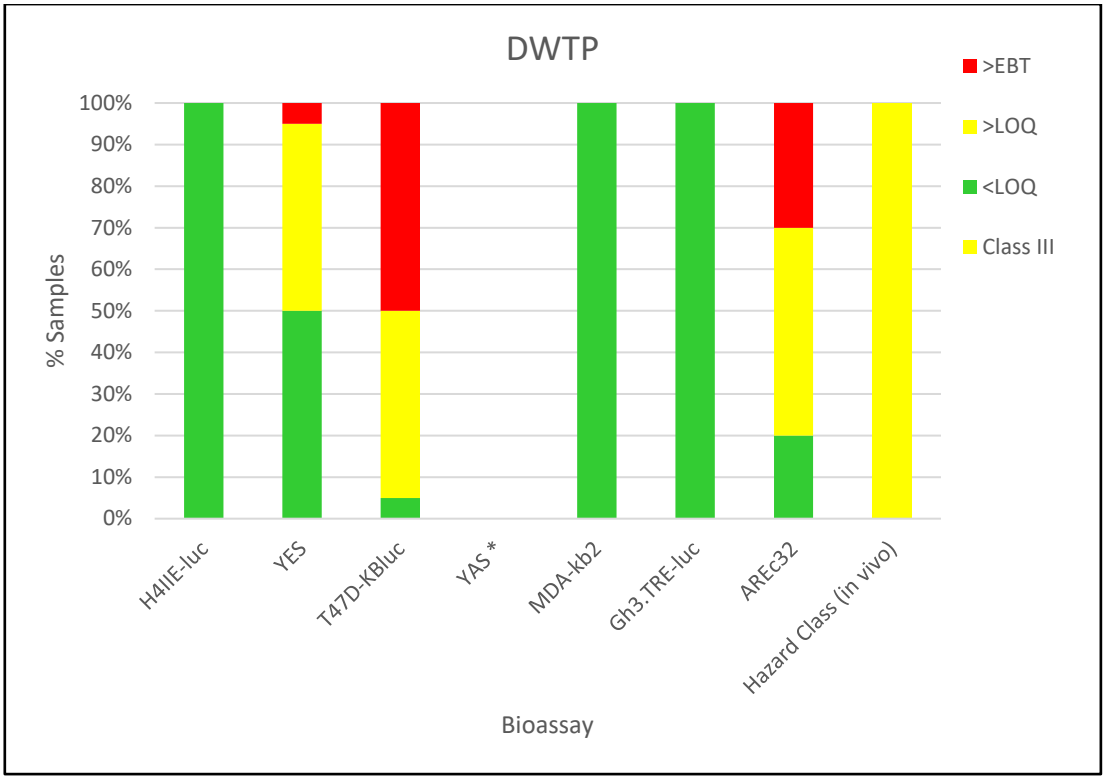


Figure 5-1: Summary of the results of DWTP samples assessed in the bioassays, expressed as the percentage of samples below LOQ, above LOQ and above EBT value. * Only samples that tested positive in the MDA-kb2 assay were analysed in the YAS assay

5.6 ASSESSING THE EFFICIENCY OF DRINKING WATER TREATMENT TECHNOLOGIES USING CHEMICAL ANALYTICAL TECHNIQUES TO COMPLEMENT *IN VITRO* AND *IN VIVO* BIOASSAYS

5.6.1 GP-DWTP1 - (conventional DWTP)

GP-DWTP1 is a conventional DWTP. Figure 5.2 summarizes the main results obtained with non-targeted chemical screening and bioassays for this water treatment plant. Estrogenic activity was detected in raw water in the YES assay, but it was below the LOQ in final treated water. However, in the T47D-KBluc reporter gene assay, estrogenic activity was detected in the raw and final treated water samples. For the first sampling season, the activity was reduced to below the EBT value, but for the second sampling event, even the final treated water was above the EBT. The estrogenic activity of backwash water going back into the environment was also above the EBT value, causing concern. In the AREc32 assay for oxidative stress, the final treated water samples showed higher activity than the raw water samples, and were above the EBT for both sampling events. The results indicate that this DWTP was not effective in removing estrogenic activity from the raw water and that the treatment process might have contributed to the increase seen in oxidative stress. Unfortunately, no results are available for targeted and non-targeted chemical analysis for this treatment plant to try and identify possible chemicals that could be responsible for the activities seen in the samples. This treatment plant should be monitored further in order to determine if the activity in final treated and backwash water will remain above the EBT, in which case, extensive chemical analysis would be recommended in order to try and identify possible causes.

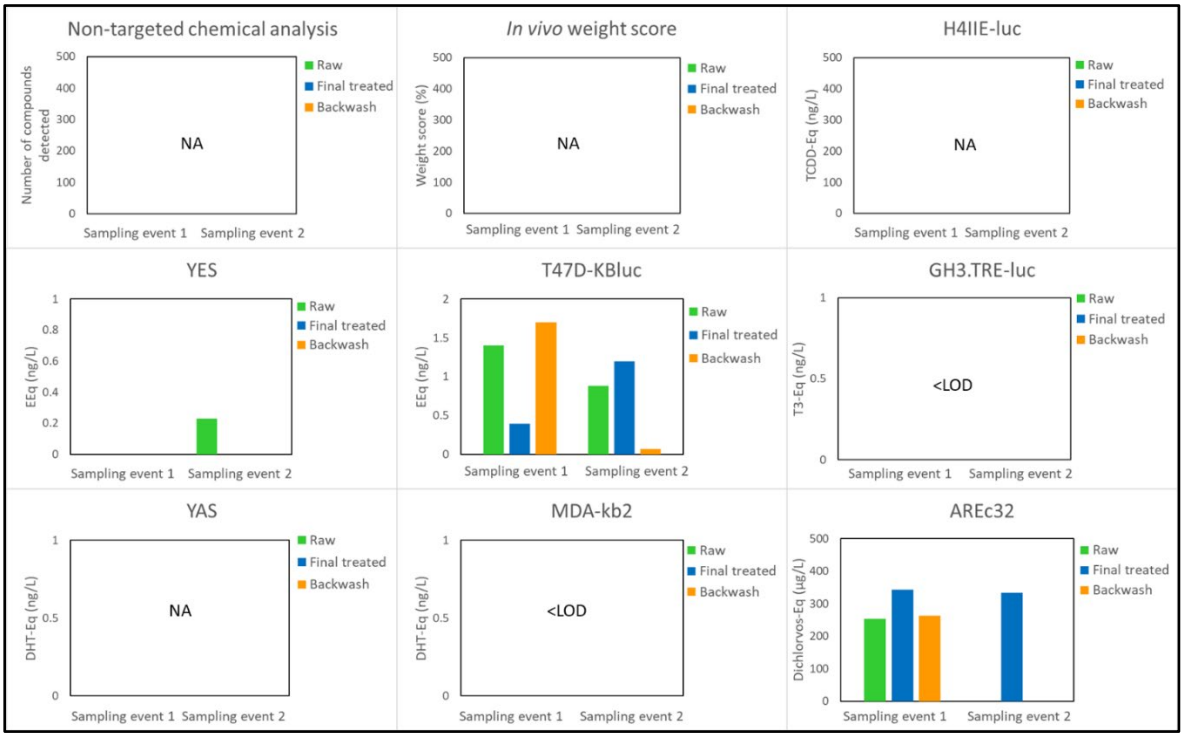


Figure 5-2: Summary of non-targeted chemical analysis and *in vitro* and *in vivo* bioassay results for GP-DWTP1. NA Not analysed; <LOD below the limit of detection

5.6.2 MP-DWTP2 - (conventional DWTP)

MP-DWTP2 is a conventional DWTP. Figure 5.3 summarises the main results obtained with non-targeted chemical screening and bioassays for this water treatment plant. Estrogenic activity was detected in raw and treated water in the T47D-KBluc assay, and oxidative stress activity in the AREc32 assay, but none of the values were above the EBT at this DWTP. The *in vivo* bioassays indicated a class III, lethal/sub-lethal hazard. Non-targeted chemical analysis detected 49-50 compounds in raw and final treated samples, but unfortunately, no results are available for targeted chemical analysis. This treatment plant should be monitored in order to determine if the activity detected in the bioassays will remain below the EBT values.

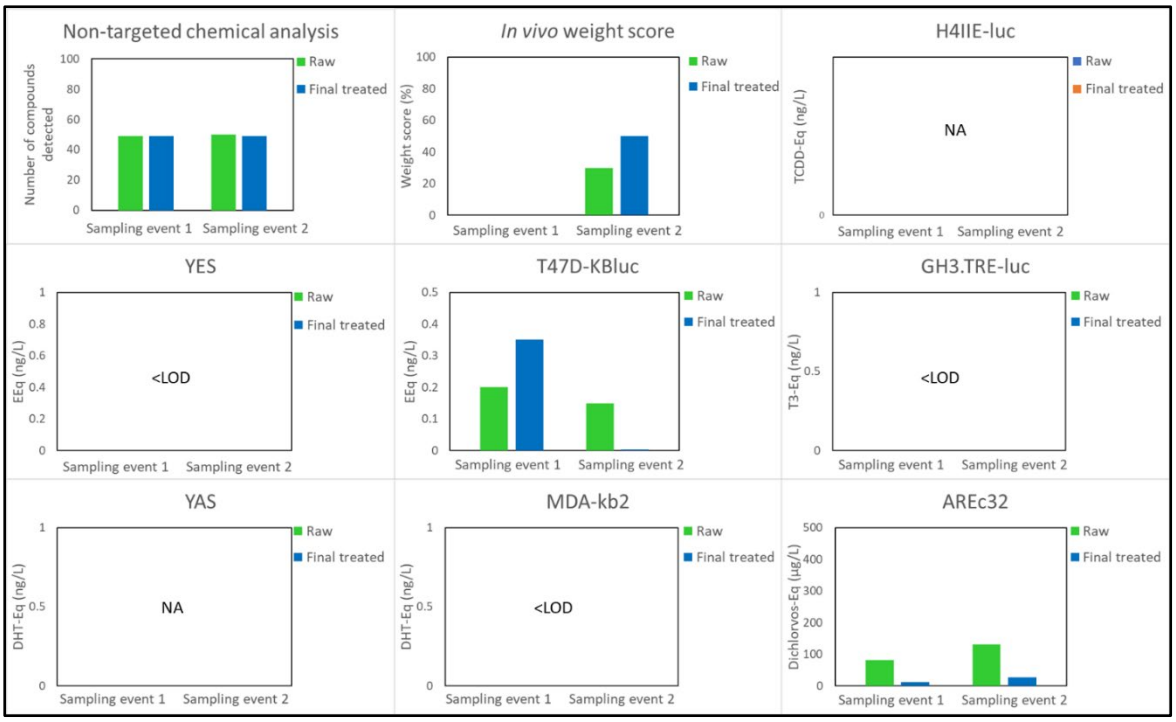


Figure 5-3: Summary of non-targeted chemical analysis and *in vitro* and *in vivo* bioassay results for MP-DWTP2. NA Not analysed; <LOD below the limit of detection

5.6.3 NW-DWTP3 - (indirect reuse DWTP)

NW-DWTP3 is an indirect reuse DWTP. Figure 5.4 summarizes the main results obtained with non-targeted chemical screening and bioassays for this water treatment plant. Estrogenic activity was detected in raw and treated water in the YES and T47D-KBluc assays, with values above the EBT in final treated water samples. The results indicate that the treatment process at this DWTP is not effective in removing estrogenic activity from the raw water samples. Non-targeted chemical analysis identified 446 compounds in raw water and 354 compounds in final treated water samples. Targeted chemical analysis detected various chemicals, including 16.5 ng/L estrone in the raw water samples, that could be responsible for some of the estrogenic activity detected in the raw water samples. However, none of the target chemicals could be quantified in the final treated water samples. These results highlight the need to do bioassays in order to complement chemical analysis.

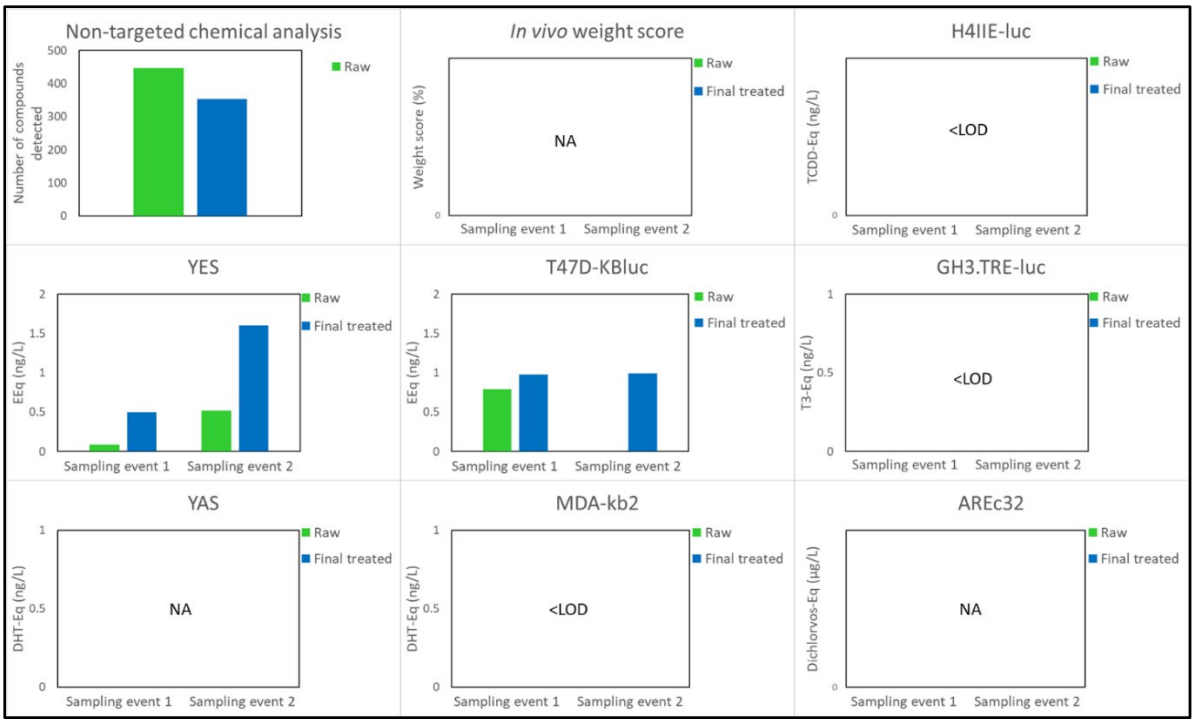


Figure 5-4: Summary of non-targeted chemical analysis and *in vitro* and *in vivo* bioassay results for NW-DWTP3. NA Not analysed; <LOD below the limit of detection

5.6.4 WC-DWTP4 - (desalination plant)

WC-DWTP4 is a desalination plant. Figure 5.5 summarizes the main results obtained with non-targeted chemical screening and bioassays for this water treatment plant. Estrogenic activity was detected in raw and final treated water in the YES and T47D-KBluc assays, but none of the values were above the EBT values. Non-targeted chemical analysis only detected 26 compounds in raw and 11 compounds in final treated water samples and target chemical analysis were below the LOQ for all samples at this site. No action is required at this DWTP, but monitoring is advised in order to confirm that samples will remain below the EBT values.

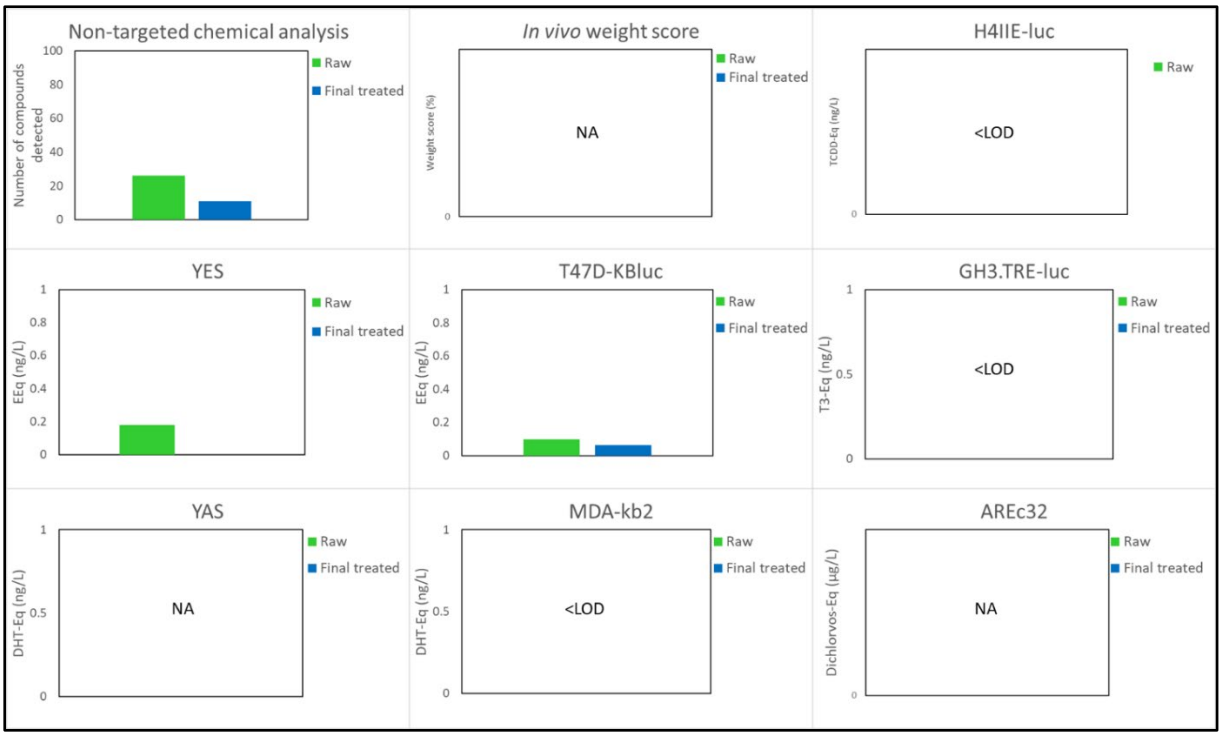


Figure 5-5: Summary of non-targeted chemical analysis and *in vitro* and *in vivo* bioassay results for WC-DWTP4. NA Not analysed; <LOD below the limit of detection

5.6.5 KZN-DWTP5 - (direct reuse)

KZN-DWTP5 is a direct reuse DWTP. Figure 5.6 summarises the main results obtained with non-targeted chemical screening and bioassays for this water treatment plant. Estrogenic activity was detected in raw and final treated water samples at this water treatment plant in the YES and T47D-KBluc bioassays. In the T47D-KBluc assay, the estrogenic activity was much higher in the final treated water compared to the raw water and exceeded the EBT value, which is a reason for concern. Non-targeted chemical analyses detected 446 compounds in the raw and 315 compounds in the final treated water, although no target chemicals could be quantified with targeted chemical analysis. This treatment plant should be monitored further in order to see if the activity in the final treated water remains above the EBT value, in which case further investigations should be done in order to establish whether the treatment process is contributing to the increase of estrogenic activity observed in the final treated water.

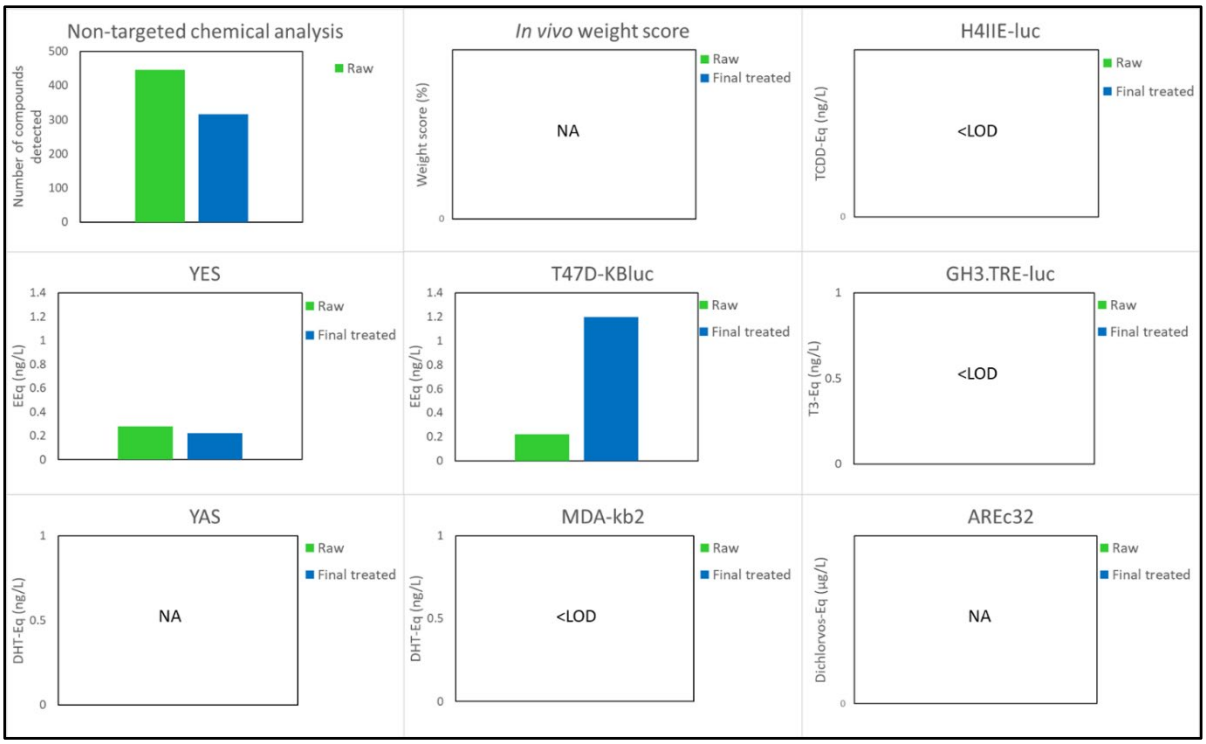


Figure 5-6: Summary of non-targeted chemical analysis and *in vitro* and *in vivo* bioassay results for KZN-DWTP5. NA Not analysed; <LOD below the limit of detection

5.6.6 GP-DWTP6 - (conventional)

GP-DWTP6 is a conventional DWTP. Figure 5.7 summarises the main results obtained with non-targeted chemical screening and bioassays for this water treatment plant. Estrogenic activity was detected in both the YES and T47D-KBluc bioassays. Although the activity was lower in the final treated water compared to the raw water, both exceeded the EBT value in the T47D-KBluc assay. Non-targeted chemical analysis revealed 94 compounds in raw and 119 compounds in final treated water, but target chemicals were all below the LOQ. *In vivo* bioassays revealed a class III, lethal/sub-lethal hazard, with a weight score of 17% for both raw and treated water. This water treatment plant should be monitored further in order to see if the activity in the final treated water remains above the EBT value, in which case further investigations are warranted.

