THE USE OF EFFECT-BASED METHODS FOR WATER SAFETY PLANNING IN SOUTH AFRICA

Volume 1: Use of bioassays for the assessment of selected modes of action (MOA) relevant for water safety planning

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Volume 1: Use of bioassays for the assessment of selected modes of action (MOA) relevant for water safety planning

Report to the
Water Research Commission

by

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This report forms part of a set of two reports. The other report is *The Use of Effect-Based Methods for Water Safety Planning in South Africa*. *Volume 2: Sample processing procedures for appropriate bioassays for the assessment of selected modes of action (MOA) relevant for water safety planning*. (WRC Report No. TT 908/2/22)

EXECUTIVE SUMMARY

BACKGROUND

The water industry in South Africa (SA) today is faced with the challenge of ensuring a sustained and safe supply of drinking water. Surface waters can contain a wide range of substances referred to as micropollutants that include industrial compounds, agricultural compounds like pesticides, pharmaceuticals, personal care products and plasticisers/microplastics. These compounds have the potential to adversely affect the ecosystems in which they are found. By identifying their sources, point (e.g. wastewater treatment plants) or diffuse (e.g. agriculture) within the ecosystem, and attempting to find solutions may help to reduce the presence and bioactivity of the compounds. These compounds are present as mixtures in the aquatic ecosystem and can affect the endocrine system, for example reproduction, cancers, neurodevelopmental effects and obesity. The focus has been mostly on the effects of endocrine disrupting chemicals (EDCs) with estrogenic activity. However, the endocrine system is not limited to estrogenic activity, other hormone-driven signalling systems like androgens, progestogens, glucocorticoids, retinoids and thyroid hormones play a critical role in maintaining processes throughout the different life-stages in humans and animals. Present in the aquatic environment, these chemicals and their degradation products are difficult to assess for risk and impact on human and environmental health. Many of the compounds are found at low doses in the environment, but may have a high potency. 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 have a similar mode of action (MOA).

RATIONALE

The Global Water Research Coalition (GWRC) has embarked on a new study investigating the use of Effect-based Monitoring (EBM) program for Water Safety Planning (WSP). For water quality assessment and risk management, carcinogenesis, adverse effects on reproduction and development, effects on xenobiotic metabolism, modulation of hormone systems, DNA reactivity and adaptive stress responses are considered the most relevant toxicological endpoints.

This project will focus on the relevant bioassays for the assessment of selected modes of action (MOA) for treated water safety in SA.

PROJECT AIMS

The aims of the project were to:

1. Develop a list of relevant stakeholders (particular reference to SA) and get an overview of ongoing pre-regulatory to fit in with protocols that will be developed for risk and effect-based monitoring plan for WP2 of the GWRC EBM in Water Safety Planning (at WRC).

2. Compile a recommended short-list of appropriate and available bioassays in SA to develop a toolbox for the assessment of selected endocrine activity endpoints relevant to the South African scenario based on the GWRC Toolbox to detect endocrine activity.

3. Select bioassays based on Mechanisms of Action (MOA) inspired by both Food Safety Approach and One-Health approach and elaborate innovative and smart combination of effect-based methods (EBM) (Found in Factsheets in the Annexures B and C).

4. Provide effect-based trigger values (EBTV) specifically relevant to ecosystem as well as human health targets (Found as a factsheet in Annexure D).

OBJECTIVES

The objectives of this project were to:

1. Compile a list of appropriate bioassays for the assessment of selected MOA and relevant for WSP.
2. Review the Estrogenic activity Toolbox from 2011.
3. Include *in vitro* assays with additional hormone receptor-mediated effects like androgenic and thyroid activity, as well other endpoints based on MOA for example, oxidative stress, and cell toxicity.
4. Include low complexity i*n vivo* bioassays for example the AMES test and FETAX.