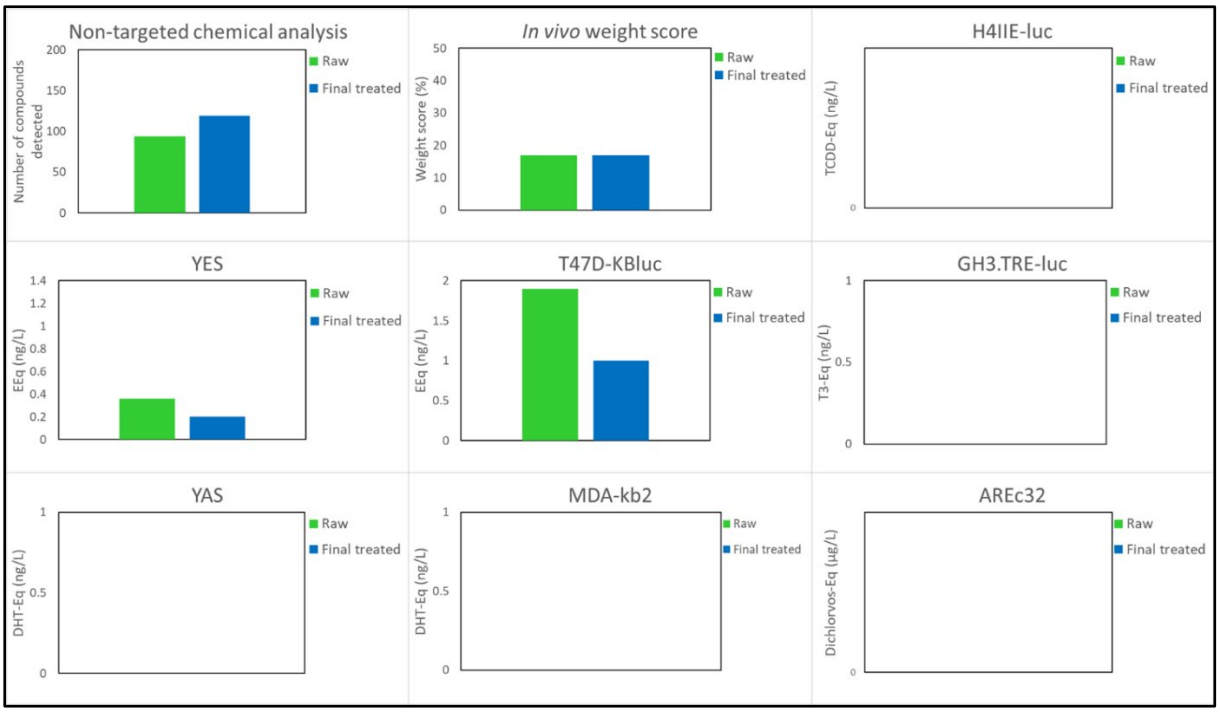


Figure 5-7: Summary of non-targeted chemical analysis and *in vitro* and *in vivo* bioassay results for GP-DWTP6. NA Not analysed; <LOD below the limit of detection

5.7 SUMMARY

This study demonstrated the value of applying *in vivo* and *in vitro* bioassays to assess the quality of drinking water as well as the efficacy of treatment processes at DWTPs. *In vivo* assays revealed class III, lethal/sub-lethal hazard class for raw and final treated drinking water. *In vitro* assays detected estrogenic activity in both the YES (yeast-based) and T47D-KBluc (mammalian cell) bioassays. In the YES assay, 50% of samples were above the LOQ, whereas in the T47D-KBluc assay, 95% of samples were above the LOQ. This discrepancy could be explained by the fact that the yeast cells only contain the estrogen receptor alpha, whereas the T47D-KBluc cells contain both estrogen receptor alpha and beta, making it a more sensitive assay. The results therefore confirm that high sensitivity bioassays (like the T47D-KBluc assay) should be used when evaluating drinking water quality. In the AREc32 assay for oxidative stress, 80% of the samples were above the LOQ, but all the samples were below the LOD in the H4IIE, MDA-kb2 and Gh3.TRE-luc assays. Although no activity was detected in the H4IIE and MDA-kb2 assays, this study had a limited number of samples, and it is recommended to still include these assays in the battery of assays for drinking water assessment.

The chemical analysis could only quantify target chemicals in the raw water from the North West drinking water treatment plant (NW-DWTP3). However, the *in vivo* hazard classification system indicated a class III hazard in some of the other raw and treated water samples. Similarly, the EBT values for some of the *in vitro* bioassays were also exceeded in several other raw and treated water samples. Therefore, the results highlight the need to include *in vivo* and *in vitro* bioassays in water quality assessment to complement chemical analysis, in order to get a more comprehensive overview of the water quality at DWTPs.

NOTE:

A factsheet on the use of *in vivo* and *in vitro* bioassays for effect-based monitoring of drinking water quality and treatment technologies is available.

CHAPTER 6: USE OF EFFECT-BASED METHODS FOR WASTEWATER ASSESSMENT – CASE STUDIES

6.1 INTRODUCTION

As an important source of mixtures of compounds, WWTPs represent an important source of chemical stressors. In order to evaluate their effective removal, a test battery covering the different AOPs was applied to influent and effluent samples of WWTPs in Gauteng and Mpumalanga. The endpoints applied to this water have human and aquatic ecosystem health relevance.

To assess product quality and treatment efficacy of wastewater treatment, battery 1 assays are suggested, and to understand wastewater treatment processes, battery 2 assays are recommended (refer to the decision-making tool in Table 3.2-3.4). Battery 1 assays consist of low sensitivity *in vitro* bioassays (yeast based bioassays), oxidative stress, AhR and *in vivo* bioassays. The recommended *in vivo* tests are *Allivibrio fischeri, Pseudokirchneriella subcapitata* (algae), *Daphnia magna, Poecillia reticulata,* with *Spirodela polyrhiza* and *Thamnocephalus platyurus* as optional assays. Battery 2 assays to assess treatment processes consist of high sensitivity *in vitro* bioassays (mammalian-based bioassays), oxidative stress, AhR and the same *in vivo* assays recommended for battery 1. For the scope of this study, we've applied the low and high sensitivity *in vitro* bioassays and all the *in vivo* assays.

There are no EBT values available specifically for wastewater. When evaluating wastewater, a 10x dilution factor is applied to the wastewater effluent samples as a conservative estimate of the dilution of wastewater effluent into a receiving water body. Then the wastewater effluent can be compared to the surface water EBT. If the dilution factor for a specific WWTP is known, that dilution factor should be applied to the effluent sample before comparing it to the surface water EBT.

6.2 APPLICATION OF *IN VIVO* BIOASSAYS FOR WASTEWATER ASSESSMENT

The WWTP *in vivo* bioassay results are summarised in Table 6.1. For sample GP-WWTP1a (influent), the highest effect (100% mortality) was noted following the vertebrate (*P. reticulata*) test. All other tests, except the micro-algae (*R. subcapitata*) test, exceeded the minimum acceptable effect level (≤10% and ≤20% respectively) for these tests. This sample was allocated a Hazard Class IV (weight score of 61). For sample GP-WWTP1b (the effluent from this site), the highest effect (68% growth inhibition) was noted following the duckweed (*S. polyrhiza*) bioassay, and other tests showing significant (although slight) effects were the *T. platyurus* bioassay and the *P. reticulata* test. This sample was allocated a Hazard Class III, with a weighting of 42, indicating a decrease in the effect noted following treatment at this facility.

For sample GP-WWTP2a (influent), the highest effects noted was for the *T. platyurus* (100% ingestion inhibition) and *P. reticulata* (100% mortality) tests, with other significant effects noted following the *D. magna*, *S. polyrhiza* and *A. fischeri* tests (≥50%). This sample was allocated a Hazard Class IV (weight score of 67). For sample GP-WWTP2b (effluent from this site), the highest effects (100%) were noted for the *A. fischeri*, *D. magna*, *T. platyurus* and *P. reticulata* tests, with a significant effect (89%) also noted following the *S. polyrhiza* – The only test not showing a significant effect (although some degree of growth inhibition was noted close to the minimum acceptable level) was the *R. subcapitata* test. This site was also allocated a Hazard Class IV but with a weight score of 78, indicating an increase in toxicity effects.

Table 6-1: Summary of *in vivo* bioassay results for WWTP samples

Sample name	*A. fischeri*	*R. subcapitata*	*S. polyrhiza*	*D. magna*	*T. platyurus*	*P. reticulata*	Hazard Class Weight
Wastewater Treatment Works							
Influent							
GP-WWTP1a	-69	+9	-65	-90	-94	-100	Class IV 61
GP-WWTP2a	-76	+6	-56	-50	-100	-100	Class IV 67
MP-WWTP3a	This site was not sampled/assessed – wastewater is collected by a honey sucker truck from the houses and then deposited in the 1st reticulation pond at the treatment plant						
Effluent							
GP-WWTP1b	+4	+18	-68	-5	-30	-17	Class III 42
GP-WWTP2b	-100	-17	-89	-100	-100	-100	Class IV 78
MP-WWTP3b	-37	-25	-52	-15	-37	-17	Class III 58

No sampling could be conducted at the MP-WWTP3a site (influent) because the water is collected by trucks from the source and pumped directly from the trucks to the treatment facility receiving area. The test showing the highest effect (52% growth inhibition) for the sample taken from the effluent of this plant (MP-WWTP3b) was the *S. polyrhiza* test, and all other tests included in the battery showed some significant (although slight) effects. This site was allocated a Hazard Class III with a weight score of 58.

The following samples were classified as a Hazard Class IV (with a percentage effect of 100% reached in at least one sample), indicating high acute toxicity to the environment: GP-WWTP1a, GP-WWTP2a and GP-WWTP2b. Of these three samples, GP-WWTP2b (with a weight score of 78) was the most toxic, followed by GP-WWTP2a (weight score of 67) and then GP-WWTP1a (with a weight score of 61) as least toxic of the three samples.

Samples collected from GP-WWTP1b and MP-WWTP3b was classed as Hazard Class III (where the percentage affect is reached or exceeded in at least one test but the effect level is below 100%), indicating an acute hazard to the environment. Based on the weight scores, the samples most toxic was MP-WWTP3b (with a weight score of 58), followed by GP-WWTP1b (with a weight score of 42).

The limited results obtained for the influents at the wastewater treatment works showed that the contents of the influents at site GP-WWTP2 showed slightly higher toxicity than those of GP-WWTP1 – it should be noted that different sources distribute influents with different contents which will show a different data trend for each plant and these contents are unknown for this study and no chemistry data were available for these samples to enable adequate interpretation. It was evident that the treatment processes at GP-WWTP1 were either more effective than those implemented at GP-WWTP2, or that the disinfection processes at GP-WWTP2 caused a greater effect on the aquatic organisms.

The best-suited test for the influents (based on data from these sites, keep in mind that each site receives different water qualities) would be the *P. reticulata* test, followed by the *T. platyurus* bioassay. For the effluents, the *S. polyrhiza* test would be best suited, but most probably due to effects of the treatment process (disinfection stage) of the GP-WWTP2 site the *A. fischeri*, *D. magna*, *T. platyurus* and *P. reticulata* tests also apply. This depends on the influent water constituency, which may be based on the area and activities taking place in the different areas.

6.3 APPLICATION OF *IN VITRO* BIOASSAYS FOR WASTEWATER ASSESSMENT

6.3.1 Xenobiotic metabolism

The H4IIE-luc reporter gene assay was used to evaluate xenobiotic metabolism and its effects on biological systems, particularly through their interaction with the aryl hydrocarbon receptor (AhR). The WWTP results obtained in the H4IIE-luc reporter gene assay are tabulated in Table 6.2. The GP-WWTP1a sample, collected during the first sample collection event, could be quantified, with a TCDD-EQ value of 0.2 ± 0.05 ng/L. This is above the proposed ecological trigger value of 0.05 ng/L for surface water. No cytotoxicity was detected in any of the WWTP samples in the H4IIE-luc cells.

Table 6-2 H4IIE-luc reporter gene assay results of WWTP samples

Sample site	TCDD-EQ (ng/L)	
	Sample collection 1	Sample collection 2
GP-WWTP1a	0.2 ± 0.05	No sample
GP-WWTP1b	<LOQ	<LOD
GP-WWTP2a	<LOQ	No sample
GP-WWTP2b	<LOD	<LOD
MP-WWTP3a	No sample	No sample
MP-WWTP3b	Not analysed	Not analysed

<LOD: Below the limit of detection of the assay. <LOQ: below the level of quantification.

6.3.2 Hormone receptor/Endocrine disruption

6.3.2.1 Yeast estrogen sceen (YES)

Water samples were collected and extracted from three WWTPs to test for estrogenic activity using the YES assay. The activity of the samples was measured against the positive control, 17β-estradiol, in order to determine the bioequivalent concentrations from the dose response curves. The results are tabulated in Table 6.3. Estrogenic activity could be quantified in all the samples. Only two influent samples could be collected, but in both cases, estrogenic activity was higher in the influent samples compared to the effluent samples, indicating that the WWTPs were able to reduce activity in wastewater samples.

Table 6-3 Estrogenic activity in WWTP samples using the yeast estrogen screen.

Sample site	EEQ (ng/L)	
	Sample collection 1	Sample collection 2
GP-WWTP1a	55 ± 3.8 *	No sample
GP-WWTP1b	2.8 ± 0.83	0.85 ± 0.054
GP-WWTP2a	54 ± 3.1 *	No sample
GP-WWTP2b	0.075 ± 0.008	2.1 ± 0.22
MP-WWTP3a	No sample	No sample
MP-WWTP3b	42.55 ± 3.71	4.96 ± 0.44

* Cytotoxicity at 50x concentration.

6.3.2.2 T47D-KBluc reporter gene assay

The WWTP samples were assessed for estrogenic and anti-estrogenic activity using the T47D-KBluc reporter gene assay. The results are tabulated in Table 6.4. Estrogenic activity could be quantified in all the samples. Only two influent samples could be collected, but in both cases, estrogenic activity was higher in the influent samples compared to the effluent samples, indicating that the WWTPs were able to reduce activity in wastewater samples. This is consistent with the YES results.

Table 6-4 Estrogenic activity in WWTP samples using the T47D-KBluc assay

Sample site	EEQ (ng/L)	
	Sample collection 1	Sample collection 2
GP-WWTP1a	87 ± 19 **	No sample
GP-WWTP1b	3.8 ± 0.4 ***	1.3 ± 0.15
GP-WWTP2a	53 ± 7.2 *	No sample
GP-WWTP2b	0.14 ± 0.03	2.7 ± 0.3
MP-WWTP3a	No sample	No sample
MP-WWTP3b	108 ± 31 ***	21 ± 0.8 ***

* Cytotoxicity at 0.3x and higher concentrations; ** Cytotoxicity at 1x and higher concentrations; *** Cytotoxicity at 10x and higher concentrations.

6.3.3 Androgenic activity

To assess androgenic activity in drinking water, two assays have been recommended: the Yeast androgen screen (YAS) and MDA-kb2 reporter gene assay. The androgenic activity of WWTP samples using the YAS assay are tabulated in Table 6.5. Androgenic activity could be quantified in four WWTP samples. Influent and effluent results were available for GP-WWTP2a, where the effluent activity was reduced compared to the influent activity, indicating that the WWTP was able to reduce androgenic activity by 94%.

Table 6-5 Androgenic activity of WWTP samples using the YAS assay

Sample site	DHT-EQ (ng/L)	
	Sample collection 1	Sample collection 2
GP-WWTP1a	1057 ± 134 **	No sample
GP-WWTP1b	Not tested	Not tested
GP-WWTP2a	1322 ± 150 *	No sample
GP-WWTP2b	73 ± 25	<LOQ
MP-WWTP3a	No sample	No sample
MP-WWTP3b	159 ± 31 **	Not tested

* Cytotoxicity above 12.5x concentration; ** Cytotoxicity above 25x concentration; <LOQ: below the level of quantification.

Table 6.6 shows a summary of the MDA-kb2 reporter gene assays for WWTP samples. Only two influent samples could be collected, but in both cases, androgenic activity was higher in the influent samples compared to the effluent samples, indicating that the WWTPs were able to reduce activity in wastewater samples. Similar to the YAS assay, the MDA-kb2 assay revealed that GP-WWTP2 was able to remove 81% of androgenic activity. No anti-androgenic or glucocorticoid activity was detected in any of the WWTP samples.

Table 6-6 Androgenic activity in WWTP samples using the MDA-Kb2 reporter gene assay

Sample site	Androgenic activity	
	Sample collection 1	Sample collection 2
GP-WWTP1a	209 ± 56	No sample
GP-WWTP1b	<LOD	<LOD
GP-WWTP2a	349 ± 62	No sample
GP-WWTP2b	67 ± 2.8	4.8 ± 0.8
MP-WWTP3a	No sample	No sample
MP-WWTP3b	4.1 ± 0.8	<LOD

<LOD: Below the limit of detection of the assay

6.3.4 Thyroid activity

No thyroid activity was detected in the WWTP samples using the GH3.TRE-Luc assay.

6.3.5 Oxidative stress

Only MP-WWTP3b samples were analysed for oxidative stress. The results are summarised in Table 6.7. Dichlorvos-EQ values could be calculated for both sample collection periods.

Table 6-7 WWTP sample analysis using the AREc32 reporter gene assay

Sample site	Sample collection 1			Sample collection 2		
	IC_{10} ± SE (REF)	$EC_{IR1.5}$ ± SE (REF)	Dichlorvos -EQ (µg/L)	IC_{10} ± SE (REF)	$EC_{IR1.5}$ ± SE (REF)	Dichlorvos -EQ (µg/L)
MP-WWTP3b	>200	1.8 ± 0.1	945.5	>200	4 ± 0.1	421.9

REF = relative enrichment factor of a water sample in the bioassay (a REF of 1 means that the concentrations of the extracted chemicals are the same as in the original water sample); IC_{10} = 10% inhibition concentration; effect concentration ($EC_{IR1.5}$), and SE = Standard error.

6.4 CHEMICAL ANALYSIS

In order to identify contaminants in the influent and effluent samples that might contribute to the effects seen in the bioassays, samples were subjected to non-targeted and targeted chemical analysis. The results are summarised in Table 6.8. Target chemicals could be quantified in the influent and effluent samples at GP-WWTP2.

Table 6-8 Non-targeted and targeted chemical analysis results in WWTP samples

Sample code	Sample collection no	Non-targeted analysis (number of compounds detected)	Targeted analysis (list of compounds and concentrations in ng/L)
GP-WWTP1a	1	378	<LOQ
GP-WWTP1b	1	402	<LOQ
GP-WWTP1b	2		

GP-WWTP2a	1	364	4-Aminobiphenyl (0.3), Acetaminophen (4.2), Atenolol (15.5), Atrazine (49.2), Benzophenone (6.0), Bisphenol A (31.3), Bufexamac (3.3), Caffeine (762.0), Carbamazepine (61.1), Danofloxacin (16.2), Digoxin (15.9), Efavirenz (37.7), Enrofloxacin (1.4), Estrone (15.5), Flumequine (0.5), Imidacloprid (33.5), Isoniazid (0.7), Ketoprofen (2.5), Levoflaxacin (739.0), Lidocaine (15.8), Lopinavir (15.3), Medroxyprogesterone (9.1), Metoprolol (321.1), Phenacetin (0.5), Pindolol (224.1), Primidone (23.9), Roxithromycin (0.6), Sulfadimethoxine (0.5), Sulfadoxine (67.3), Sulfamethoxazole (60.3), Sulfisoxazol (0.2), Terbuthylazine (39.7), Terbutryn (1.4), Thiabendazole (3.3), Thiacloprid (0.2), Tramadol (188.9), Trimethoprim (58.4), Valsartan (93.8)
GP-WWTP2b	1	290	4-Aminobiphenyl (0.4), Acetaminophen (2.3), Atenolol (24.4), Atrazine (46.0), Benzophenone (14.9), Bisphenol A (7.7), Bufexamac (1.6), Caffeine (36.7), Carbamazepine (68.0), Danofloxacin (23.0), Digoxin (9.4), Efavirenz (58.4), Enrofloxacin (2.4), Estrone (11.4), Flumequine (0.6), Imidacloprid (19.5), Isoniazid (1.4), Ketoprofen (3.3), Levoflaxacin (652.4), Lidocaine (21.3), Lopinavir (7.2), Medroxyprogesterone (6.8), Metoprolol (221.0), Phenacetin (1.7), Pindolol (131.6), Primidone (27.6), Roxithromycin (0.7), Sulfadimethoxine (0.2), Sulfadoxine (24.4), Sulfamethoxazole (29.7), Sulfisoxazol (0.2), Terbuthylazine (45.0), Terbutryn (2.0), Thiabendazole (3.7), Thiacloprid (0.2), Tramadol (161.0), Trimethoprim (54.8), Valsartan (6.0)
GP-WWTP2b	2		
MP-WWTP3b	1	49	NA
MP-WWTP3b	2	50	NA

NA Not analysed; <LOQ Below the level of quantification

6.5 APPLYING EBT VALUES TO BIOASSAY RESULTS FOR WASTEWATER QUALITY MONITORING

The percentage of WWTP samples below the LOQ, above the LOQ and above the EBT in the various bioassays are graphically displayed in Figure 6.1. For androgenic activity, there is only an EBT value available for surface water, but not for wastewater. In order to compare the WWTP effluent samples to the surface water EBT, a 10x dilution factor was applied to wastewater effluent samples as a

conservative estimate of the dilution of wastewater effluent into a receiving water body. All WWTP samples were below the LOD in the GH3.TRE-luc (thyroid) assays. Only one WWTP sample could be quantified in the H4IIE-luc bioassay. In this assay, the TCDD equivalent value of the influent sample for GP-WWTP1a was 0.2 ± 0.05 ng/L. This is above the EBT trigger value of 0.05 ng/L TCDD-EQ for surface water. All the WWTP samples were above the LOQ in the YES and T47D-KBluc bioassays for estrogenic activity, and in both assays, 50% of the samples were above the EBT value.

Androgenic activity could be quantified in 5 of the 6 WWTP samples using the MDA-kb2 assay and 4 of these samples could be quantified in the YAS assay. In the MDA-kb2 assay, 2 of the samples were above the EBT value and 3 samples were above the EBT in the YAS assay. Only two of the WWTP samples were assessed in the AREc32 assay for oxidative stress, and both were below the EBT value. In the *in vivo* bioassays, two of the samples were classified as Class III and three samples were classified class IV with the hazard classification system. Two of the samples that were classified as class IV (high lethal/sub-lethal hazard), were also above the EBT in at least two of the *in vitro* bioassays. Target chemical analyses were done for GP-WWTP1 and GP-WWTP2. None of the target chemicals could be quantified in the influent or effluent samples from GP-WWTP1, but various target chemicals could be quantified in influent and effluent samples from GP-WWTP2 that could be responsible for the estrogenic and androgenic activity reported in these samples, including bisphenol A, estrone and medroxyprogesterone.

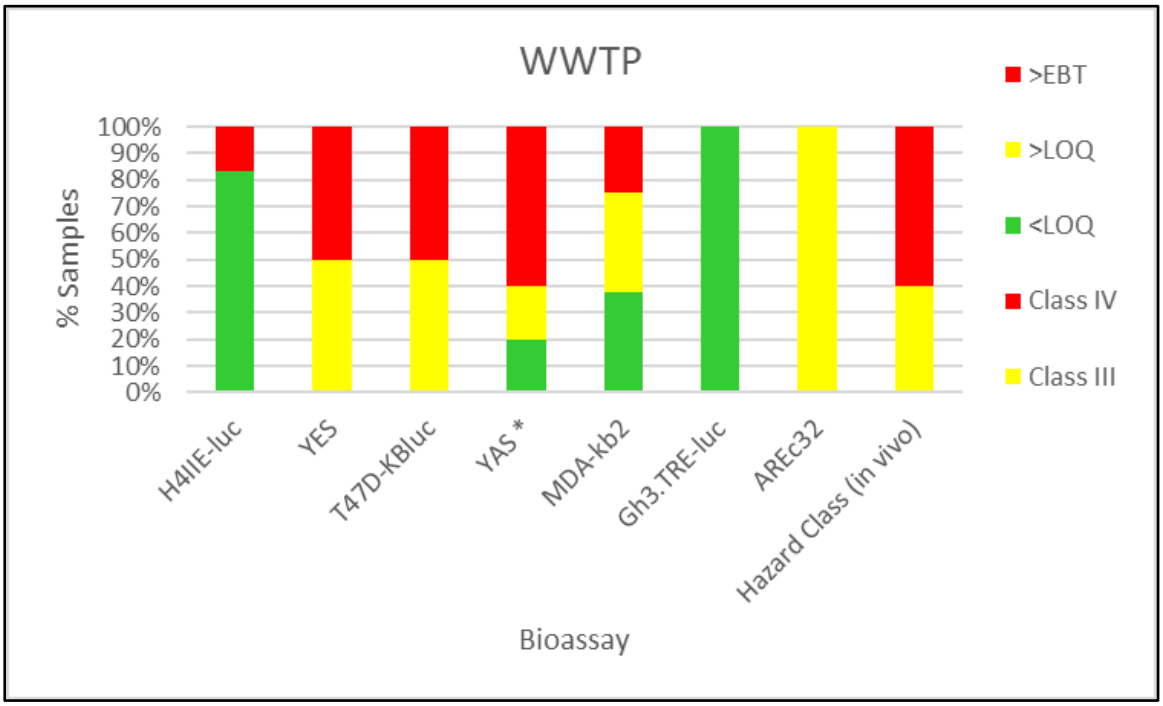


Figure 6-1: Summary of the results of WWTP samples assessed in the bioassays, expressed as the percentage of samples below LOQ, above LOQ and above EBT value. * Only samples that tested positive in the MDA-kb2 assay were analysed in the YAS assay

6.6 ASSESSING THE EFFICIENCY OF WASTEWATER TREATMENT TECHNOLOGIES USING CHEMICAL ANALYTICAL TECHNIQUES TO COMPLEMENT *IN VITRO* AND *IN VIVO* BIOASSAYS

6.6.1 GP-WWTP1

Figure 6.2 summarises the main results obtained with non-targeted chemical screening and bioassays for GP-WWTP1. In the H4IIE-luc assay, the TCDD-EQ value was above the EBT value in influent water, but below the LOQ in the effluent. In both the YES and T47D-KBluc assays, estrogenic activity was above the EBT in influent water samples, but was reduced to levels below the EBT in effluent samples, when assuming a 10x dilution factor for wastewater effluent into a receiving water body. Androgenic activity was above the EBT value in influent water in the YAS and MDA-kb2 assays, but was reduced to below the LOD in effluent water. Non-targeted chemical analysis revealed 378 compounds in influent and 402 compounds in effluent water, although no target chemicals could be quantified with the targeted chemical analysis. The *in vivo* bioassays revealed a class IV high lethal/sub-lethal hazard for influent water that was reduced to class II, lethal/sub-lethal hazard in effluent water samples. The weight score reduced from 61% in influent water to 42% in effluent water. The results indicate that GP-WWTP1 was functioning effectively and was able to reduce EDC activity to acceptable levels.

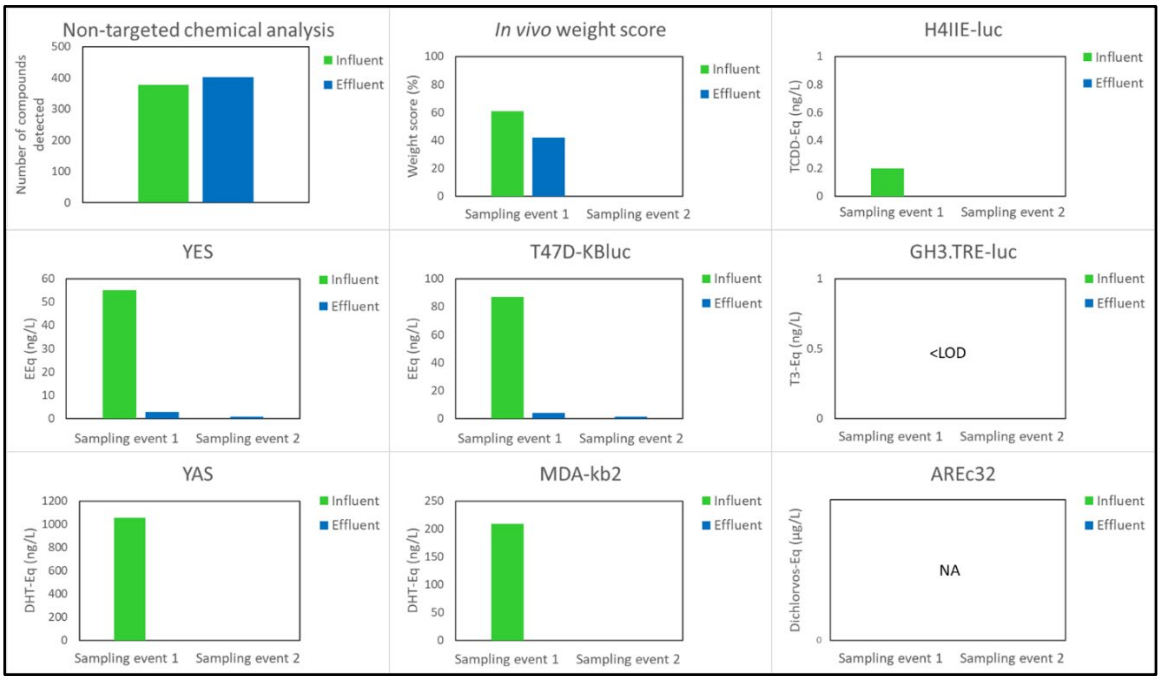


Figure 6-2: Summary of non-targeted chemical analysis and *in vitro* and *in vivo* bioassay results for GP-WWTP1. NA Not analysed; <LOD below the limit of detection

6.6.2 GP-WWTP2

Figure 6.3 summarizes the main results obtained with non-targeted chemical screening and bioassays for GP-WWTP2. Influent and effluent samples showed estrogenic and androgenic activities above the EBT values in influent samples, but were below the EBT values in effluent samples. *In vivo* bioassays also revealed a class IV, high lethal/sub-lethal hazard in influent and effluent samples. With non-targeted chemical analysis, 364 compounds were detected in influent and 290 compounds in effluent samples. Targeted chemical analysis could quantify various chemicals, including bisphenol A, estrone

and medroxyprogesterone that could contribute to the EDC activities seen in influent and effluent samples. The results indicate that GP-WWTP2 was functioning effectively in order to reduce EDC activity to acceptable levels.

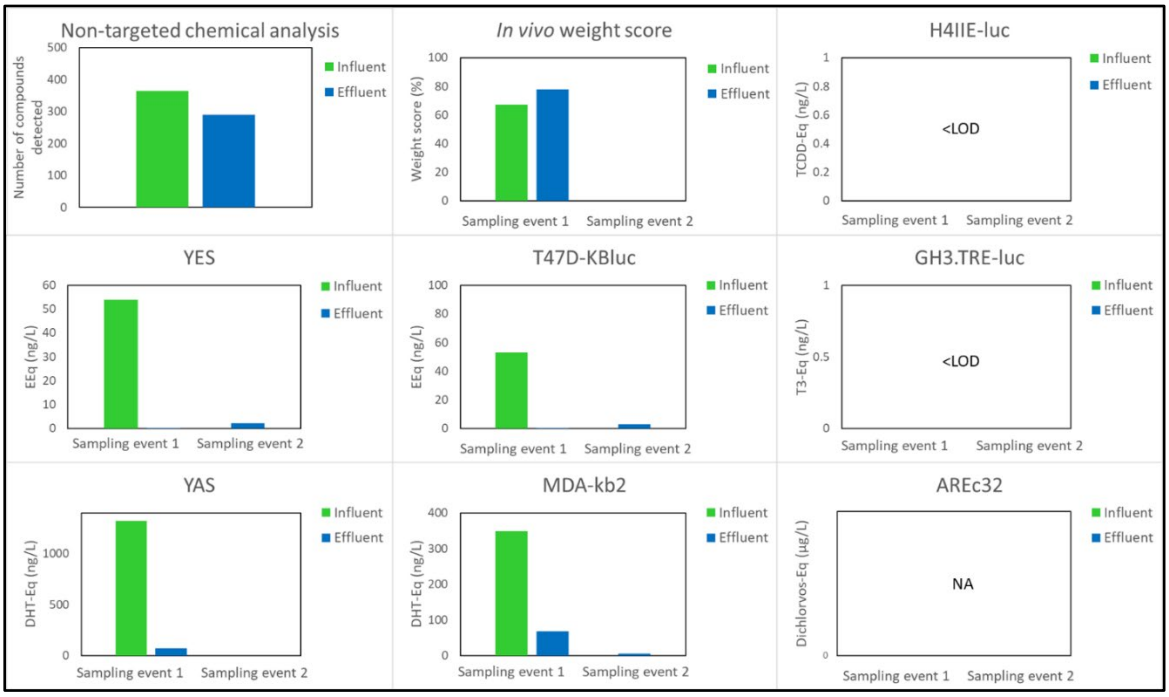


Figure 6-3: Summary of non-targeted chemical analysis and *in vitro* and *in vivo* bioassay results for GP-WWTP2. NA Not analysed; <LOD below the limit of detection

6.6.3 MP-WWTP3

Figure 6.4 summarises the main results obtained with non-targeted chemical screening and bioassays for MP-WWTP3. It was not possible to obtain influent water samples for MP-WWTP3, but in the effluent samples, estrogenic activity was above the EBT value for both the YES and T47D-KBluc bioassays for both sampling events. Androgenic activity was also above the EBT value for the first sampling event in the YAS assay. In the AREc32 bioassay, the dichlorvos-EQ was below the EBT value for both sampling events. Non-targeted chemical analysis revealed 49 and 50 compounds, respectively for the first and second sampling event, but unfortunately, there are no results available for targeted chemical analysis. The *in vivo* bioassays were only done for the second sampling event and revealed a class III, lethal/sub-lethal hazard. From the results, it is clear that this WWTP warrants further investigation.

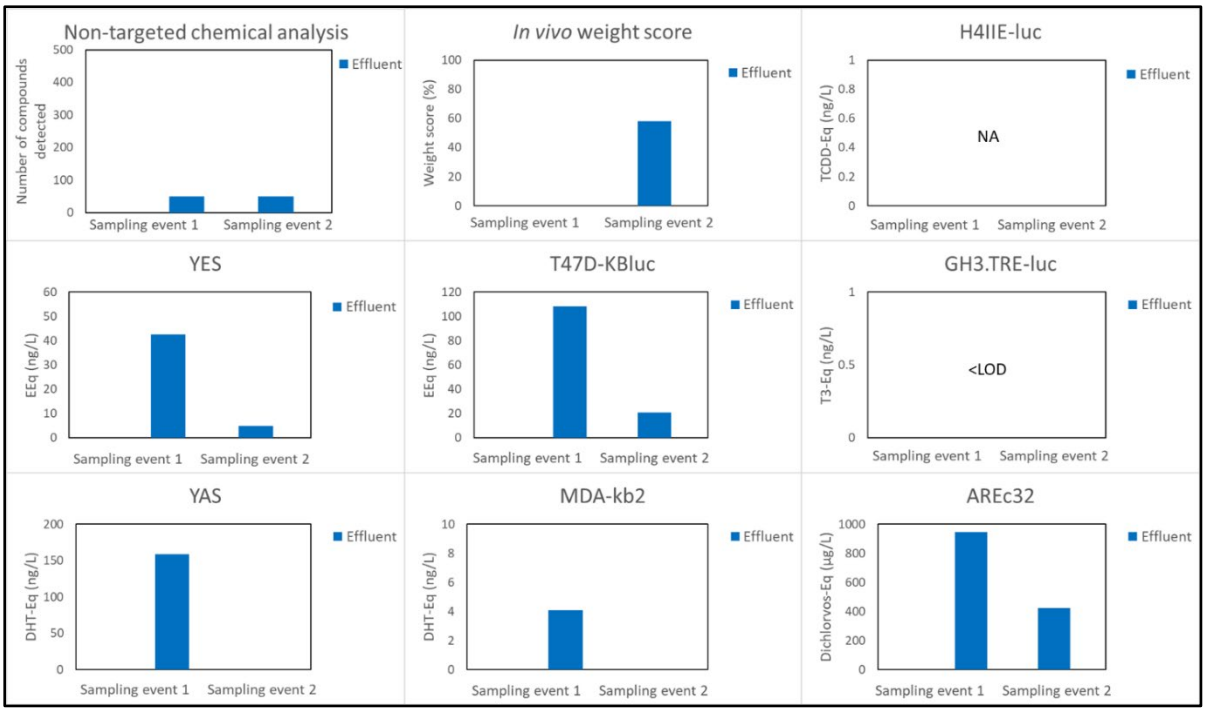


Figure 6-4: Summary of non-targeted chemical analysis and *in vitro* and *in vivo* bioassay results for MP-WWTP3. NA Not analysed; <LOD below the limit of detection

6.7 SUMMARY

This study demonstrated the value of applying *in vivo* and *in vitro* bioassays to assess the quality of wastewater as well as the efficacy of treatment processes at WWTPs. In the *in vivo* assays, two influent and one effluent sample were classified as class IV, high lethal/sub-lethal hazard, and two effluent samples were classified as class III, lethal/sub-lethal hazard. In the *in vivo* bioassays, activities could be quantified in one sample in the H4IIE bioassay, all samples in the YES and T47D-KBluc bioassays, 4 samples in the YAS, 5 samples in the MDA-kb2 and 2 samples in the AREc32 bioassay. The results indicated that the low sensitivity bioassays (YES and YAS) were sensitive enough to be used for the wastewater samples. None of the samples were above the LOD in the GH3. TRE-luc bioassay for thyroid activity, indicating that this assay might not be sensitive enough and other relevant bioassays for thyroid activity should be considered.

Two of the samples that were classified as class IV (high lethal/sub-lethal hazard) in the *in vivo* bioassays, were also above the EBT in at least two of the *in vitro* bioassays. Target chemicals were above the LOQ in the influent and effluent samples of the second WWTP (GP-WWTP2) in Gauteng. At this WWTP, the EBT values were also exceeded in four of the *in vitro* bioassays for the influent water sample, and the hazard classification system for the *in vivo* assays classified both the influent and effluent water samples as a class IV hazard. However, target chemicals could not be quantified in the influent water from GP-WWTP1, which was above the EBT in five *in vitro* assays and classified as class IV hazard in the *in vivo* assays. The results highlight the need to include *in vivo* and *in vitro* bioassays in water quality assessment to complement chemical analysis, in order to get a more comprehensive overview of the water quality at WWTPs.

NOTE:

A factsheet on the use of *in vivo* and *in vitro* bioassays for effect-based monitoring of wastewater quality and treatment technologies is available.

CHAPTER 7: USE OF EFFECT BASED METHODS FOR GROUNDWATER ASSESSMENT – CASE STUDIES

7.1 INTRODUCTION

Aquifers and groundwater-dependent ecosystems (GDEs) are facing increasing pressure from water consumption, irrigation and climate change. These pressures modify groundwater levels and their temporal patterns and threaten vital ecosystem services such as arable land irrigation and ecosystem water requirements, especially during droughts (Kløve et al., 2014). Groundwater samples in this study included water from boreholes (MP-GW1 and WC-GW3) and natural springs (MP-GW2 and MP-GW4). For groundwater that is used for drinking water, battery 3 assays are recommended (refer to decision-making tool in Table 3.2-3.4), consisting of high sensitivity *in vitro* bioassays (mammalian-based bioassays), oxidative stress, AhR and mutagen or genotoxicity assays. In addition to the suite of assays recommended for drinking water, the *in vivo* bioassays applicable to environmental water are also recommended. The recommended *in vivo* assays are *Allivibrio fischeri, Pseudokirchneriella subcapitata* (algae), *Daphnia magna, Poecillia reticulata,* with *Spirodela polyrhiza* and *Thamnocephalus platyurus* as optional assays. For the scope of this study, we've applied the low and high sensitivity *in vitro* bioassays and all the *in vivo* assays. When evaluating groundwater, surface water EBT values can be applied.

7.2 APPLICATION OF *IN VIVO* BIOASSAYS FOR GROUNDWATER ASSESSMENT

The *in vivo* bioassay results from analysis of groundwater samples are summarised in Table 7.1. The sample tested from MP-GW1 (borehole) indicated highest effect (56% growth inhibition) with *S. polyrhiza* test. Effects exceeding the minimum acceptable effect levels were also noted for the *A. fischeri*, *T. platyurus* and *P. reticulata* bioassays. MP-GW2 (natural spring) sample tested also indicated highest effect (68% growth inhibition) with the *S. polyrhiza* test. Effects exceeding the minimum acceptable effect levels were also noted for the *A. fischeri*, *R. subcapitata* and *P. reticulata* bioassays. Results from the MP-GW4 (bottled water from natural spring) sample indicated highest effect (65% growth inhibition) with *S. polyrhiza* test. Effects exceeding the minimum acceptable effect levels were also noted for the *A. fischeri*, *R. subcapitata* and *P. reticulata* bioassays.

Table 7-1: Summary of *in vivo* bioassay results for GW samples

Sample name	*A. fischeri*	*R. subcapitata*	*S. polyrhiza*	*D. magna*	*T. platyurus*	*P. reticulata*	Hazard Class Weight
Groundwater							
MP-GW1	-31	-6	-56	-5	-29	-25	Class III 42
MP-GW2	-33	-36	-68	0	-11	-33	Class III 42
MP-GW4	-37	-38	-65	0	-15	-25	Class III 42

All three samples were allocated a Hazard Class III (where the percentage affect is reached or exceeded in at least one test but the effect level is below 100%), indicating an acute hazard to the environment and a weight score of 42.

The test showing the highest effect and deemed the most sensitive and most suitable for the three groundwater samples was the *S. polyrhiza* bioassay. The *A. fischeri* bioassay and the *P. reticulata* test showed slight, but significant effects for all three groundwater samples, and the *R. subcapitata* bioassay for groundwater samples MP-GW2 and MP-GW4 while the *T. platyurus* bioassay showed the effect for sample MP-GW1.

7.3 APPLICATION OF *IN VITRO* BIOASSAYS FOR GROUNDWATER ASSESSMENT

7.3.1 Xenobiotic metabolism

Only one of the GW samples (WC-GW3) was analysed in the H4IIE-luc reporter gene assay, but the sample was below the detection limit and showed no toxicity in the H4IIE-luc cells.

7.3.2 Hormone receptor/ Endocrine disruption

7.3.2.1 Yeast estrogen screen (YES)

The estrogenic activity of the samples using the YES assay are tabulated in Table 7.2.

Table 7-2 EEQ concentrations (ng/L) in ground water using the yeast estrogen screen.

Sample site	EEQ (ng/L)	
	Dry season	Wet season
MP-GW1	0.10 ± 0.028	<LOD
MP-GW2	<LOQ	<LOD
WC-GW3	6.8 ± 0.55	No sample
MP-GW4	0.23 ± 0.050	<LOD

<LOD: Below the limit of detection of the assay. <LOQ: below the level of quantification.

7.3.2.2 T47D-KBluc reporter gene assay

The estrogenic activity of ground water samples using the T47D-KBluc reporter gene assay is tabulated in Table 7.3.

Table 7-3 Estrogenic activity of ground water samples using the T47D-KBluc assay

Sample site	EEQ (ng/L)	
	Dry season	Wet season
MP-GW1	0.72 ± 0.05	0.014 ± 0.003
MP-GW2	1.5 ± 0.2	0.099 ± 0.014
WC-GW3	1.4 ± 0.6	No sample
MP-GW4	0.99 ± 0.08	0.022 ± 0.003

7.3.3 Androgenic activity

7.3.3.1 Yeast androgen screen (YAS)

The androgenic activity of groundwater samples was not assessed in the YAS assay.

7.3.3.2 MDA-kb2 reporter gene assay

No androgenic or glucocorticoid activity was detected in any of the GW samples.

7.3.4 Thyroid activity

No thyroid activity was detected in the groundwater samples using the GH3.TRE-Luc assay.

7.3.5 Oxidative stress

MP-GW1, MP-GW2 and MP-GW4 samples were analysed for oxidative stress responses. Dichlorvos-EQ values could be calculated for all samples, except for MP-GW1 at the second collection. The results are summarised in Table 7.4.

Table 7-4 Oxidative stress response results of ground water samples using the AREc32 reporter gene assay

Sample site	Sample collection 1 (Dry Season)			Sample collection 2 (Wet Season)		
	IC_{10} ± SE (REF)	$EC_{IR1.5}$ ± SE (REF)	Dichlorvos-EQ (µg/L)	IC_{10} ± SE (REF)	$EC_{IR1.5}$ ± SE (REF)	Dichlorvos-EQ (µg/L)
MP-GW1	>200	135.5 ± 9	12.6	20.0 ± 6.4	>200	-
MP-GW2	53.4 ± 5.3	7.8 ± 0.4	217.9	>200	38.8 ± 1.7	43.8
MP-GW4	>200	29.3 ± 3.1	57.9	>200	82.9 ± 6.9	20.5

REF = relative enrichment factor of a water sample in the bioassay (a REF of 1 means that the concentrations of the extracted chemicals are the same as in the original water sample); IC_{10} = 10% inhibition concentration; effect concentration ($EC_{IR1.5}$), and SE = Standard error.

7.4 CHEMICAL ANALYSIS

Only non-targeted chemical analysis was performed on groundwater samples. The results are summarised in Table 7.5.

Table 7-5 Chemical analysis results of GW samples

Sample code	Sample collection no	Non-targeted analysis (number of compounds detected)	Targeted analysis
MP-GW1	1	49	NA
MP-GW1	2	50	NA
MP-GW2	1	49	NA
MP-GW2	2	49	NA
WC-GW3	1	NA	NA
MP-GW4	1	NA	NA
MP-GW4	2	NA	NA

NA Not analysed

7.5 APPLYING EBT VALUES TO BIOASSAY RESULTS FOR GROUNDWATER QUALITY MONITORING

Groundwater (GW) samples included samples from boreholes and springs. The percentage of GW samples below the LOQ, above the LOQ and above the EBT in the various bioassays are graphically displayed in Figure 7.1. All GW samples were below the LOD in the H4IIE-luc, MDA-kb2 and GH3.TRE-luc assays. Estrogenic activity could be quantified in 43% samples in the YES assay, with 14% samples above the EBT value. In the T47D-KBluc assay, all samples were above the LOQ, with 57% above the EBT. In the AREc32 assay, 83% samples were above the LOQ and 17% were above the EBT value. Three GW samples were assessed in the *in vivo* bioassays, and all three samples were classified as class III (lethal/sub-lethal hazard). Only non-targeted chemical analysis was done on the groundwater samples, but no targeted chemical analysis.

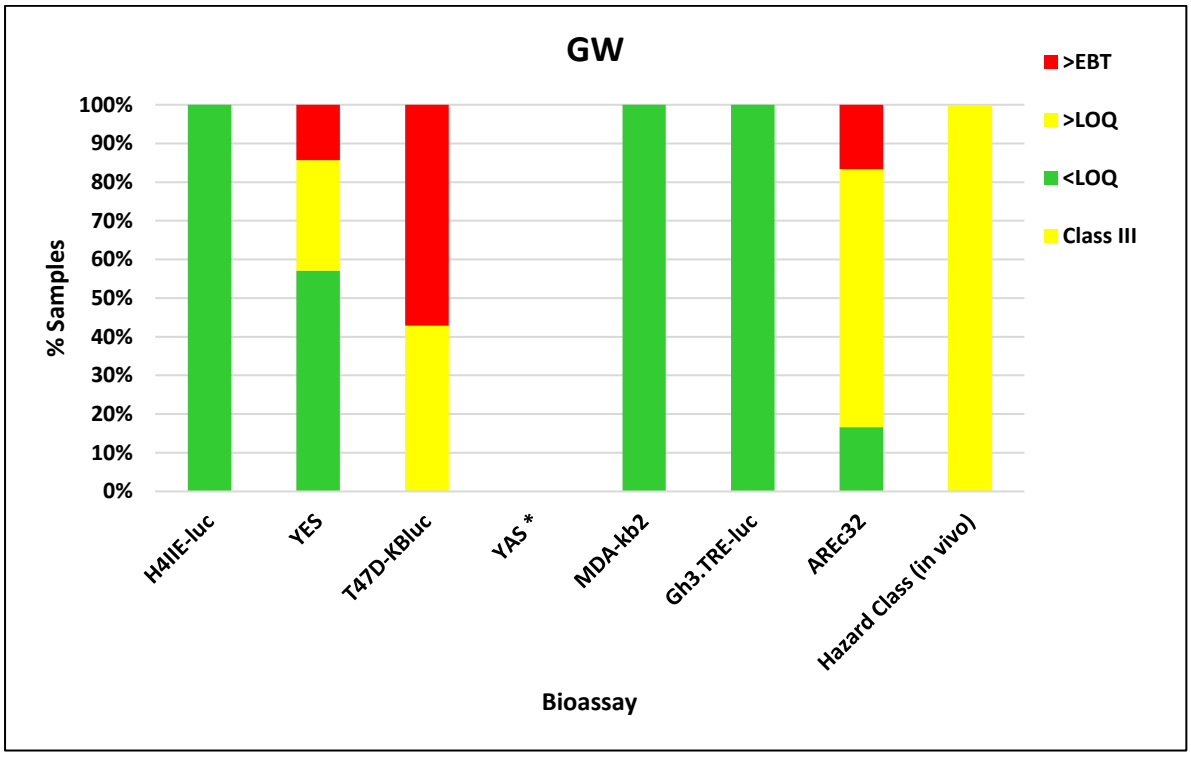


Figure 7-1: Summary of the results of GW samples assessed in the bioassays, expressed as the percentage of samples below LOQ, above LOQ and above EBT value. * Only samples that tested positive in the MDA-kb2 assay were analysed in the YAS assay

7.6 SUMMARY

This study demonstrated the value of applying *in vivo* and *in vitro* bioassays to assess the quality of groundwater. All the samples were below the LOD in the H4IIE-luc, MDA-kb2 and GH3.TRE-luc assays. Estrogenic activity could be quantified in 3 samples in the YES assay and all the samples using the T47D-KBluc assay. One sample was above the EBT value using the YES assay and 4 samples were above the EBT in the T47D-KBluc assay. In the AREc32 assay, five samples could be quantified and one sample was above the EBT value. Three GW samples were assessed in the *in vivo* bioassays, and all three samples were classified as class III (lethal/sub-lethal hazard). Unfortunately, no targeted chemical analysis results are available for the groundwater samples in order to compare the chemical analysis with bioassay results. However, the results show that *in vivo* and *in vitro* bioassays can add valuable information when assessing the quality of groundwater.

NOTE:

A factsheet on the use of *in vivo* and *in vitro* bioassays for effect-based monitoring of groundwater quality is available.

CHAPTER 8: USE OF EFFECT BASED METHODS FOR ASSESSING THE IMPACT OF DISCHARGES ONTO SURFACE WATER RESOURCES– CASE STUDIES

8.1 INTRODUCTION

Surface waters, including water from upstream and downstream of a WWTP, are contaminated with an increasing diversity of anthropogenic compounds, which can also be influenced by seasonal changes and rainfall events. This gives rise to the presence of complex contaminant mixtures that can cause serious harm to aquatic ecosystems and also to communities using this as a source of drinking water (De Baat et al., 2020, Escher et al., 2021). For this study, surface water samples were collected from various sources such as rivers, dams and wetlands (MP-SW1-GP-SW13). Battery 2 assays are recommended for surface water (refer to Table 3.2-3.4). Battery 2 assays consist of high sensitivity *in vitro* bioassays (mammalian-based bioassays), oxidative stress, AhR and *in vivo* bioassays. The recommended *in vivo* tests are *Allivibrio fischeri, Pseudokirchneriella subcapitata* (algae), *Daphnia magna, Poecillia reticulata,* with *Spirodela polyrhiza* and *Thamnocephalus platyurus* as optional assays. If the surface water is used for drinking water, mutagen or genotoxicity assays should also be included. For the scope of this study, we've applied the low and high sensitivity *in vitro* bioassays and all the *in vivo* assays.

8.2 APPLICATION OF *IN VIVO* BIOASSAYS FOR SURFACE WATER RESOURCES ASSESSMENT

The *in vivo* bioassay results for the different surface water samples (including dams & rivers and wetlands) are summarised in Table 8.1. The upstream sample from the wetland was allocated a Hazard Class III (with a weight score of 58) and the middle of the wetland sample (MP-SW3) was allocated a Hazard Class IV (with a weight score of 22). The downstream sample, clearly showing the cleaning effect of the wetland, was allocated a Hazard Class II (with a weight score of 33). Literature is available indicating the sensitivity of the *T. platyurus* bioassay to cyanobacteria toxins (as noted for MP-SW2 and MP-SW3), although it cannot be confirmed as the source of the effects noted in this case. However, it should be noted that odours and discolouration of these sites were reported by the community at site MP-SW3, which is consistent with the presence of the algae producing these toxins.

For Dam System 1, the highest effect noted was the slight *S. polyrhiza* growth inhibition effect, and the only other test showing a slight effect (much lower) was the *R. subcapitata* bioassay. This sample was allocated a Hazard Class II with a weight score of 40.

For Dam System 2, the 2 river samples flowing into the dam (L-SW5 and L-SW6) both showed the *S. polyrhiza* bioassay as being the most sensitive, and for the dam outlet sample (L-SW7) the *T. platyurus* bioassay showed the highest effect. All 3 samples were allocated a Hazard Class III (weight scores of the river samples being 42 & 50, and for the outlet sample being 33, showing the dilution effect of the water flowing into the dam). It should be noted that there is no other information on any other sources that may enter this dam apart from these 2 rivers. Other tests showing some significant (although slight) effects that could be noted for this dam system were the *A. fischeri* (for all 3 samples), the *P. reticulata* (for the river samples (L-SW5 & L-SW6)) test. The *R. subcapitata* bioassay showed a slight effect for sample L-SW6 and the *S. polyrhiza* bioassay showed a slight effect for the outlet sample (L-SW7).

For Dam System 3, the most sensitive test for the inlet sample (as for dam system 2) was the *S. polyrhiza* bioassay and for the outlet (as for dam system 2) the *T. platyurus* bioassay was the most sensitive. The other tests that showed a slight (but significant) effect was the *A. fischeri* bioassay and the *P. reticulata* test. The inlet sample was allocated a Hazard Class II (with a weight score of 67) while the outlet sample was allocated a Hazard Class III (with a weight score of 42) – again it should be noted that other sources entering this dam which may have an impact on its water quality, are not known.

Table 8-1: Summary of *in vivo* bioassay results for SW samples

Sample name	*A. fischeri*	*R. subcapitata*	*S. polyrhiza*	*D. magna*	*T. platyurus*	*P. reticulata*	Hazard Class Weight
Surface water							
Wetland samples							
MP-SW2	-21	-36	-53	-5	-82	-17	Class III 58
MP-SW3	-3	-19	-34	-5	-100	-8	Class IV 22
MP-SW4	-28	-15	-39	-5	-13	-8	Class II 33
Dam system 1							
MP-SW1	+2	-22	-37	0	-	0	Class II 40
Dam system 2							
L-SW5	-44	+2	-56	0	-26	-33	Class III 42
L-SW6	-46	-34	-50	-10	-36	-25	Class III 50
L-SW7	-45	+1	-38	-5	-63	-8	Class III 33
Dam system 3							
L-SW8	-38	+2	-49	-5	-46	-25	Class II 67
L-SW9	-41	+2	-43	0	-51	-33	Class III 42
Dam system 4							
GP-SW10	-19	+13	-36	0	-18	-8	Class II 17
GP-SW11	-8	+14	-21	0	-100	0	Class IV 22

For Dam System 4, the most sensitive test for the upstream sample (as for dam systems 2 & 3) was the *S. polyrhiza* bioassay, and for the downstream sample (as for dam systems 2 & 3) it was the *T. platyurus* bioassay. No other tests showed any significant effects for the upstream sample (GP-SW10), but the *S. polyrhiza* bioassay showed a slight (but significant) effect for the downstream sample. The upstream sample (GP-SW10) was allocated a Hazard Class II (with a weight score of 17) while the downstream sample (GP-SW11 – located in an area of significant anthropological activity) was allocated a Hazard Class IV (with a weight score of 22) – again it should be noted that other sources entering this

dam which may have an impact on its water quality, are not known and the chemical constituents of these waters are also unknown.

Ranging from the highest to the lowest Hazard Class, the samples was classified as follows:
-MP-SW3 and GP-SW11 was in Hazard Class IV with weight score of 22
-MP-SW2, L-SW6, L-SW5, L-SW9 and L-SW7 was in Hazard Class III with respective weight scores of 58, 50, 42, 42 and 33.
-L-SW8, MP-SW1, MP-SW4 and GP-SW10 was in Hazard Class II with respective weight scores of 67, 40, 33 and 17.

For the wetland system samples the most sensitive test was the *T. platyurus* bioassay followed by *S.polyrhiza*. The other bioassay showing effects (although slight) is the *A. fischeri* bioassay followed by *P. reticulata* and *S. subcapitata*. This trend was also seen in the dam samples collected.

The highest effect of 100% ingestion inhibition for MP-SW3 (middle of wetland) and GP-SW11 (downstream GP-WWTP2), followed by 82% ingestion inhibition for MP-SW2 (upstream wetland) and 63% ingestion inhibition for L-SW7 (dam 1 outlet) as well 51% ingestion inhibition for the L-SW9 (dam 2 outlet) was recorded for the *P. platyurus* test.

For L-SW5, L-SW6, L-SW8, MP-SW4, MP-SW1 and GP-SW10 the highest effect of 56%, 50%, 49%, 39%, 37% and 36% growth inhibition was recorded for the *S.polyrhiza* test respectively.

8.3 APPLICATION OF *IN VITRO* BIOASSAYS

8.3.1 Xenobiotic metabolism

Only four of the surface water samples, GP-SW10, GP-SW11, GP-SW12 and GP-SW13 were tested in the H4IIE-luc reporter gene assay. All of the tested samples were below the LOD and no cytotoxicity observed.

8.3.2 Hormone receptor/ Endocrine disruption

8.3.2.1 Yeast estrogen screen (YES)

The estrogenic activity of the surface water samples using the YES assay are presented in Table 8.2.

Table 8-2 Estrogenic activity of surface water samples using the yeast estrogen screen.

Sample site	EEQ (ng/L)	
	Sample collection 1	Sample collection 2
MP-SW1	<LOD	<LOD
MP-SW2	1.4 ± 0.12	<LOD
MP-SW3	<LOD	<LOD
MP-SW4	<LOD	<LOD
L-SW5	0.11 ± 0.020	<LOD
L-SW6	<LOQ	<LOD
L-SW7	<LOQ	23 ± 6.8
L-SW8	0.33 ± 0.023	<LOD
L-SW9	<LOQ	<LOD
GP-SW10	0.55 ± 0.048	0.78 ± 0.060
GP-SW11	1.4 ± 0.26	1.6 ± 0.13

GP-SW12	0.71 ± 0.11	2.6 ± 0.16
GP-SW13	No sample	0.62 ± 0.021

<LOD: Below the limit of detection of the assay. <LOQ: below the level of quantification.

8.3.2.2 T47D-KBluc reporter gene assay

The estrogenic activity of surface water samples using the T47D-KBluc assay is presented in Table 8.3.

Table 8-3 Estrogenic activity of surface water samples using the T47D-KBluc assay

	EEQ (ng/L)	
Sample site	**Sample collection 1**	**Sample collection 2**
MP-SW1	0.18 ± 0.02	0.026 ± 0.003
MP-SW2	32 ± 2.8	0.077 ± 0.023
MP-SW3	1.4 ± 0.4	0.17 ± 0.01
MP-SW4	0.71 ± 0.03	0.028 ± 0.008
L-SW5	0.65 ± 0.10	0.11 ± 0.02
L-SW6	0.56 ± 0.19	0.077 ± 0.006
L-SW7	0.57 ± 0.02	99 ± 30
L-SW8	1.6 ± 0.2	0.20 ± 0.02
L-SW9	0.93 ± 0.2	0.13 ± 0.01
GP-SW10	0.63 ± 0.06 *	1.3 ± 0.24
GP-SW11	5.5 ± 0.2 *	2.2 ± 0.2
GP-SW12	1.7 ± 0.2	1.6 ± 0.07
GP-SW13	No sample	1.4 ± 0.29

* Cytotoxicity when concentrated 10x and above

8.3.3 Androgenic activity

8.3.3.1 Yeast androgen screen (YAS)

Only GP-SW11 were tested in the YAS assay. The DHT-EQ value of this sample was 152 ± 30 ng/L. Due to time and facility constraints only the samples that tested positive in the more sensitive MDA-kb2 assay were assessed in the YAS assay.

8.3.3.2 MDA-kb2 reporter gene assay

The androgenic activity of surface water samples using the MDA-kb2 assay are shown in Table 8.4. No glucocorticoid activity were detected in any of the SW samples.

Table 8-4 Androgenic activity in WWTP samples using the MDA-Kb2 reporter gene assay.

	Androgenic activity	
Sample site	**Sampling 1**	**Sampling 2**
MP-SW1	<LOD	<LOD
MP-SW2	<LOD	<LOD
MP-SW3	<LOD	<LOD
MP-SW4	<LOD	<LOD
L-SW5	<LOD	<LOD
L-SW6	<LOD	<LOD

L-SW7	<LOD	<LOD
L-SW8	<LOD	<LOD
L-SW9	<LOD	<LOD
GP-SW10	<LOD	<LOD
GP-SW11	26 ± 4	<LOD
GP-SW12	<LOD	<LOD
GP-SW13	<LOD	<LOD

<LOD: Below the limit of detection of the assay

8.3.4 Thyroid activity

No thyroid activity was detected in the DWTP samples using the GH3.TRE-Luc assay.

8.3.5 Oxidative stress

The oxidative stress results are summarised in Table 8.5. Dichlorvos-EQ values ranged from 13.8 – 1106 µg/L.

Table 8-5 Oxidative stress response results of surface water samples using the AREc32 reporter gene assay

Sample site	Sample collection 1 (Dry Season)			Sample collection 2 (Wet Season)		
	IC_{10} ± SE (REF)	$EC_{IR1.5}$ ± SE (REF)	Dichlorvos -EQ (µg/L)	IC_{10} ± SE (REF)	$EC_{IR1.5}$ ± SE (REF)	Dichlorvos -EQ (µg/L)
MP-SW1	72.4 ± 22.4	5.7 ± 0.3	299.9	>200	113.3 ± 17.2	15
MP-SW2		4.6 ± 0.1	367.2	>200	84.3 ± 7.5	20.2
MP-SW3		1.5 ± 0.1	1106		4.9 ± 0.1	345
MP-SW4		4.5 ± 0.2	376		75.5 ± 7.7	22.6
L-SW5	>200	37.4 ± 1	45.4	>200	21.1 ± 2.2	80.4
L-SW6	188.2 ± 57	19 ± 0.9	89.7	30.7 ± 3.6	>30	-
L-SW7	66.8 ± 10.2	24 ± 1.1	70.9	14.2 ± 1.1	>14	-
L-SW8	23.8 ± 2	29.4 ± 2.3	57.9	13.8 ± 1.5	>14	-
L-SW9	52.6 ± 9.2	41.1 ± 2.6	41.4	22.8 ± 1.6	47.2 ± 7.8	36

REF = relative enrichment factor of a water sample in the bioassay (a REF of 1 means that the concentrations of the extracted chemicals are the same as in the original water sample); IC_{10} = 10% inhibition concentration; effect concentration ($EC_{IR1.5}$), and SE = Standard error.

8.4 CHEMICAL ANALYSIS

In order to identify contaminants in the surface water samples that might contribute to the effects seen in the bioassays, samples were subjected to non-targeted and targeted chemical analysis. Target chemicals could be quantified in GP-SW10 and GP-SW11, which are located upstream and downstream of GP-WWTP2. The results are summarised in Table 8.6.

Table 8-6 Non-targeted and targeted chemical analysis of SW samples

Sample code	Sample collection no	Non-targeted analysis (number of compounds detected)	Targeted analysis (list of compounds and concentrations in ng/L)
MP-SW1	1	1739	NA
MP-SW1	2	50	NA
MP-SW2	1	49	NA
MP-SW2	2	50	NA
MP-SW3	1	49	NA
MP-SW3	2	50	NA
MP-SW4	1	49	NA
MP-SW4	2	50	NA
L-SW5	1	NA	NA
L-SW5	2	NA	NA
L-SW6	1	NA	NA
L-SW6	2	NA	NA
L-SW7	1	NA	NA
L-SW7	2	NA	NA
L-SW8	1	NA	NA
L-SW8	2	NA	NA
L-SW9	1	NA	NA
L-SW9	2	NA	NA
GP-SW10	1	314	4-Aminobiphenyl (0.6), Acetaminophen (5.0), Atenolol (12.1), Atrazine (45.5), Benzophenone (8.1), Bisphenol A (25.3), Bufexamac (2.5), Caffeine (582.7), Carbamazepine (90.8), Danofloxacin (13.0), Digoxin (7.3), Efavirenz (30.5), Enrofloxacin (4.0), Estrone (14.0), Flumequine (2.0), Imidacloprid (16.6), Isoniazid (1.4), Ketoprofen (2.9), Levoflaxacin (711.2), Lidocaine (13.4), Lopinavir (4.2), Medroxyprogesterone (5.5), Metoprolol (504.6), Phenacetin (1.7), Pindolol (61.0), Primidone (30.6), Roxithromycin (0.4), Sulfadimethoxine (0.3), Sulfadoxine (52.3), Sulfamethoxazole (129.4), Sulfisoxazol (0.4), Terbuthylazine (42.3), Terbutryn (1.3), Thiabendazole (1.3), Thiacloprid (0.3), Tramadol (117.8), Trimethoprim (35.2), Valsartan (10.7)
GP-SW10	2		
GP-SW11	1	311	4-Aminobiphenyl (0.5), Acetaminophen (1.3), Atenolol (8.3), Atrazine (52.5), Benzophenone (6.9), Bisphenol A (32.5), Bufexamac (3.1), Caffeine (736.4), Carbamazepine (52.2), Danofloxacin (8.9), Digoxin (12.0), Efavirenz (36.5), Enrofloxacin (1.0), Estrone (15.8), Flumequine (0.3), Imidacloprid (40.7), Isoniazid (0.7), Ketoprofen (2.1), Levoflaxacin (170.2), Lidocaine (4.0), Lopinavir (29.6), Medroxyprogesterone (5.9), Metoprolol (293.8), Phenacetin (2.1), Pindolol (210.8),
GP-SW11	2		

			Primidone (10.4), Roxithromycin (0.2), Sulfadimethoxine (0.1), Sulfadoxine (19.5), Sulfamethoxazole (21.0), Sulfisoxazol (0.4), Terbuthylazine (31.7), Terbutryn (0.5), Thiabendazole (1.8), Thiacloprid (5.6), Tramadol (53.6), Trimethoprim (36.2), Valsartan (21.2)
GP-SW12	1	392	<LOQ
GP-SW12	2		
GP-SW13	2	388	<LOQ

NA Not analysed; <LOQ Below the level of quantification

8.5 APPLYING EBT VALUES TO BIOASSAY RESULTS FOR ASSESSING THE IMPACT OF DISCHARGES ONTO SURFACE WATER REOURCES

The percentage of SW samples below the LOQ, above the LOQ, and above the EBT in the various bioassays is graphically displayed in Figure 8.1. All SW samples were below the LOD in the H4IIE-luc and GH3.TRE-luc assays. Estrogenic activity could be quantified in 11 of the 25 SW samples using the YES assay, and all samples could be quantified in the T47D-KBluc assay. In the YES assay, 36% of the SW samples were above the EBT value and 64% of the samples were above the EBT in the T47D-KBluc assay. Androgenic activity was detected in one SW sample (GP-SW11) using the MDA-kb2 assay. The same sample also showed androgenic activity in the YAS assay. For both assays, the androgenic activity was above the EBT value. In the AREc32 assay, 28% samples were above the EBT value. In the *in vivo* bioassays, 36% of samples were classified as class II (slight sub-lethal hazard), 45% class III (Lethal/sub-lethal hazard) and 18% class IV (high lethal/sub-lethal hazard) hazard rating. Target chemical analyses were completed for four of the SW samples.

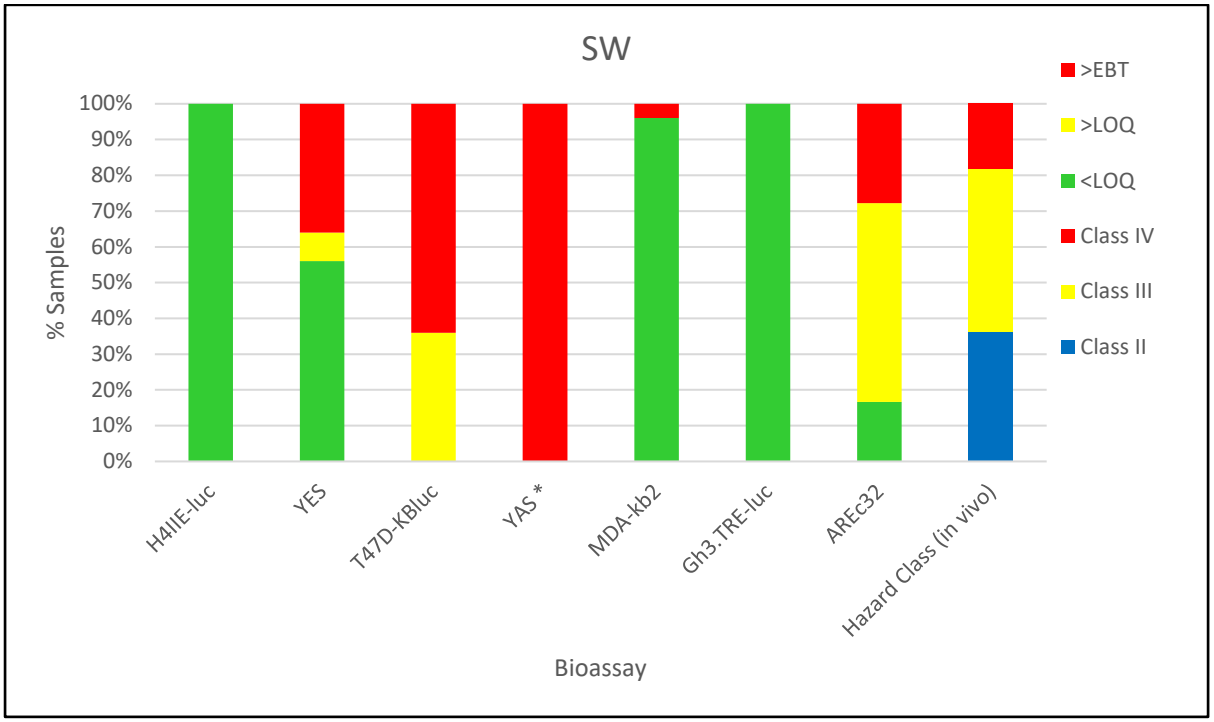


Figure 8-1: Summary of the results of SW samples assessed in the bioassays, expressed as the percentage of samples below LOQ, above LOQ and above EBT value. * Only samples that tested positive in the MDA-kb2 assay were analysed in the YAS assay

8.6 SUMMARY

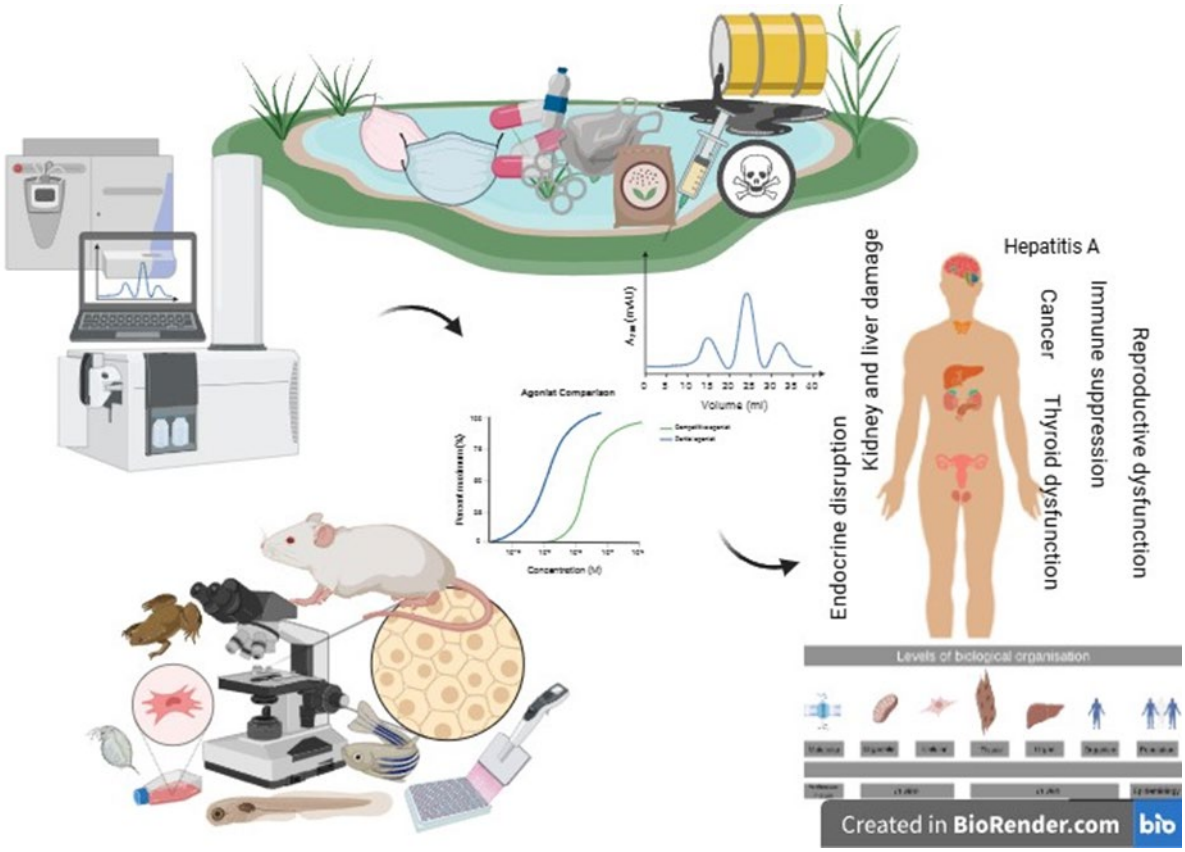
This study demonstrated the value of applying *in vivo* and *in vitro* bioassays to assess the quality of surface water. Estrogenic-, androgenic- and oxidative stress activity were detected in some of the SW samples. The EBT values were exceeded in 36 % of the YES, 64% of the T47D-KBluc, 4% of the MDA-kb2 and 28% of the AREc32 samples. In the *in vivo* bioassays, 36% of samples were classified as class II (slight sub-lethal hazard), 45% class III (Lethal/sub-lethal hazard) and 18% class IV (high lethal/sub-lethal hazard) hazard rating. Target chemical analyses were completed for four of the SW samples and could be quantified for GP-SW10 and GP-SW11. These two sites are located upstream and downstream of GP-WWTP2. Estrogenic activity was above the EBT value for the upstream sample. Estrone was detected in this sample at 14 ng/L, which may have contributed to the estrogenic activity in this sample. Estrogenic and androgenic activity above the EBT value were detected in the downstream sample. Target chemicals that were quantified at this site and might have contributed to the estrogenic and androgenic activities, include bisphenol A (32.5 ng/L), estrone (15.8 ng/L) and medroxyprogesterone (5.9 ng/L). The results highlight the need to include *in vivo* and *in vitro* bioassays in water quality assessment to complement chemical analysis, in order to get a more comprehensive overview of surface water quality.

NOTE:

A factsheet on the use of *in vivo* and *in vitro* bioassays for effect-based monitoring of surface water quality is available.

CHAPTER 9: FACTSHEETS

9.1 FACTSHEET: THE USE OF *IN VIVO* AND *IN VITRO* BIOASSAYS FOR EFFECT-BASED MONITORING OF DRINKING WATER QUALITY AND TREATMENT TECHNOLOGIES



9.1.1 Why use bioassays for water quality monitoring?

More than 87 000 compounds exist that may end up in natural water sources. It is almost an impossible task to quantify each of them due to: cost, lack of highly skilled personnel, availability of infrastructure, and reference standards. Even if it were viable to do chemical analysis for each of these, the biological effects the mixture of compounds would elicit on biota and human health are still unknown. This gap is addressed by using biological entities such as fish, daphnids, algae, bacteria, and mammalian cells. These entities are exposed to a mixture of compounds found in the sample, and the total biological response is assessed.

Bioassays support chemical analysis because they respond to unknown compounds present in the water sample and also show a collective response to a complex mixture. Bioassays designed to detect specific modes of action are referred to as effect-based bioassays (EBAs) and when used in water

quality monitoring the process is known as effect-based monitoring (EBM). Results from the EBM can be used as input for risk-based monitoring programs (Brack et al., 2019).

9.1.2 *In vivo* and *in vitro* bioassays applicable to drinking water

Battery 3 bioassays are recommended for drinking water, consisting of high sensitivity *in vitro* bioassays, oxidative stress, AhR and mutagen or genotoxicity assays. The high sensitivity bioassays are mammalian-based assays, as yeast-based assays are not sensitive enough for drinking water. Although the low complexity *in vivo* assays were previously not indicated for drinking water, a recent study showed the applicability of these assays for drinking water (WRC 2020/2021-00348). Therefore, it is recommended to also include the low complexity *in vivo* assays for drinking water quality monitoring. Recommended in vivo and *in vitro* bioassays for drinking water that are currently available in South Africa include the following:

9.1.2.1 In vivo assays:

Allivibrio fischeri bioluminescence test (ISO/SANS 11348-3: 2013)
Pseudokirchneriella subcapitata growth inhibition test (ISO/SANS 8692: 2015)
Spirodela polyrhiza growth inhibition test (ISO/SANS 20227: 2017)
Daphnia magna/pulex acute toxicity test (immobilisation test) (ISO/SANS 6341: 2015)
Thamnocephalus platyurusacute toxicity test (ISO/SANS 14380: 2011)
Poecilia reticulata acute toxicity test (ISO/SANS 7346-1: 2013)

9.1.2.2 In vitro assays:

Xenobiotic metabolism: H4IIE-luc reporter gene assay
Estrogenic activity: T47D-KBluc assay (estrogenic and anti-estrogenic activity)
Androgenic activity: MDA-Kb2 assay (androgenic and anti-androgenic activity)
Oxidative stress: AREc32 assay

NOTE:
Factsheets for the *in vivo* and *in vitro* assays can be found in the Annexure section of the WRC 2020/2021-00165, bioanalytical toolbox report.

9.1.3 Information that will be obtained from the bioassays

The bioassays can be used to assess product quality, to assess treatment efficacy or to understand treatment processes.

The *in vivo* tests are all toxicity tests, developed to measure the influence of toxicants in the water sample on the viability of whole organisms. The organisms are representative of different trophic levels in the food web: bacteria (*Allivibrio fisheri, Salmonella typhimurium*), photosynthesising plants are represented by the algae *Pseudokirchneriella subcapitata*, lower invertebrates by *Daphnia magna/pulex* and vertebrates by fish, usually guppies (*Poecilia reticulata*) or zebra fish (*Brachydanio rerio*) and frogs (*Xenopus*). The *Heterocypris incongruens* represent invertebrates in the sediment specifically.

The *in vitro* bioassays indicate whether toxicants interfere with normal steroid hormone processes; whether xenobiotic metabolism is taking place or whether adaptive stress responses such as oxidative stress is being triggered.

9.1.4 Sampling of drinking water

For drinking water, grab samples are sufficient. The purpose of the sampling will determine what samples should be collected.

- To evaluate the product of a drinking water treatment plant (DWTP), the only sample required would be the product water and an appropriate blank and control sample.
- In order to evaluate treatment process efficiency, the source water and product water (including the blank and control) will be required.
- To understand treatment processes, samples from every step of the treatment process should be collected in addition to the source and product water.

For *in vivo* bioassays, samples are collected in clean plastic or glass bottles that were rinsed with source water before being filled with sample. No air space should be left between the contents and the lid, in order to minimise the loss of toxicity due to volatilization of toxic constituents.

For *in vitro* bioassays, samples should be collected in clean glass bottles with polytetrafluoroethylene (PTFE) lined caps, or alternatively in uncoated aluminium or stainless steel bottles. Before collection, bottles and caps should be rinsed with small quantities of an appropriate organic solvent (acetone or hexane for aryl hydrocarbon receptor ligands and methanol for steroid hormone receptor ligands). Samples should be protected from photo-degradation or stored in dark containers.

All samples should be transported and stored at 0–6°C, and analysed or extracted as soon as possible (within 36 h for *in vivo* and 48h for *in vitro* assays). Samples for *in vitro* bioassays may be frozen if they cannot be extracted immediately.

NOTE:
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, for more detailed sample collection procedures.

9.1.5 Pre-treatment and processing of drinking water samples for *in vitro* bioassays

After collection, the pH of the water samples is adjusted to pH 3 to reduce degradation of contaminants. Chlorinated samples should be treated with sodium thiosulphate or ascorbic acid to quench the residual free chlorine. Appropriate extraction methods should be used for the different targeted pollutants. For many of the water extraction methods, solid phase extraction (SPE) is typically used, using SPE sorbents such as Oasis HLB (Waters), Chromabond HX-R (Macherey-Nagel) and Strata-X (Phenomenex).

NOTE:
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, and WRC 2020/2021-00348 for more detailed sample pre-treatment and processing procedures.

9.1.6 Interpretation of *in vivo* bioassay results

Lethal or sub-lethal toxicity testing is applied by exposing biota to water sources in order to determine the potential risk of such waters to the biota/biological integrity of the receiving water bodies. A risk category (hazard class) is determined based on the percentage of mortalities (lethal) or inhibition (sub-lethal) of the exposed biota. It is important to note that the hazard class is based on the standardised battery of selected test biota and therefore represents the risk/hazard towards similar biota in the receiving aquatic environment. The toxicity hazard is therefore in terms of the aquatic biotic integrity and does in no way represent toxicology towards humans or other mammals.

A risk/hazard category is determined by using a hazard classification system developed by Persoone et al. (2003) whereby one can classify sites using the toxicity data of the non-diluted samples. The percentage effect (PE) of toxicity (mortalities, growth inhibition, luminescence inhibition, ingestion inhibition) is used to rank the sample into one of five classes (Table 9-1) based on the highest toxic response obtained in at least one of the tests applied.

Table 9-1: Hazard classification system for natural waters/screening samples (Persoone et al., 2003)

Class	Symbol	Hazard rating	PE	Percentage effect
I	☺	No lethal/sub-lethal hazard	≤10/20%	None of the tests show a toxic effect (i.e. an effect value that is significantly higher than that noted in the controls)
II	☹	Slight lethal/sub-lethal hazard	10/20%≤PE<50%	A statistically significant (P<0.05) PE is reached in at least one test, but the effect level is below 50%
III	☠	Lethal/sub-lethal hazard	50%≤PE<100%	The 50% effect level is reached or exceeded in at least one test but the effect level is below 100%
IV	☠☠	High lethal/sub-lethal hazard	PE 100% in at least one test	The 100% effect is reached or exceeded in at least one test
V	☠☠☠	Very high lethal/ sub-lethal hazard	PE 100% in all tests	The 100% effect is exceeded in all the tests

Each sample is furthermore weighted (Table 2) according to its relative toxicity level (out of 100%). Higher values indicate that more of the individual tests indicated toxicity within a specific class.

Table 9-2: Weight score allocation for each test type (Persoone et al., 2003)

Test score	Category
0	No significant toxicity effect
1	Significant toxicity effect < PE50
2	Toxicity effect >PE50 but <PE100
3	The PE100 is reached
Class weight score calculated as follows: Class weight score = (∑ all test scores)/n) where n is the number of tests performed Class weight score % = (class score) / (maximum class weight score) x 100	

9.1.7 Interpretation of *in vitro* bioassay results

In vitro bioassay results are expressed as bioequivalent (BEQ) concentrations. For all types of monitoring, the observed effect should be compared to an effect-based trigger (EBT) value. Available EBTs for drinking water are provided on a factsheet from WRC 2020/2021-00165 and are also available in the WRC 2020/2021-00348 report. Figure 9-1 shows a flow diagram of the process to assess the outcome of the bioassay (BEQ) versus the EBT of a water sample.

- If the BEQ of the sample is lower than the EBT-BEQ then no further action is required.
- If the measured BEQ value exceeds the EBT-BEQ, the first step is to check the bioassay quality control (QC) and collect another water sample from the same site and re-test.
- If the BEQ of the second sample is below the EBT-BEQ, then no further action is required.

- If the second test confirms the initial positive result and both samples report a BEQ > EBT-BEQ, then further action is needed. The magnitude of the response should depend on the magnitude of the exceedance and regulatory advice.

NOTE:
Available EBTs for drinking water are provided on a factsheet from WRC 2020/2021-00165 and is also available in the WRC 2020/2021-00348 report.
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, and WRC 2020/2021-00348 for more details on the interpretation of *in vitro* and *in vivo* bioassay results and actions that need to be taken when EBT-BEQ values are exceeded.

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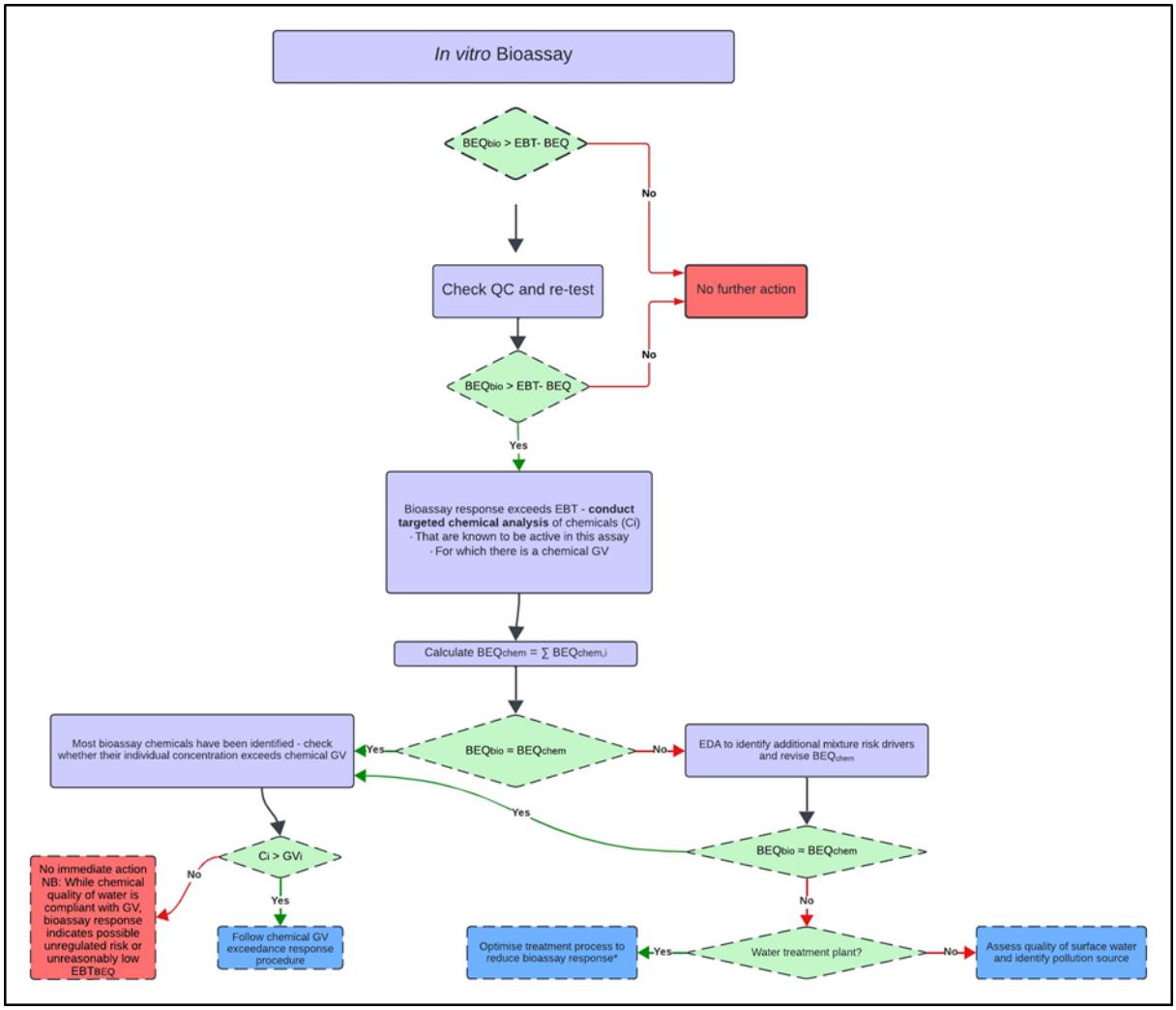

Figure 9-1: Flow diagram to represent the process of assessing the outcome of the bioassay (BEQ) versus the EBT of a water sample. (Adapted from Escher et al, 2021).

9.1.8 References

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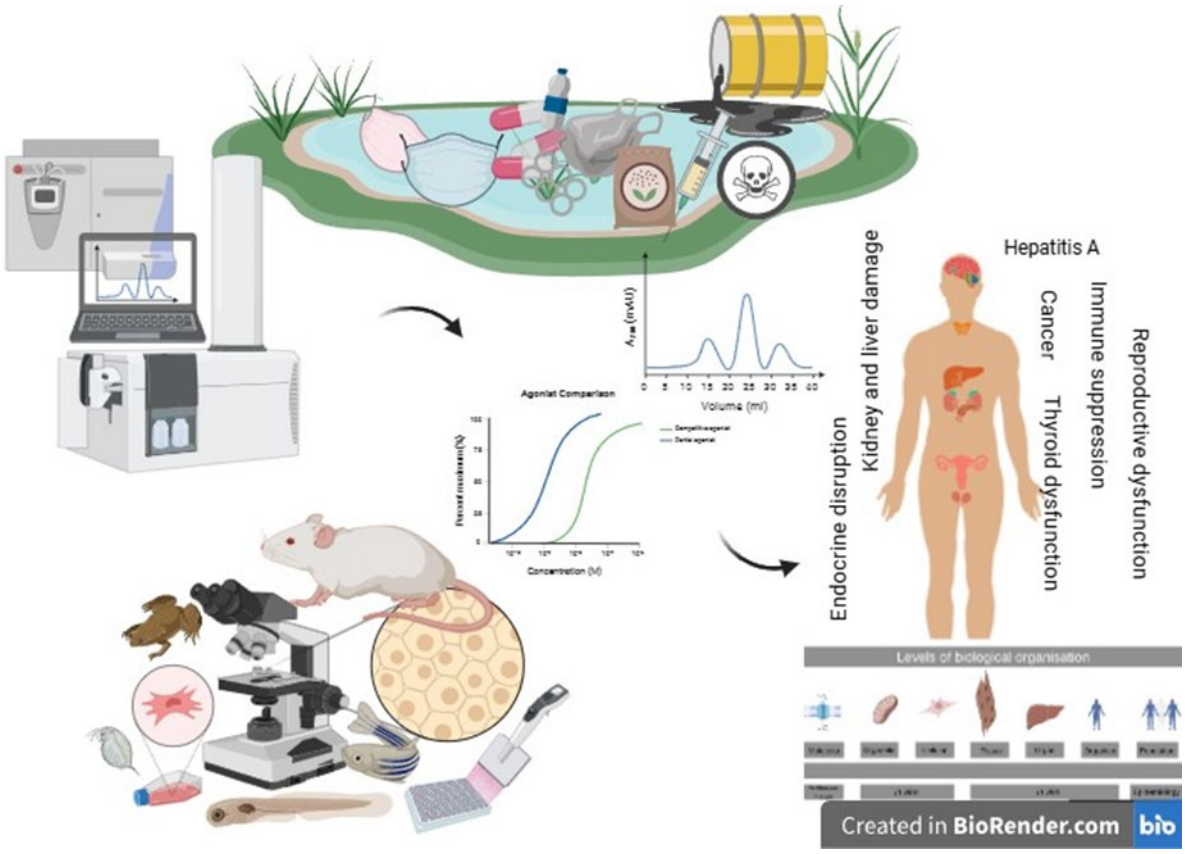
9.2 FACTSHEET: THE USE OF *IN VIVO* AND *IN VITRO* BIOASSAYS FOR EFFECT-BASED MONITORING OF GROUNDWATER QUALITY



9.2.1 Why use bioassays for water quality monitoring?

More than 87 000 compounds exist that may end up in natural water sources. It is almost an impossible task to quantify each of them due to: cost, lack of highly skilled personnel, availability of infrastructure and reference standards. Even if it was viable to do chemical analysis for each of these, the biological effects the mixture of compounds would elicit on biota and human health is still unknown. This gap is addressed by using biological entities such as fish, daphnids, algae, bacteria and mammalian cells. These entities are exposed to a mixture of compounds found in the sample and the total biological response is assessed.

Bioassays support chemical analysis because they respond to unknown compounds present in the water sample and also show a collective response to a complex mixture. Bioassays designed to detect specific modes of action are referred to as effect-based bioassays (EBAs) and when used in water quality monitoring the process is known as effect-based monitoring (EBM). Results from the EBM can be used as input for risk-based monitoring programs (Brack et al., 2019).

9.2.2 *In vivo* and *in vitro* bioassays applicable to groundwater

Battery 3 bioassays are recommended for groundwater that is used for drinking water. The battery of assays consists of low complexity *in vivo* bioassays, high sensitivity *in vitro* bioassays, oxidative stress, AhR and mutagen or genotoxicity assays. Recommended *in vivo* and *in vitro* bioassays for groundwater that are currently available in South Africa include the following:

9.2.2.1 In vivo assays

Allivibrio fischeri bioluminescence test (ISO/SANS 11348-3: 2013)
Pseudokirchneriella subcapitata growth inhibition test (ISO/SANS 8692: 2015)
Spirodela polyrhiza growth inhibition test (ISO/SANS 20227: 2017)
Daphnia magna/pulex acute toxicity test (immobilisation test) (ISO/SANS 6341: 2015)
Thamnocephalus platyurusacute toxicity test (ISO/SANS 14380: 2011)
Poecilia reticulata acute toxicity test (ISO/SANS 7346-1: 2013)

9.2.2.2 In vitro assays

Xenobiotic metabolism: H4IIE-luc reporter gene assay
Estrogenic activity: YES assay T47D-KBluc assay (estrogenic and anti-estrogenic activity)
Androgenic activity: YAS assay MDA-Kb2 assay (androgenic and anti-androgenic activity)
Oxidative stress: AREc32 assay

NOTE:
Factsheets for the *in vivo* and *in vitro* assays can be found in the Annexure section of the WRC 2020/2021-00165, bioanalytical toolbox report.

9.2.3 Information that will be obtained from the bioassays

The *in vivo* tests are all toxicity tests, developed to measure the influence of toxicants in the water sample on the viability of whole organisms. The organisms are representative of different trophic levels in the food web: bacteria (*Allivibrio fisheri, Salmonella typhimurium*), photosynthesising plants are represented by the algae *Pseudokirchneriella subcapitata*, lower invertebrates by *Daphnia magna/pulex* and vertebrates by fish, usually guppies (*Poecilia reticulata*) or zebra fish (*Brachydanio rerio*) and frogs (*Xenopus*). The *Heterocypris incongruens* represent invertebrates in the sediment specifically.

The *in vitro* bioassays indicate whether toxicants interfere with normal steroid hormone processes; whether xenobiotic metabolism is taking place or whether adaptive stress responses such as oxidative stress is being triggered.

9.2.4 Sampling of groundwater

For *in vivo* bioassays, samples are collected in clean plastic or glass bottles, that were rinsed with source water before being filled with the sample. No air space should be left between the contents and the lid, in order to minimise the loss of toxicity due to volatilization of toxic constituents.

For *in vitro* bioassays, samples should be collected in clean glass bottles with polytetrafluoroethylene (PTFE) lined caps, or alternatively in uncoated aluminium, or stainless steel bottles. Before collection, bottles and caps should be rinsed with small quantities of an appropriate organic solvent (acetone or hexane for aryl hydrocarbon receptor ligands and methanol for steroid hormone receptor ligands). Samples should be protected from photo-degradation or stored in dark containers.

All samples should be transported and stored at 0–6°C, and analysed or extracted as soon as possible (within 36 h for *in vivo* and 48h for *in vitro* assays). Samples for *in vitro* bioassays may be frozen if they cannot be extracted immediately.

NOTE:
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, for more detailed sample collection procedures.

9.2.5 Pre-treatment and processing of groundwater samples for *in vitro* bioassays:

After collection, the pH of water samples is adjusted to pH 3 to reduce degradation of contaminants. Samples should be filtered using glass fibre filters prior to extraction. Appropriate extraction methods should be used for the different targeted pollutants. For many of the water extraction methods solid phase extraction (SPE) is typically used, using SPE sorbents such as Oasis HLB (Waters), Chromabond HX-R (Macherey-Nagel) and Strata-X (Phenomenex).

NOTE:
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, and WRC 2020/2021-00348 for more detailed sample pre-treatment and processing procedures.

9.2.6 Interpretation of *in vivo* bioassay results

Lethal or sub-lethal toxicity testing is applied by exposing biota to water sources in order to determine the potential risk of such waters to the biota/biological integrity of the receiving water bodies. A risk category (hazard class) is determined, based on the percentage of mortalities (lethal) or inhibition (sub-

lethal) of the exposed biota. It is important to note that the hazard class is based on the standardised battery of selected test biota and therefore represents the risk/hazard towards similar biota in the receiving aquatic environment. The toxicity hazard is therefore in terms of the aquatic biotic integrity and does in no way represent toxicology towards humans or other mammals.

A risk/hazard category is determined by using a hazard classification system developed by Persoone et al. (2003) whereby one can classify sites using the toxicity data of the non-diluted samples. The percentage effect (PE) of toxicity (mortalities, growth inhibition, luminescence inhibition, ingestion inhibition) is used to rank the sample into one of five classes (Table 9-2) based on the highest toxic response obtained in at least one of the tests applied.

Figure 9-2: Hazard classification system for natural waters/screening samples (Persoone et al., 2003)

Class	Symbol	Hazard rating	PE	Percentage effect
I	☺	No lethal/sub-lethal hazard	≤10/20%	None of the tests show a toxic effect (i.e. an effect value that is significantly higher than that noted in the controls)
II	☹	Slight lethal/sub-lethal hazard	10/20%≤PE<50%	A statistically significant (P<0.05) PE is reached in at least one test, but the effect level is below 50%
III	☠	Lethal/sub-lethal hazard	50%≤PE<100%	The 50% effect level is reached or exceeded in at least one test but the effect level is below 100%
IV	☠☠	High lethal/sub-lethal hazard	PE 100% in at least one test	The 100% effect is reached or exceeded in at least one test
V	☠☠☠	Very high lethal/ sub-lethal hazard	PE 100% in all tests	The 100% effect is exceeded in all the tests

Each sample is furthermore weighted (Table 9-3) according to its relative toxicity level (out of 100%). Higher values indicate that more of the individual tests indicated toxicity within a specific class.

Figure 9-3: Weight score allocation for each test type (Persoone et al., 2003)

Test score	Category
0	No significant toxicity effect
1	Significant toxicity effect < PE50
2	Toxicity effect >PE50 but <PE100
3	The PE100 is reached
Class weight score calculated as follows: Class weight score = (∑ all test scores)/n) where n is the number of tests performed Class weight score % = (class score) / (maximum class weight score) x 100	

9.2.7 Interpretation of *in vitro* bioassay results

In vitro bioassay results are expressed as bioequivalent (BEQ) concentrations. For all types of monitoring, the observed effect should be compared to an effect-based trigger (EBT) value. Groundwater may be compared to surface water EBT values. Available EBTs for surface water are

provided on a factsheet from WRC 2020/2021-00165 and is also available in the WRC 2020/2021-00348 report. Figure 1 shows a flow diagram of the process to assess the outcome of the bioassay (BEQ) versus the EBT of a water sample.

- If the BEQ of the sample is lower than the EBT-BEQ then no further action is required.
- If the measured BEQ_{bio} value exceeds the EBT-BEQ, the first step is to check the bioassay quality control (QC) and collect another water sample from the same site and re-test.
- If the BEQ_{bio} of the second sample is below the EBT-BEQ, then no further action is required.
- If the second test confirms the initial positive result and both samples report a BEQ > EBT-BEQ, then further action is needed. The magnitude of the response should depend on the magnitude of the exceedance and regulatory advice.

NOTE:
Available EBTs are provided on a factsheet from WRC 2020/2021-00165 and is also available in the WRC 2020/2021-00348 report.
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, and WRC 2020/2021-00348 for more details on the interpretation of *in vitro* and *in vivo* bioassay results and actions that need to be taken when EBT-BEQ values are exceeded.

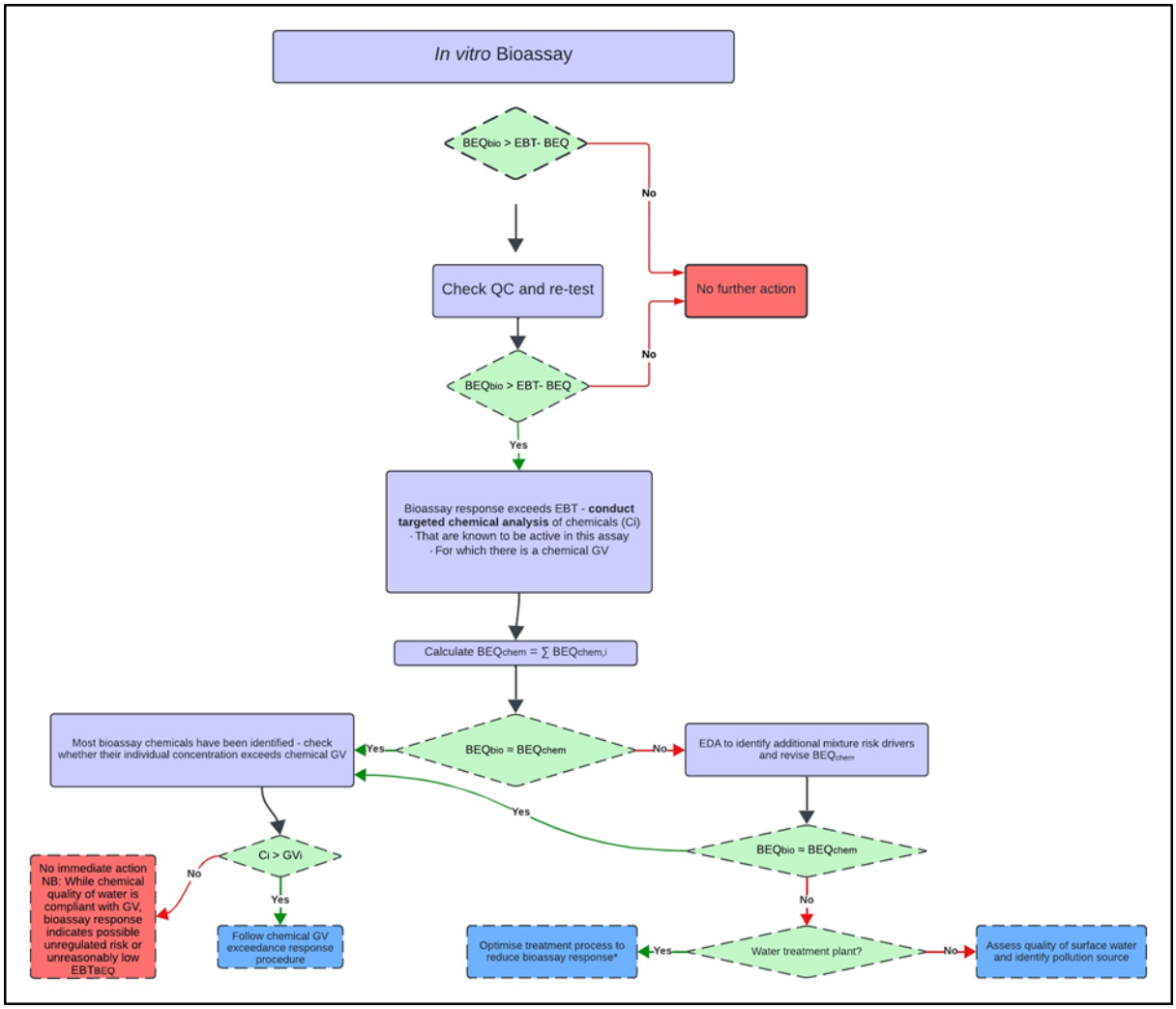


Figure 9-4: Flow diagram to represent the process of assessing the outcome of the bioassay (BEQ) versus the EBT of a water sample. (Adapted from Escher et al, 2021).

9.2.8 References

BRACK, W., AÏSSA, S.A., BACKHAUS, T., DULIO, V., ESCHER, B.I., FAUST, M., HILSCHEROVA K.,HOLLENDER, J., HOLLERT, H., MÜLLER, C., MUNTHE, J., POSTHUMA, L., SEILER, T.B., SLOBODNIK J., TEODOROVIC, I., TINDALL, A.J., DE ARAGÃO UMBUSEIRO, G., SHANG, X., and ALTENBURGER, R. 2019. Effect-based methods are key: The European collaborative project SOLUTIONS recommends integrating effect-based methods for diagnosis and monitoring of water quality. *Environmental Sciences Europe*, 31(1):4–9. DOI:10.1186/s12302-019-0192-2

ESCHER, B., NEALE, P. & LEUSCH, F. 2021. Bioanalytical Tools in Water Quality Assessment, IWA Publishing.

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ISO 2012 (INTERNATIONAL STANDARD ORGANISATION 14371 2012. Water quality — Determination of fresh water sediment toxicity to *Heterocypris incongruens* (*Crustacea, Ostracoda*).

PERSOONE, G., MARSALEK, B., BLINOVA, I., TÖRÖKNE, A., ZARINA, D., MANUSADZIANAS, L., NALECZ-JAWECKI, G., TOFAN, L., STEPANOVA, N., TOTHOVA, L. & KOLAR, B. 2003. A practical and user-friendly toxicity classification system with microbiotests for natural waters and wastewaters. *Environ Toxicol,* 18**,** 395-402.

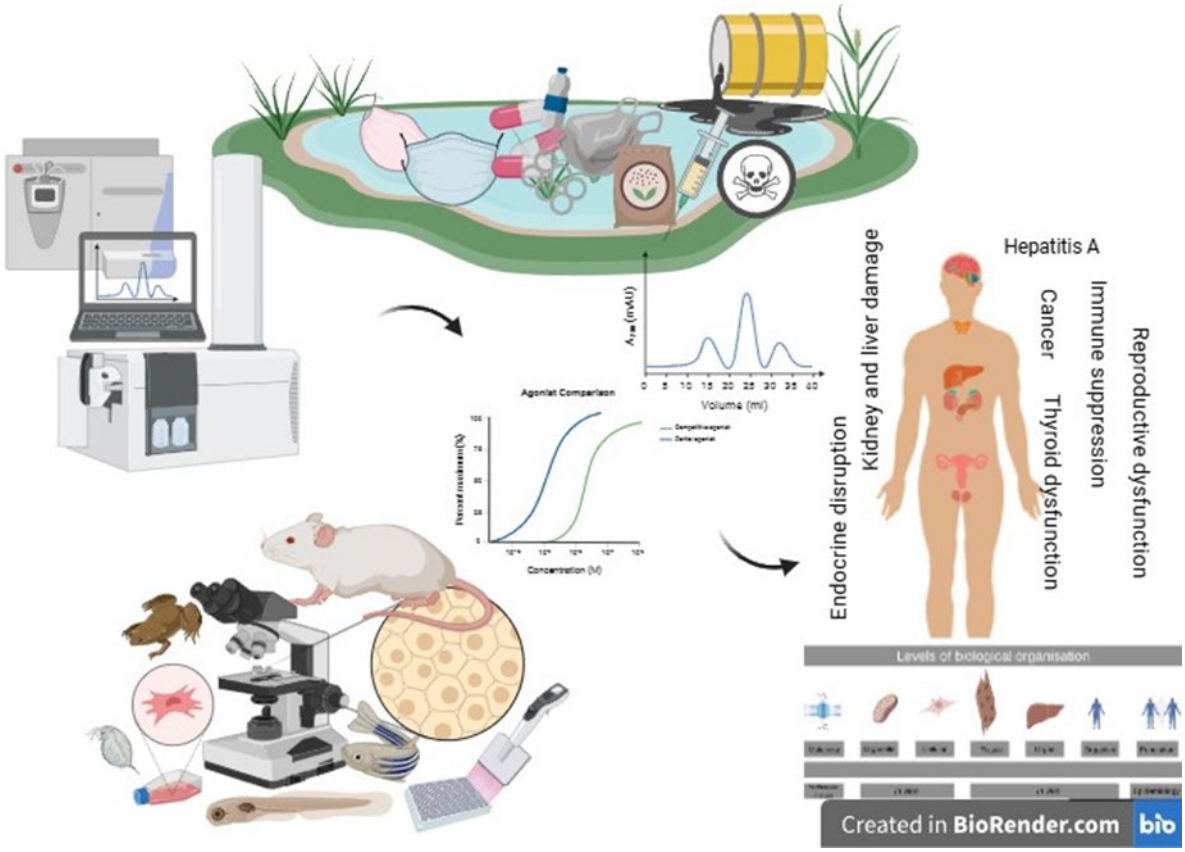
SANS 2013a (SOUTH AFRICAN NATIONAL STANDARD) 11348-3: 2013. “Water quality — Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test). Part 3: Method using freeze-dried bacteria

SANS 2013b (SOUTH AFRICAN NATIONAL STANDARD) 7346-1:2013. “Water quality — Determination of the acute lethal toxicity of substances to a freshwater fish *Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyrinidae) Part 1: Static method – also applicable to *Poecilia reticulata* (Teleostei, Poeciliidae)

SANS 2015a (SOUTH AFRICAN NATIONAL STANDARD) 6341:2015. “Water quality — Determination of the inhibition of the mobility of *Daphnia magna* Straus (*Cladocera, Crustacea*) — Acute toxicity test.

SANS 2015b (SOUTH AFRICAN NATIONAL STANDARD) 8692: 2015. “Water quality — Fresh water algal growth inhibition test with unicellular green algae

9.3 FACTSHEET: THE USE OF *IN VIVO* AND *IN VITRO* BIOASSAYS FOR EFFECT-BASED MONITORING OF SURFACE WATER QUALITY



9.3.1 Why use bioassays for water quality monitoring?

More than 87 000 compounds exist that may end up in natural water sources. It is almost an impossible task to quantify each of them due to: cost, lack of highly skilled personnel, availability of infrastructure, and reference standards. Even if it were viable to do chemical analysis for each of these, the biological effects the mixture of compounds would elicit on biota and human health is still unknown. This gap is addressed by using biological entities such as fish, daphnids, algae, bacteria, and mammalian cells. These entities are exposed to a mixture of compounds found in the sample, and the total biological response is assessed.

Bioassays support chemical analysis because they respond to unknown compounds present in the water sample and also show a collective response to a complex mixture. Bioassays designed to detect specific modes of action are referred to as effect-based bioassays (EBAs), and when used in water quality monitoring, the process is known as effect-based monitoring (EBM). Results from the EBM can be used as input for risk-based monitoring programs (Brack et al., 2019).

9.3.2 *In vivo* and *in vitro* bioassays applicable to surface water:

Battery 2 bioassays are recommended for surface water, consisting of high sensitivity *in vitro* bioassays (mammalian based bioassays), oxidative stress, AhR and *in vivo* bioassays. If the surface water is used for drinking water, mutagen or genotoxicity assays should also be included.
Recommended *in vivo* and *in vitro* bioassays for surface water that are currently available in South Africa include the following:

9.3.2.1 In vivo assays:

Allivibrio fischeri bioluminescence test (ISO/SANS 11348-3: 2013)
Pseudokirchneriella subcapitata growth inhibition test (ISO/SANS 8692: 2015)
Spirodela polyrhiza growth inhibition test (ISO/SANS 20227: 2017)
Daphnia magna/pulex acute toxicity test (immobilisation test) (ISO/SANS 6341: 2015)
Thamnocephalus platyurusacute toxicity test (ISO/SANS 14380: 2011)
Poecilia reticulata acute toxicity test (ISO/SANS 7346-1: 2013)

9.3.2.2 In vitro assays:

Xenobiotic metabolism: H4IIE-luc reporter gene assay
Estrogenic activity: T47D-KBluc assay (estrogenic and anti-estrogenic activity)
Androgenic activity: MDA-Kb2 assay (androgenic and anti-androgenic activity)
Oxidative stress: AREc32 assay

NOTE:
Factsheets for the *in vivo* and *in vitro* assays can be found in the Annexure section of the WRC 2020/2021-00165, bioanalytical toolbox report.

9.3.3 Information that will be obtained from the bioassays:

The *in vivo* tests are all toxicity tests, developed to measure the influence of toxicants in the water sample on the viability of whole organisms. The organisms are representative of different trophic levels in the food web: bacteria (*Allivibrio fisheri, Salmonella typhimurium*), photosynthesising plants are represented by the algae *Pseudokirchneriella subcapitata*, lower invertebrates by *Daphnia magna/pulex* and vertebrates by fish, usually guppies (*Poecilia reticulata*) or zebra fish (*Brachydanio rerio*) and frogs (*Xenopus*). The *Heterocypris incongruens* represents invertebrates in the sediment specifically.

The *in vitro* bioassays indicate whether toxicants interfere with normal steroid hormone processes; whether xenobiotic metabolism is taking place, or whether adaptive stress responses, such as oxidative stress, are being triggered.

9.3.4 Sampling of surface water:

For *in vivo* bioassays, samples are collected in clean plastic or glass bottles that were rinsed with source water before being filled with sample. No air space should be left between the contents and the lid, in order to minimize the loss of toxicity due to volatilization of toxic constituents.

For *in vitro* bioassays, samples should be collected in clean glass bottles with polytetrafluoroethylene (PTFE) lined caps, or alternatively in uncoated aluminium, or stainless steel bottles. Prior to collection, bottles and caps should be rinsed with small quantities of an appropriate organic solvent (acetone or hexane for aryl hydrocarbon receptor ligands and methanol for steroid hormone receptor ligands). Samples should be protected from photo-degradation or stored in dark containers.

All samples should be transported and stored at 0–6°C, and analysed or extracted as soon as possible (within 36 h for *in vivo* and 48h for *in vitro* assays). Samples for *in vitro* bioassays may be frozen if they cannot be extracted immediately.

NOTE:
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, for more detailed sample collection procedures.

9.3.5 Pre-treatment and processing of surface water samples for *in vitro* bioassays:

After collection, the pH of water samples is adjusted to pH 3 to reduce degradation of contaminants. Samples should be filtered using glass fibre filters prior to extraction. Appropriate extraction methods should be used for the different targeted pollutants. For many of the water extraction methods solid phase extraction (SPE) is typically used, using SPE sorbents such as Oasis HLB (Waters), Chromabond HX-R (Macherey-Nagel) and Strata-X (Phenomenex).

NOTE:
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, and WRC 2020/2021-00348 for more detailed sample pre-treatment and processing procedures.

9.3.6 Interpretation of *in vivo* bioassay results

Lethal or sub-lethal toxicity testing is applied by exposing biota to water sources in order to determine the potential risk of such waters to the biota/biological integrity of the receiving water bodies. A risk category (hazard class) is determined, based on the percentage of mortalities (lethal) or inhibition (sub-lethal) of the exposed biota. It is important to note that the hazard class is based on the standardised

battery of selected test biota and therefore represents the risk/hazard towards similar biota in the receiving aquatic environment. The toxicity hazard is therefore in terms of the aquatic biotic integrity and does in no way represent toxicology towards humans or other mammals.

A risk/hazard category is determined by using a hazard classification system developed by Persoone et al. (2003) whereby one can classify sites using the toxicity data of the non-diluted samples. The percentage effect (PE) of toxicity (mortalities, growth inhibition, luminescence inhibition, ingestion inhibition) is used to rank the sample into one of five classes (Table 9-3) based on the highest toxic response obtained in at least one of the tests applied.

Table 9-3: Hazard classification system for natural waters/screening samples (Persoone et al., 2003)

Class	Symbol	Hazard rating	PE	Percentage effect
I	☺	No lethal/sub-lethal hazard	≤10/20%	None of the tests show a toxic effect (i.e. an effect value that is significantly higher than that noted in the controls)
II	☹	Slight lethal/sub-lethal hazard	10/20%≤PE<50%	A statistically significant (P<0.05) PE is reached in at least one test, but the effect level is below 50%
III	☠	Lethal/sub-lethal hazard	50%≤PE<100%	The 50% effect level is reached or exceeded in at least one test but the effect level is below 100%
IV	☠☠	High lethal/sub-lethal hazard	PE 100% in at least one test	The 100% effect is reached or exceeded in at least one test
V	☠☠☠	Very high lethal/ sub-lethal hazard	PE 100% in all tests	The 100% effect is exceeded in all the tests

Each sample is furthermore weighted (Table 9-4) according to its relative toxicity level (out of 100%). Higher values indicate that more of the individual tests indicated toxicity within a specific class.

Table 9-4: Weight score allocation for each test type (Persoone et al., 2003)

Test score	Category
0	No significant toxicity effect
1	Significant toxicity effect < PE50
2	Toxicity effect >PE50 but <PE100
3	The PE100 is reached
Class weight score calculated as follows: Class weight score = (∑ all test scores)/n) where n is the number of tests performed Class weight score % = (class score) / (maximum class weight score) x 100	

9.3.7 Interpretation of *in vitro* bioassay results

In vitro bioassay results are expressed as bioequivalent (BEQ) concentrations. For all types of monitoring, the observed effect should be compared to an effect-based trigger (EBT) value. Available EBTs for surface water are provided on a factsheet from WRC 2020/2021-00165 and is also available

in the WRC 2020/2021-00348 report. Figure 9-5 shows a flow diagram of the process to assess the outcome of the bioassay (BEQ) versus the EBT of a water sample.

- If the BEQ of the sample is lower than the EBT-BEQ then no further action is required.
- If the measured BEQ value exceeds the EBT-BEQ, the first step is to check the bioassay quality control (QC) and collect another water sample from the same site and re-test.
- If the BEQ of the second sample is below the EBT-BEQ, then no further action is required.
- If the second test confirms the initial positive result and both samples report a BEQ > EBT-BEQ, then further action is needed. The magnitude of the response should depend on the magnitude of the exceedance and regulatory advice.

NOTE:
Available EBTs for surface water are provided on a factsheet from WRC 2020/2021-00165 and is also available in the WRC 2020/2021-00348 report.
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, and WRC 2020/2021-00348 for more details on the interpretation of *in vitro* and *in vivo* bioassay results and actions that need to be taken when EBT-BEQ values are exceeded.

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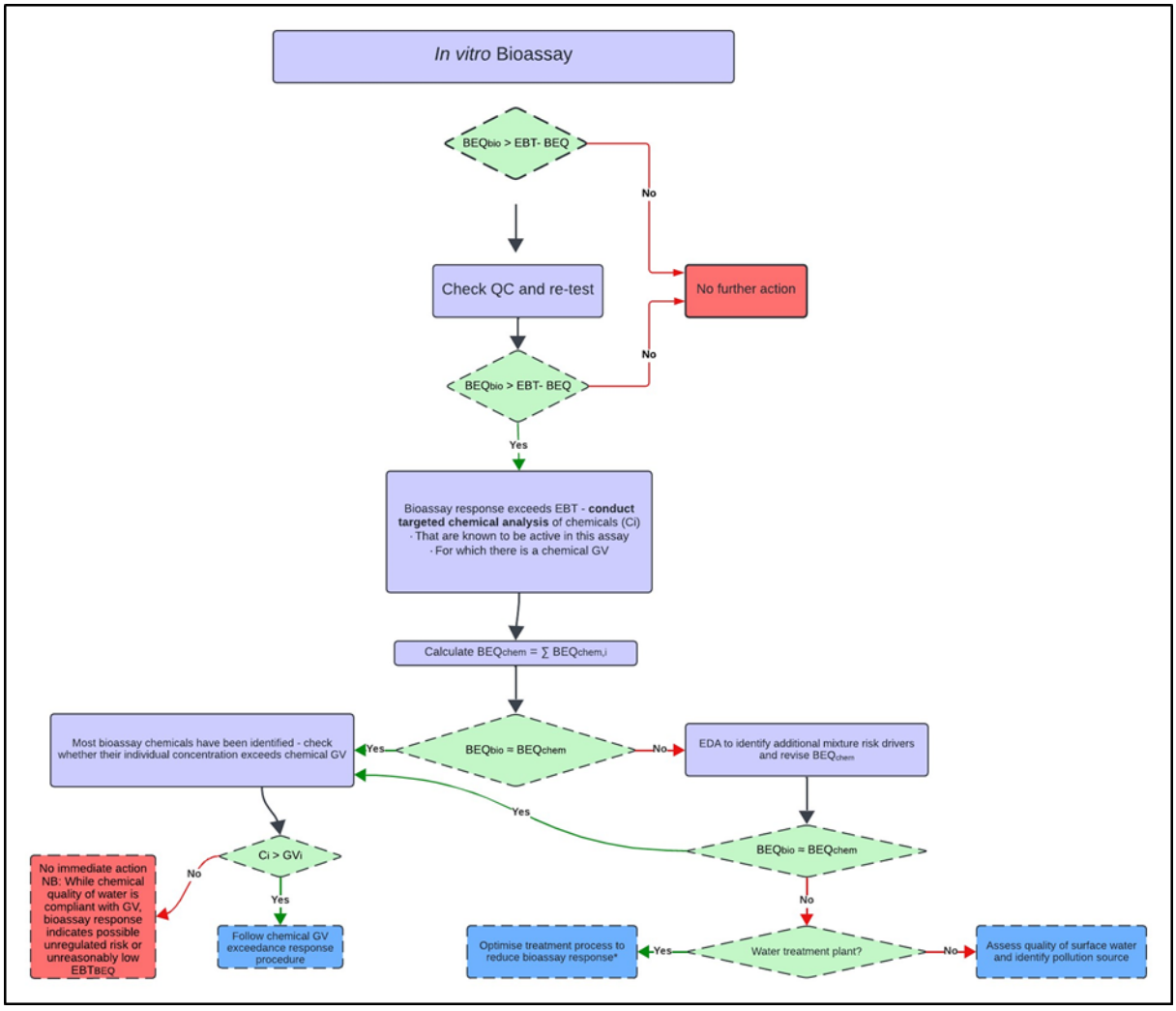

Figure 9-5: Flow diagram to represent the process of assessing the outcome of the bioassay (BEQ) versus the EBT of a water sample. (Adapted from Escher et al, 2021).

9.3.8 References:

BRACK, W., AÏSSA, S.A., BACKHAUS, T., DULIO, V., ESCHER, B.I., FAUST, M., HILSCHEROVA K.,HOLLENDER, J., HOLLERT, H., MÜLLER, C., MUNTHE, J., POSTHUMA, L., SEILER, T.B., SLOBODNIK J., TEODOROVIC, I., TINDALL, A.J., DE ARAGÃO UMBUSEIRO, G., SHANG, X., and ALTENBURGER, R. 2019. Effect-based methods are key: The European collaborative project SOLUTIONS recommends integrating effect-based methods for diagnosis and monitoring of water quality. *Environmental Sciences Europe*, 31(1):4–9. DOI:10.1186/s12302-019-0192-2

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SANS 2013b (SOUTH AFRICAN NATIONAL STANDARD) 7346-1:2013. “Water quality — Determination of the acute lethal toxicity of substances to a freshwater fish *Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyrinidae) Part 1: Static method – also applicable to *Poecilia reticulata* (Teleostei, Poeciliidae)

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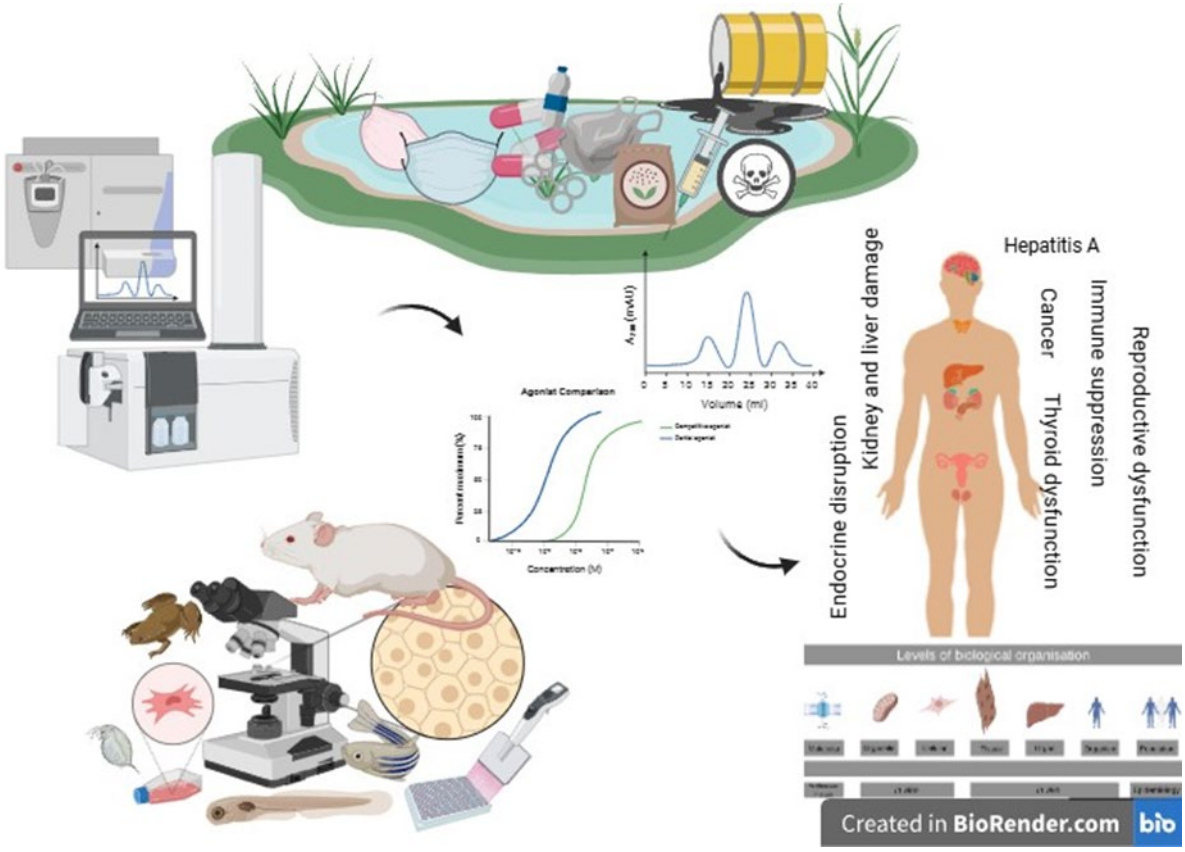
SANS 2015b (SOUTH AFRICAN NATIONAL STANDARD) 8692: 2015. “Water quality — Fresh water algal growth inhibition test with unicellular green algae

9.4 FACTSHEET: THE USE OF *IN VIVO* AND *IN VITRO* BIOASSAYS FOR EFFECT-BASED MONITORING OF WASTEWATER QUALITY AND TREATMENT TECHNOLOGIES



9.4.1 Why use bioassays for water quality monitoring?

More than 87 000 compounds exist that may end up in natural water sources. It is almost an impossible task to quantify each of them due to: cost, lack of highly skilled personnel, availability of infrastructure and reference standards. Even if it were viable to do chemical analysis for each of these, the biological effects the mixture of compounds would elicit on biota and human health are still unknown. This gap is addressed by using biological entities such as fish, daphnids, algae, bacteria and mammalian cells. These entities are exposed to a mixture of compounds found in the sample and the total biological response is assessed.

Bioassays support chemical analysis because they respond to unknown compounds present in the water sample and also show a collective response to a complex mixture. Bioassays designed to detect specific modes of action are referred to as effect-based bioassays (EBAs), and when used in water quality monitoring, the process is known as effect-based monitoring (EBM). Results from the EBM can be used as input for risk-based monitoring programs (Brack et al., 2019).

9.4.2 *In vivo* and *in vitro* bioassays applicable to wastewater:

To assess product quality and treatment efficacy of wastewater treatment, **battery 1** assays are suggested, and to understand wastewater treatment processes, **battery 2** assays are recommended.

- **Battery 1** assays consist of low sensitivity *in vitro* bioassays (yeast based bioassays), oxidative stress, AhR and *in vivo* bioassays.
- **Battery 2** assays to assess treatment processes consist of high sensitivity *in vitro* bioassays (mammalian-based bioassays), oxidative stress, AhR and the same *in vivo* assays recommended for battery 1.

Recommended in vivo and *in vitro* bioassays for wastewater that are currently available in South Africa include the following:

9.4.2.1 In vivo assays:

Allivibrio fischeri bioluminescence test (ISO/SANS 11348-3: 2013)
Pseudokirchneriella subcapitata growth inhibition test (ISO/SANS 8692: 2015)
Spirodela polyrhiza growth inhibition test (ISO/SANS 20227: 2017)
Daphnia magna/pulex acute toxicity test (immobilisation test) (ISO/SANS 6341: 2015)
Thamnocephalus platyurusacute toxicity test (ISO/SANS 14380: 2011)
Poecilia reticulata acute toxicity test (ISO/SANS 7346-1: 2013)

9.4.2.2 In vitro assays:

Xenobiotic metabolism: H4IIE-luc reporter gene assay
Estrogenic activity: YES assay (battery 1), T47D-KBluc assay (battery 2)
Androgenic activity: YAS assay (battery 1), MDA-Kb2 assay (battery 2)
Oxidative stress: AREc32 assay

NOTE:
Factsheets for the *in vivo* and *in vitro* assays can be found in the Annexure section of the WRC 2020/2021-00165, bioanalytical toolbox report.

9.4.3 Information that will be obtained from the bioassays:

The bioassays can be used to assess product quality, to assess treatment efficacy or to understand treatment processes.

The *in vivo* tests are all toxicity tests, developed to measure the influence of toxicants in the water sample on the viability of whole organisms. The organisms are representative of different trophic levels in the food web: bacteria (*Allivibrio fisheri, Salmonella typhimurium*), photosynthesising plants are represented by the algae *Pseudokirchneriella subcapitata*, lower invertebrates by *Daphnia magna/pulex*

and vertebrates by fish, usually guppies (*Poecilia reticulata*) or zebra fish (*Brachydanio rerio*) and frogs (*Xenopus*). The *Heterocypris incongruens* represent invertebrates in the sediment specifically.

The *in vitro* bioassays indicate whether toxicants interfere with normal steroid hormone processes; whether xenobiotic metabolism is taking place or whether adaptive stress responses such as oxidative stress is being triggered.

9.4.4 Sampling of wastewater:

The purpose of the sampling will determine what samples should be collected.

- To evaluate the product of a wastewater treatment plant (WWTP), the only sample required would be the treated effluent and an appropriate blank and control sample.
- In order to evaluate treatment process efficiency, influent and effluent samples (including the blank and control) will be required.
- To understand treatment processes, samples from every step of the treatment process should be collected in addition to the influent and effluent samples.
- In order to capture diurnal variation, composite samples should be collected.

For *in vivo* bioassays, samples are collected in clean plastic or glass bottles, that were rinsed with source water before being filled with sample. No air space should be left between the contents and the lid, in order to minimize the loss of toxicity due to volatilization of toxic constituents.

For *in vitro* bioassays, samples should be collected in clean glass bottles with polytetrafluoroethylene (PTFE) lined caps, or alternatively in uncoated aluminium, or stainless steel bottles. Prior to collection, bottles and caps should be rinsed with small quantities of an appropriate organic solvent (acetone or hexane for aryl hydrocarbon receptor ligands and methanol for steroid hormone receptor ligands). Samples should be protected from photo-degradation or stored in dark containers.

All samples should be transported and stored at 0–6°C, and analysed or extracted as soon as possible (within 36 h for *in vivo* and 48h for *in vitro* assays). Samples for *in vitro* bioassays may be frozen if they cannot be extracted immediately.

NOTE:
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, for more detailed sample collection procedures.

9.4.5 Pre-treatment and processing of wastewater samples for *in vitro* bioassays:

After collection, the pH of the water samples is adjusted to pH 3 to reduce degradation of contaminants. Chlorinated samples should be treated with sodium thiosulphate or ascorbic acid to quench the residual free chlorine. Wastewater samples should be filtered using glass fibre filters before extraction. Appropriate extraction methods should be used for the different targeted pollutants. For many of the water extraction methods, solid phase extraction (SPE) is typically used, using SPE sorbents such as Oasis HLB (Waters), Chromabond HX-R (Macherey-Nagel) and Strata-X (Phenomenex).

NOTE:
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, and WRC 2020/2021-00348 for more detailed sample pre-treatment and processing procedures.

9.4.6 Interpretation of *in vivo* bioassay results

Lethal or sub-lethal toxicity testing is applied by exposing biota to water sources in order to determine the potential risk of such waters to the biota/biological integrity of the receiving water bodies. A risk category (hazard class) is determined based on the percentage of mortalities (lethal) or inhibition (sub-lethal) of the exposed biota. It is important to note that the hazard class is based on the standardised battery of selected test biota and therefore represents the risk/hazard towards similar biota in the receiving aquatic environment. The toxicity hazard is therefore in terms of the aquatic biotic integrity and does in no way represent toxicology towards humans or other mammals.

A risk/hazard category is determined by using a hazard classification system developed by Persoone et al. (2003) whereby one can classify sites using the toxicity data of the non-diluted samples. The percentage effect (PE) of toxicity (mortalities, growth inhibition, luminescence inhibition, ingestion inhibition) is used to rank the sample into one of five classes (Table 9-5) based on the highest toxic response obtained in at least one of the tests applied.

Table 9-5: Hazard classification system for natural waters/screening samples (Persoone et al., 2003)

Class	Symbol	Hazard rating	PE	Percentage effect
I	☺	No lethal/sub-lethal hazard	≤10/20%	None of the tests show a toxic effect (i.e. an effect value that is significantly higher than that noted in the controls)
II	☹	Slight lethal/sub-lethal hazard	10/20%≤PE<50%	A statistically significant (P<0.05) PE is reached in at least one test, but the effect level is below 50%
III	☠	Lethal/sub-lethal hazard	50%≤PE<100%	The 50% effect level is reached or exceeded in at least one test but the effect level is below 100%
IV	☠☠	High lethal/sub-lethal hazard	PE 100% in at least one test	The 100% effect is reached or exceeded in at least one test

V	☠☠☠	Very high lethal/ sub-lethal hazard	PE 100% in all tests	The 100% effect is exceeded in all the tests

Each sample is furthermore weighted (Table 9-6) according to its relative toxicity level (out of 100%). Higher values indicate that more of the individual tests indicated toxicity within a specific class.

Table 9-6: Weight score allocation for each test type (Persoone et al., 2003)

Test score	Category
0	No significant toxicity effect
1	Significant toxicity effect < PE50
2	Toxicity effect >PE50 but <PE100
3	The PE100 is reached
Class weight score calculated as follows: Class weight score = (∑ all test scores)/n) where n is the number of tests performed Class weight score % = (class score) / (maximum class weight score) x 100	

9.4.7 Interpretation of *in vitro* bioassay results

In vitro bioassay results are expressed as bioequivalent (BEQ) concentrations. For all types of monitoring, the observed effect should be compared to an effect-based trigger (EBT) value. Effect-based trigger values are not readily available for wastewater effluent. The effect in wastewater effluent can be compared to surface water EBT after dilution. If the dilution factor is not known, a dilution factor of 10 can be used as a conservative estimate of wastewater dilution into a receiving water body. Available EBTs for surface water are provided on a factsheet from WRC 2020/2021-00165 and are also available in the WRC 2020/2021-00348 report. Figure 9-6 shows a flow diagram of the process to assess the outcome of the bioassay (BEQ) versus the EBT of a water sample.

- If the BEQ of the sample is lower than the EBT-BEQ then no further action is required.
- If the measured BEQ value exceeds the EBT-BEQ, the first step is to check the bioassay quality control (QC) and collect another water sample from the same site and re-test.
- If the BEQ of the second sample is below the EBT-BEQ, then no further action is required.
- If the second test confirms the initial positive result and both samples report a BEQ > EBT-BEQ, then further action is needed. The magnitude of the response should depend on the magnitude of the exceedance and regulatory advice.

NOTE:
Available EBTs are provided on a factsheet from WRC 2020/2021-00165 and is also available in the WRC 2020/2021-00348 report.
Please refer to WRC 2020/2021-00165, bioanalytical toolbox report, and WRC 2020/2021-00348 for more details on the interpretation of *in vitro* and *in vivo* bioassay results and actions that need to be taken when EBT-BEQ values are exceeded.

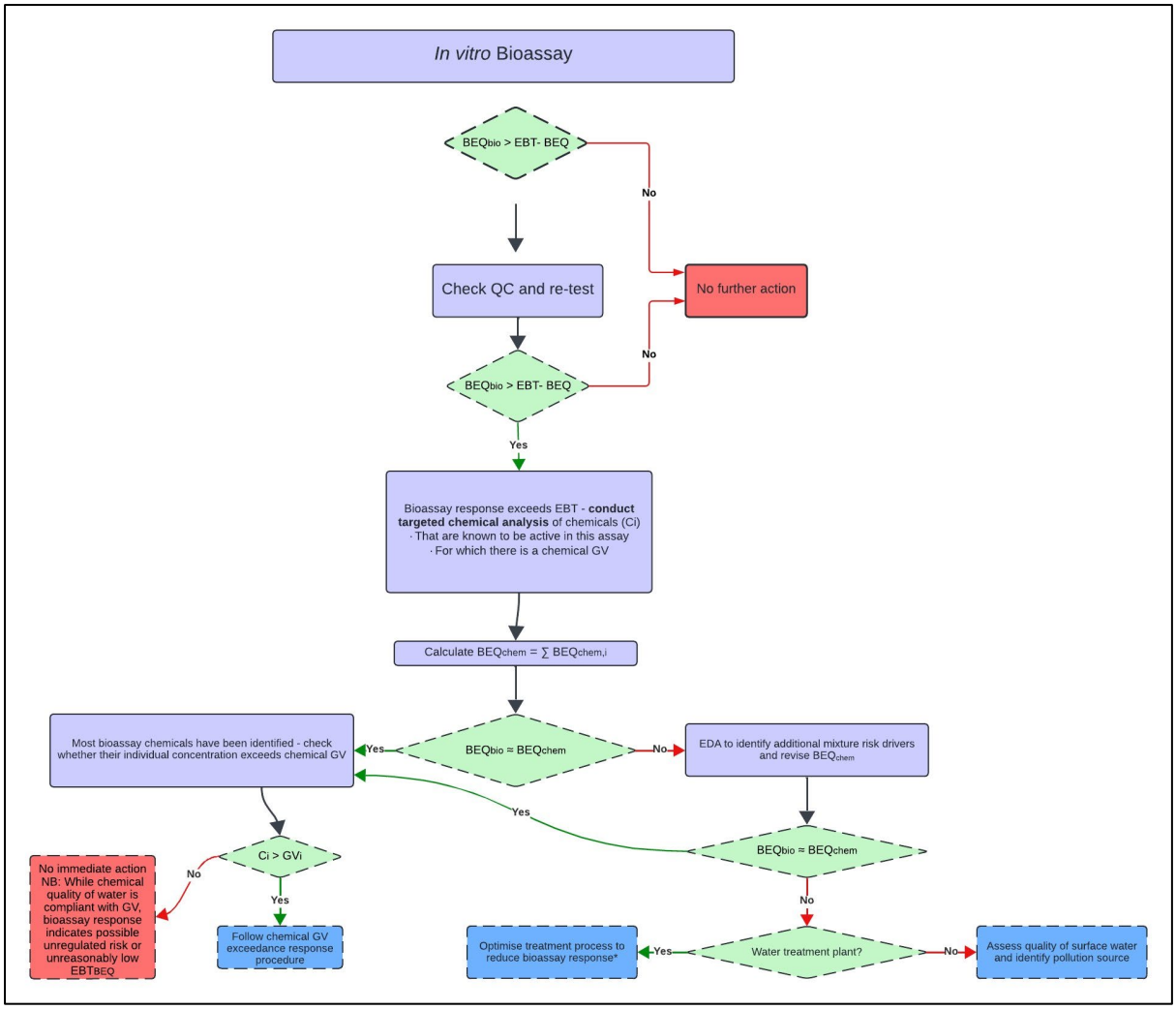


Figure 9-6: Flow diagram to represent the process of assessing the outcome of the bioassay (BEQ) versus the EBT of a water sample. (Adapted from Escher et al, 2021).

9.4.8 References:

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CHAPTER 10: CONCLUSIONS, RECOMMENDATIONS AND WAY FORWARD

10.1 CONCLUSIONS

The Department of Water and Sanitation (DWS) has previously controlled water pollution by managing levels of single substances in water. It has been noted that substance-specific methods are not in themselves able to fully assess the ecological and toxicity hazard that may be posed by these waters. Such bioassays are not effective in assessing the direct environmental toxicity hazard of discharges containing complex mixtures of substances. Therefore, a more comprehensive approach to monitor the potential toxicity hazard of complex mixtures/waters in a holistic manner as a means to protect the ecological integrity of aquatic ecosystems is required.

This is particularly relevant in light of Chapter 3 of the National Water Act (Act 36 of 1998) which focuses on the protection of the water resource itself. Internationally, various countries are currently applying these bioassays to assess and control water pollution. Locally, the demand for these tests is rapidly increasing. As a result, Pearson. et al. (2015) completed a Water Research Commission (WRC) project titled: “Development of Research Support to Enable the Issuing of Aquatic Toxicity-based Water Use Licenses,” followed by a WRC report by Griffen. et al. (2019) on “Benchmarking a Decision Support System for Aquatic Toxicity Testing”. During this project, locally available toxicity bioassays were applied to different types of water to evaluate the efficiency and applicability of the tests, and the study concluded that all tests (except for one test) have a viable role to play in water quality monitoring and control.

Furthermore, another document, “Aquatic toxicity testing in South Africa: status of aquatic toxicity testing in South Africa) was compiled by Chapman. et al. (2011), with the aims of developing an implementation plan for the DWS for routine toxicity testing and developing a guideline to promote a sustainable network between toxicity testing laboratories. A previous document, “Guidelines for toxicity bioassaying of drinking and environmental waters in South Africa,” by Slabbert. et al. (1998) compiled and outlined the specific guidelines on test methodologies, data analysis, sampling and application. Currently, no national guideline/standard is available for aquatic toxicity testing; thus, the decision remains with the DWS to set the guidelines based on these documents.

The assessment of aquatic toxicity is an important component of the environmental hazard and risk assessment of all types of chemicals and receiving waters and effluents. Aquatic toxicity in general refers to the effects of a sample on whole organisms living in water and is determined with organisms representing different trophic levels (with different sensitivities to different components) in the natural environment (e.g., bacteria, algae or plants as primary producers, invertebrates as primary consumers/secondary producers and vertebrates representing secondary consumers. In general, there are acute/lethal and chronic/sub-lethal endpoints in aquatic toxicity. Acute/lethal toxicity is usually determined with short-term exposure of organisms to determine lethal effects, whereas chronic/sub-lethal is about longer-term exposure, covering effects on hatching, growth and survival.

Historically, these tests were performed using in-house cultures, but more recently, commercial test kits have been produced, which simplify testing by removing the requirement for cultures by supplying immobilized test taxa as part of the kit. The results from the same tests using cultured taxa and taxa from kits have been compared, and while there are exceptions, the consensus seems to be that no significant differences exist in the endpoints from these methods. These kits have therefore been adopted as a tool in aquatic toxicology because they are often fast, require little sample and are mostly

cost effective as a screening tool. It is important to note that these tests cannot provide information on the source/contributors of/to the effects noted, but can serve as an excellent, cost-effective and time-saving screening tool for environmental samples.

A summary of the main results obtained in the case studies is presented in Table 10.1.

Table 10-1: A summary of the main results obtained in the case studies

			In vitro bioassays							*In vivo* bioassays							
Sample site	Sampling event no	Non-targeted analysis (number of compounds detected)	H4IIE-luc TCDD-Eq (ng/L)	YES EEq (ng/L)	T47D-KBluc EEq (ng/L)	YAS DHTEq (ng/L)	MDA-kb2 DHTEq (ng/L)	Gh3.TRE-luc T3Eq (ng/L)	AREc32 Dichlorvos-Eq (µg/L)	*Aliivibrio fischeri*	*Pseudokirchneriella subcapitata*	*Spirodela polyrhiza*	*Daphnia magna*	*Thamnocephalus platyurus*	*Poecilia reticulata*	Hazard Class	Weight score (%)
Drinking Water Treatment Plant (DWTP)																	
DWTP1a	1	NA	NA	<LOD	1.4 ± 0.3	NA	<LOD	<LOD	253.9	NA	NA	NA	NA	NA	NA	NA	NA
DWTP1a	2	NA	NA	0.23 ± 0.02	0.88 ± 0.07	NA	<LOD	<LOD	NQ	NA	NA	NA	NA	NA	NA	NA	NA
DWTP1b	1	NA	NA	<LOQ	0.39 ± 0.07	NA	<LOD	<LOD	342.4	NA	NA	NA	NA	NA	NA	NA	NA
DWTP1b	2	NA	NA	<LOQ	1.2 ± 0.05	NA	<LOD	<LOD	333.4	NA	NA	NA	NA	NA	NA	NA	NA
DWTP1c	1	NA	NA	<LOQ	1.7 ± 0.27	NA	<LOD	<LOD	263.1	NA	NA	NA	NA	NA	NA	NA	NA
DWTP1c	2	NA	NA	<LOD	0.07 ± 0.03	NA	<LOD	<LOD	NQ	NA	NA	NA	NA	NA	NA	NA	NA
DWTP2a	1	49	NA	<LOD	0.20 ± 0.04	NA	<LOD	<LOD	81.2	NA	NA	NA	NA	NA	NA	NA	NA
DWTP2a	2	50	NA	<LOD	0.15 ± 0.04	NA	<LOD	<LOD	131.7	-4	-26	-53	5	NA	0	Class III, Lethal/sub-lethal hazard	30
DWTP2b	1	49	NA	<LOQ	0.35 ± 0.01	NA	<LOD	<LOD	12.5	NA	NA	NA	NA	NA	NA	NA	NA
DWTP2b	2	49	NA	<LOD	0.004 ± 0.001	NA	<LOD	<LOD	28	-16	-26	-57	0	-86	25	Class III, Lethal/sub-lethal hazard	50
DWTP3a	1	446	<LOD	0.09 ± 0.006	0.79 ± 0.18	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
DWTP3a	2		<LOD	0.52 ± 0.06	<LOQ	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
DWTP3b	1	354	<LOD	0.50 ± 0.03	0.98 ± 0.24	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
DWTP3b	2		<LOD	1.6 ± 0.20	0.99 ± 0.62	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
DWTP4a	1	26	<LOD	0.18 ± 0.04	0.098 ± 0.02	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
DWTP4b	1	11	<LOD	<LOD	0.064 ± 0.002	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
DWTP5a	1	446	<LOD	0.28 ± 0.07	0.22 ± 0.02	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
DWTP5b	1	315	<LOD	0.22 ± 0.04	1.2 ± 0.3	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
DWTP6a	1	94	<LOD	0.36 ± 0.03	1.9 ± 0.63	NA	<LOD	<LOD	NA	-11	-3	-54	0	-1	0	Class III, Lethal/sub-lethal hazard	17
DWTP6b	1	119	<LOD	0.20 ± 0.07	1.0 ± 0.03	NA	<LOD	<LOD	NA	-11	-6	-86	0	-14	0	Class III, Lethal/sub-lethal hazard	17
Wastewater Treatment Plant (WWTP)																	
WWTP1a	1	378	0.2 ± 0.05	55 ± 3.8	87 ± 19	1057 ± 134	209 ± 56	<LOD	NA	-69	9	-65	90	-94	100	Class IV, High lethal/sub-lethal hazard	61
WWTP1b	1	402	<LOQ	2.8 ± 0.8	3.8 ± 0.4	NA	<LOD	<LOD	NA	4	18	-68	5	-30	17	Class III, Lethal/sub-lethal hazard	42
WWTP1b	2		<LOD	0.85 ± 0.05	1.3 ± 0.15	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
WWTP2a	1	364	<LOQ	54 ± 3.1	53 ± 7.2	1322 ± 150	349 ± 62	<LOD	NA	-67	6	-56	50	-100	100	Class IV, High lethal/sub-lethal hazard	67
WWTP2b	1	290	<LOD	0.075 ± 0.008	0.14 ± 0.03	73 ± 25	67 ± 2.8	<LOD	NA	-100	-17	-89	100	-100	100	Class IV, High lethal/sub-lethal hazard	78
WWTP2b	2		<LOD	2.1 ± 0.22	2.7 ± 0.3	<LOQ	4.8 ± 0.8	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
WWTP3b	1	49	NA	42.55 ± 3.71	108 ± 31	159 ± 31	4.1 ± 0.8	<LOD	945.5	NA	NA	NA	NA	NA	NA	NA	NA
WWTP3b	2	50	NA	4.96 ± 0.44	21 ± 0.8	NA	<LOD	<LOD	421.9	-37	-25	-52	15	-37	17	Class III, Lethal/sub-lethal hazard	58
Borehole, spring water, ground water (GW)																	
GW1	1	49	NA	0.10 ± 0.03	0.72 ± 0.05	NA	<LOD	<LOD	12.6	NA	NA	NA	NA	NA	NA	NA	NA
GW1	2	50	NA	<LOD	0.014 ± 0.003	NA	<LOD	<LOD	NQ	-31	-6	-56	5	-29	25	Class III, Lethal/sub-lethal hazard	42
GW2	1	49	NA	<LOQ	1.5 ± 0.2	NA	<LOD	<LOD	217.9	NA	NA	NA	NA	NA	NA	NA	NA
GW2	2	49	NA	<LOD	0.099 ± 0.01	NA	<LOD	<LOD	43.8	-33	-36	-68	0	-11	33	Class III, Lethal/sub-lethal hazard	42
GW3	1	NA	<LOD	6.8 ± 0.55	1.4 ± 0.6	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
GW4	1	NA	NA	0.23 ± 0.05	0.99 ± 0.08	NA	<LOD	<LOD	57.9	NA	NA	NA	NA	NA	NA	NA	NA
GW4	2	NA	NA	<LOD	0.022 ± 0.003	NA	<LOD	<LOD	20.5	-37	-38	-65	0	-15	25	Class III, Lethal/sub-lethal hazard	42
Surface water (rivers dams and wetland) (SW)																	
SW1	1	1739	NA	<LOD	0.18 ± 0.02	NA	<LOD	<LOD	299.9	NA	NA	NA	NA	NA	NA	NA	NA
SW1	2	50	NA	<LOD	0.03 ± 0.003	NA	<LOD	<LOD	15	2	-22	-37	0	NS	0	Class II, Slight sub-lethal hazard	40
SW2	1	49	NA	1.4 ± 0.12	32 ± 2.8	NA	<LOD	<LOD	367.2	NA	NA	NA	NA	NA	NA	NA	NA
SW2	2	50	NA	<LOD	0.08 ± 0.02	NA	<LOD	<LOD	20.2	-21	-36	-53	5	-82	17	Class III, Lethal/sub-lethal hazard	58
SW3	1	49	NA	<LOD	1.4 ± 0.4	NA	<LOD	<LOD	1106	NA	NA	NA	NA	NA	NA	NA	NA
SW3	2	50	NA	<LOD	0.17 ± 0.01	NA	<LOD	<LOD	345	-3	-19	-34	5	-100	8	Class IV, High lethal/sub-lethal hazard	22
SW4	1	49	NA	<LOD	0.71 ± 0.03	NA	<LOD	<LOD	376	NA	NA	NA	NA	NA	NA	NA	NA
SW4	2	50	NA	<LOD	0.03 ± 0.008	NA	<LOD	<LOD	22.6	-28	-15	-39	5	-13	8	Class II, Slight sub-lethal hazard	33
SW5	1	NA	NA	0.11 ± 0.020	0.65 ± 0.10	NA	<LOD	<LOD	45.4	NA	NA	NA	NA	NA	NA	NA	NA
SW5	2	NA	NA	<LOD	0.11 ± 0.02	NA	<LOD	<LOD	80.4	-44	2	-56	0	-26	33	Class III, Lethal/sub-lethal hazard	42
SW6	1	NA	NA	<LOQ	0.56 ± 0.19	NA	<LOD	<LOD	89.7	NA	NA	NA	NA	NA	NA	NA	NA
SW6	2	NA	NA	<LOD	0.08 ± 0.006	NA	<LOD	<LOD	NQ	-46	-34	-50	10	-36	25	Class III, Lethal/sub-lethal hazard	50
SW7	1	NA	NA	<LOQ	0.57 ± 0.02	NA	<LOD	<LOD	70.9	NA	NA	NA	NA	NA	NA	NA	NA
SW7	2	NA	NA	23 ± 6.8	99 ± 30	NA	<LOD	<LOD	NQ	-45	1	-38	5	-63	8	Class III, Lethal/sub-lethal hazard	33
SW8	1	NA	NA	0.33 ± 0.023	1.6 ± 0.2	NA	<LOD	<LOD	57.9	NA	NA	NA	NA	NA	NA	NA	NA
SW8	2	NA	NA	<LOD	0.20 ± 0.02	NA	<LOD	<LOD	NQ	-38	2	-49	5	-46	25	Class II, Slight sub-lethal hazard	67
SW9	1	NA	NA	<LOQ	0.93 ± 0.2	NA	<LOD	<LOD	41.4	NA	NA	NA	NA	NA	NA	NA	NA
SW9	2	NA	NA	<LOD	0.13 ± 0.01	NA	<LOD	<LOD	36	-41	2	-43	0	-51	33	Class III, , Lethal/sub-lethal hazard	42
SW10	1	314	<LOD	0.55 ± 0.05	0.63 ± 0.06	NA	<LOD	<LOD	NA	-19	13	-36	0	-18	8	Class II, Slight sub-lethal hazard	17
SW10	2		<LOD	0.78 ± 0.06	1.3 ± 0.24	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
SW11	1	311	<LOD	1.4 ± 0.26	5.5 ± 0.2	152 ± 30	26 ± 4	<LOD	NA	-8	14	-21	0	-100	0	Class IV, High lethal/sub-lethal hazard	22
SW11	2		<LOD	1.6 ± 0.13	2.2 ± 0.2	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
SW12	1	392	<LOD	0.71 ± 0.11	1.7 ± 0.2	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
SW12	2		<LOD	2.6 ± 0.16	1.6 ± 0.07	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA
SW13	2	388	<LOD	0.62 ± 0.02	1.4 ± 0.29	NA	<LOD	<LOD	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA Not analysed; <LOD Below the level of detection; <LOQ Below the level of quantification; NQ Could not be quantified due to cytotoxicity

In vitro bioassay results highlighted in red indicate that the value is above the EBT value (wastewater effluent was compared with surface water EBT after a 10x dilution factor)

It is important to note that toxicity testing presents information on a “snapshot” in time basis, and for this project, very limited data are available (one set of data per site), and thus these data do not represent any seasonal variation. The tests were performed on a screening level (using only the 100% concentration of the samples) and therefore the dilution effect (e.g., when this water is discharged/spilled to the environment or where waters enter dams) cannot be commented on or taken into consideration when discussing the impact of the samples on the environment. These results should be interpreted alongside chemistry results to get a better understanding of the effects observed – this should correlate with results obtained during chemistry tests.

It should also be noted that these results are discussed in isolation – for more accurate interpretation of the results, information should be available on the upstream anthropological and industrial activities/impacts as well as other sources in the areas, and it should be kept in mind that different treatment plants receive raw water from different impacted sites therefore containing different constituents which may have different effects on different organisms (different organisms are sensitive to different constituents).

As indicated per area, the battery of tests is site specific, and tests must be conducted at least once per season and at least 2-3 times to get representative results. At least 3 trophic levels should be covered per battery of tests to cover all potential impacts of the samples on the environment, but this will depend on the different activities taking place in each area. It was clear from the tests that these samples do have an impact on the environment, and especially from the water treatment plants (drinking water and wastewater) due to the disinfection activities of the water before release to the environment.

In vivo tests should be used as screening tools to prioritize impacted areas. Following the interpretation of the results from these tests, it was concluded that there are not enough data to recommend a set battery of tests, and that results from each area are different due to the different activities taking place in each individual area. Based on the results obtained while performing the aquatic toxicity tests, the pH levels of these waters were within the acceptable (6-9) range where pH can be excluded as a contributing factor for toxicity. The dissolved oxygen levels for all sites were >4 mg/L (standard aquatic toxicology test limit) except for the wastewater treatment plant influents and the toxicity effects noted on the *P. reticulata* (vertebrate) test may have been due to the low oxygen levels.

This traditional battery of *in vivo* tests are not normally used for drinking water, however, effects were noted for these type of samples following the *S. polyrhiza* and the *T. platyurus* bioassays, which can be of great value to the screening of drinking water quality. Based on the results obtained using this limited set of data, it was also evident that the battery for drinking water, surface water, groundwater and effluents will differ.

The Hazard Classes obtained showed a definitive impact of these waters on the environment, but this may not necessarily be due to pollution; it may be due to disinfection processes (at the treatment plants). The Hazard Classification protects the environment because we use the most sensitive Hazard Class of the battery of tests performed.

It is also important to note that not only the battery of aquatic toxicity tests normally implemented and required by the DWS showed effects, but also, and in most cases, more so, additional newly developed tests showed higher sensitivity levels than the traditional battery of tests (*D. magna*, *P. reticulata*, *R. subcapitata* and *A. fischeri*) and these newly tested kits should be considered during licensing processes.

Estrogenic activity and oxidative stress responses were detected in all types of water, indicating the importance of including these assays in an EBM program. Although the yeast-based assays performed very well for wastewater samples, overall, the T47D-KBluc assay detected estrogenic activity in more samples compared to the YES assay, indicating that the T47D-KBluc assay is a more sensitive assay to use. In this study, no thyroid activity was detected in any of the samples, indicating that the GH3.TRE-luc assay might not be sensitive enough to incorporate into the toolbox of assays. Alternative assays for thyroid activity should be investigated. Androgenic activity was detected in one surface water sample, but mostly in WWTP samples. The only response above the LOQ in the H4IIE-luc assay was also in a WWTP sample. These assays are therefore recommended for WWTP samples, but due to the limited number of samples that could be done in this project, we still recommend including these assays in the battery of assays for other types of water as well.

The samples that showed a class IV, high lethal/sub-lethal hazard in the *in vivo* bioassays, were also above the EBT value for at least one of the *in vitro* bioassays. The *in vivo* bioassays can therefore give a good indication if there is a problem with the water quality. However, many of the samples that were above the trigger value did not indicate a high lethal/sub-lethal hazard in the *in vivo* assays, and therefore, it is not recommended to use the *in vivo* assays as a screening tool to decide whether it is necessary to do the *in vitro* bioassays. The *in vitro* and *in vivo* assays are rather used to complement one another.

Regarding the chemical analysis, the number of chemical compounds detected with non-targeted chemical analysis could not be used to predict the outcome in the bioassays. Furthermore, in many of the samples that were above the EBT, no target chemicals could be quantified using targeted chemical analysis.

The bioassay EBT values for drinking and surface waters is generally in the ng/L range, whereas the LOQ for targeted chemical analysis was in the µg/L range. The chemical analysis method might therefore not be sensitive enough to detect EDC compounds at the low levels required to protect human and ecological health.

In summary, this data highlights the important role that bioassays can play in water quality monitoring. Chemical analysis, *in vitro* and *in vivo* bioassays should therefore all be used to complement one another in order to get a complete profile of the water system and the potential hazards.

10.2 RECOMMENDATIONS AND WAY FORWARD

South Africans have a right to access safe and healthy water. In order to ensure that this is possible, there needs to be strategies and actions in place to do this. The project has highlighted the need to use EBMs together with monitoring to improve our water quality and safety in a positive way. However, there are several aspects that need to be addressed before we can proceed to policy, legislation and implementation, and these are as follows:

- The first challenge is to build capacity in the private or government sectors.
- Currently capacity to do the bioassays lies in academic institutions, particularly for the *in vitro* assays. This is also research based. In order to implement EBMs in the laboratory, the correct infrastructure will be required to cope with the high throughput of doing these batteries of assays on a larger scale. For example, in Europe, many of these laboratories are using robotics to perform the high throughput assays of assays for EBM.
- An additional challenge is that many of the assays require a user licence for commercial use, which can have an economic impact on what we can do in SA.

- Although these assays are useful, they cannot replace the analytical analyses associated with water quality and safety, therefore additional funds will be required from Government to set up the appropriate laboratories to complement the current requirements set out in the guidelines. However, we also understand that if there is not some sort of legal requirements the situation will not change and that will present health risks to human and the aquatic environment.
- In this project we proposed a decision-making tool but there is a necessity to refine the process requiring more data and information. In order to achieve this a comprehensive and strategic monitoring program will need to be designed and implemented. This will include frequent sampling and monitoring over an extended period for the different types of water systems as indicated in Figure 3.2.
- In order for this to happen multiple government departments, municipalities, water consumers, researchers and private sector water stakeholders will need to work together.

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ANNEXURE 1 - PROJECT OUTPUTS

1. **WORKSHOPS**

a. Knowledge Dissemination Workshop: The use of EBM for water quality and safety, was held on 7 September 2022, at Future Africa at the University of Pretoria, Pretoria.

b. Workshop and Community engagement on the use of EBM for water quality and safety was held at BirdLife SA in Wakkerstroom on the 18 October 2022.

2. WEBINARS

a. Global Water Research Coalition (GWRC) webinar “Case-studies of micropollutants bioassays-the real practice of effect-based monitoring”, on the 26th of April 2022, from 9.00-10.30 am (GMT+2). Aneck-Hahn NH & Van Zijl MC were invited to present on the SA perspective. The title of the talk was “The development and application of EBM: A South African perspective”

3. CONFERENCES

a. The 5th Water Research Commission (WRC) Biennial Symposium, 20-22 September 2021. Fifteen years of EDC research - tracking progress on science to Policy. Tuesday 21 September 2021. Dr NH Aneck-Hahn presented the following talk, "Towards development of an EDC toolbox for water quality assessment"

b. 9th Water Forum, in Dakar Senegal, 21-26 March 2022. Dr Sean Patrick was an invited speaker to the One Water, One Heath session. He presented the following talk, "SSP39-1- Effect based monitoring (of water chemical mixtures) in water safety planning's (POC project 2018-2022)

c. SETAC Europe 32nd Annual meeting in Copenhagen, Denmark, from 15-19 May 2022. Ms Annika Kruger is presenting the following talk "Androgenic activity of water and sediment up- and downstream of a wetland" A. Kruger, N. Mmekwa, S. Horn, R. Pieters

d. Aneck-Hahn N, Van Zijl C, Pieters R, Horn S, Mmekwa N, Kruger A, Swart L, Pearson H, Archer E, Truter C. The development and application of effect-based methods (EBM) for water quality and safety: A South African perspective. Water Institute of South Africa (WISA) 2022

e. Aneck-Hahn N, Van Zijl C, Pieters R, Horn S, Mmekwa N, Kruger A, Swart L, Pearson H, Archer E, Truter C. A South African perspective on the need for the development of toolbox of effect-based methods for water quality: Does the evidence highlight the need? 21 September 2022, UFZ, Hemholtz Center for Environmental Research, Leipzig, Germany

f. Faculty of Health Sciences Research Day, 24 August 2023. The MSc student Ms Naledi Mmekwa was selected to present her work on her project. Her talk was titled "Assessing oxidative stress and identifying pharmaceuticals in water sources of Mpumalanga province, South Africa".

g. Environmental Endocrine Disruptors Gordon Research Conference, Addressing Environmental Endocrine Disruptors to Improve Planetary Health, Luca (Barga), Italy from June 23 - 28, 2024. Dr Natalie Aneck-Hahn has been invited to serve on the organising committee for the conference and is also an invited speaker. Her talk entitled “Endocrine disrupting chemicals in Africa -30 years on” will include the information generated from the project.

4. PUBLICATIONS

a. Kruger, A., Pieters, R., Horn, S. et al. The role of effect-based methods to address water quality monitoring in South Africa: a developing country's struggle. Environ Sci Pollut Res 29, 84049–84055 (2022). https://doi.org/10.1007/s11356-022-23534-3
b. Prof Rialet Pieters wrote a piece for ENVIRA a NWU publication on the knowledge dissemination workshop that was held for Wakkerstroom community members on 18 October 2022
c. Factsheets (assays and stakeholders)
d. The project was also announced in the local Wakkerstroom National Heritage Association (WNHA) newsletter (Attached PDF)
e. A follow up community communication was done in the WNHA newsletter

5. SOCIAL MEDIA

a. Prof Rialet Pieters one of the team members hosts a podcast on RSG radio (Radio without borders) on a Saturday morning on the Breakfast show with Derrik Gardner called Omgewings praaitjies (Environmental chat). On 27 August 2022 she hosted her first podcast where she highlighted the importance and impact of this project (https://www.rsg.co.za/rsg/potgooi-resultaat/?_sf_s=omgewing&post_date=20220827)

6. CAPACITY BUILDING

a. University of Pretoria:

i. Ms Naledi Mmekwa graduated in May 2024 (MSc)

b. North West University (Potchefstroom):

i. Ms Annika Kruger graduated in May 2024 (MSc)

c. Training

i. A senior scientist from Prof Beate Escher's laboratory at the Department of Cell toxicology, Helmholtz-Zentrum für Umweltforschung (Helmholtz Centre for Environmental Research), Leipzig, Germany visited the University of Pretoria the EDC laboratory in the Environmental Chemical Pollution and Health – Research Unit from 6 – 17 November 2023. The purpose of the visit was to assist the lab to establish the AREc-32 cell line for oxidative stress. It was an extremely busy time and we ran experiments to refine the method and instrumentation. But importantly taking the data generated and interpreting the results in the same way to ensure standardisation between the two laboratories.

ii. Ms Ratanang Mlaba spent time in the ECPH-Research Unit at UP learning and observing how to do the YES assay from Dr Catherina Van Zijl

iii. In April Ms Matjomane a TUT student (MSc)in the ECPH-Research Unit and staff member in the Department of Urology at Steve Biko Academic Hospital spent time working on the bioassays and other aspects of the project. She was selected to do a PhD in collaboration with the Department of Pharmacy at Copenhagen University. She represented the ECPH-RU and spent 2 weeks in Copenhagen to learn how to work on the H295R cell line for steroidogenesis (also relevant to EBM). She was sponsored through a collaboration and capacity exchange fund from the Danish Environmental Agency in Denmark. This cell line will be included in any further studies on water quality, thereby adding to the capacity available in the country.