METHODOLOGY

A comprehensive literature review was done by the GWRC (May 2020 report) to identify applicable bioassays for EBM. This report was used as a guideline to select the relevant bioassays applicable to the South African scenario. The list of assays was dependent on capacity, infrastructure and which assays are currently available or under development within SA. The report addressing the aims and objectives stated in Section 1.1.2 is presented in two volumes:

- Volume 1 – covering literature review on the use of effect-based methods for water safety planning in South Africa and factsheets on potential bioassays for assessing different modes of action (MoAs) relevant for water safety planning in South Africa (this report).
- Volume 2 – contains a toolbox for sample processing for selected *in vitro* and *in vivo* bioassays

CONCLUSIONS AND RECOMMENDATIONS

Chemical analysis comes with limitations and challenges (capacity, cost and infrastructure, etc.), but also with the vast number of chemicals that may be present in the environment, particularly in SA. The approach of effect-based monitoring using *in vitro* bioassays and well plate-based *in vivo* assays has been recommended for water quality assessment. *In vitro* bioassays can be used to investigate different stages of cellular toxicity pathways, including induction of xenobiotic metabolism, receptor-mediated effects, adaptive stress responses and cytotoxicity. These assays are useful as high-throughput screens which is essential for routine water quality monitoring. These assays can be used to develop a toolbox of EBMs not just estrogenic activity for water quality and safety in SA. One of the aims of developing a toolbox of assays is to allow water quality laboratories to build capacity and use these assays to test water quality from different water sources, from treated water to surface and groundwater on a regular basis. It will also enable water stakeholders to design a suitable fit-for-purpose bioassay test battery for a particular water type or source.

While a battery of three to four bioassays is recommended, there are some situations or constraints at specific locations that may not allow for this. Therefore even a simple cytotoxicity assay can be considered. At the same time, depending on the type of water being investigated, other assay endpoints, for example androgenic activity, thyroid activity and other receptor-mediated endpoints, should also be included in the battery of assays.

Effect-based method use in SA is limited. This may be for a number of reasons, for example, the cost of the bioassays and the infrastructure required such as cost, capacity and policy. The current SANS 241 that is currently under revision should include EBMs and the accompanying EBTVs for drinking water. This stands true for other water quality guidelines and WSPs. It is clear from the literature that SA's water is contaminated with chemicals of emerging concern (CECs), pharmaceutical and personal care products (PPCP) and EDCs. Using EBMs in monitoring programmes is important. Case studies looking at using the toolbox in parallel with chemical analysis will facilitate the development of these tools to assess the health risk of these compounds to humans and animals. The factsheets, together with the updated toolbox, should be used by water stakeholders in order to improve and increase the sustainability of water quality and use in SA.

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ACRONYMS & ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AHTN	Tonalide
AOPs	Adverse outcome pathways
AR	Androgen receptor
BPA	Bisphenol A
BSA	Bovine serum albumin
CAF	caffeine
CBZ	Carbamazepine
CECs	Chemicals of emerging concern
DBP	Disinfection by-products
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DOHaD	Developmental Origins of Health and Disease
DWTP	Drinking water treatment plant
EASZY	Embryonic zebrafish assay
EBM	Effect-based monitoring
EC	Effect concentration
EDC	Endocrine disrupting chemicals
EQ	Equivalent concentration
ER	Estrogen receptor
FETAX	Frog Teratogenesis Assay of *Xenopus*
GR	Glucocorticoid receptor
GWRC	Global Water Research Coalition
HHCB	Galaxolide
IARC	International Agency for Research on Cancer
IPCS	International Programme on Chemical Safety
KCs	Key characteristics
LLE	Liquid-liquid extraction
MOA	Modes of action
MR	Mineralocorticoid receptor
NHRs	Nuclear hormone receptors
NP	4-Nonylphenol
NTMP	National Toxicity Monitoring Program
NWS	National Water Services
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
PPAR	Peroxisome proliferator-activated receptor
PPCP	Pharmaceutical and personal care products
PR	Progesterone receptor
PXR	Pregnane X receptor

RAR	Retinoic acid receptor
REACH	Registration, evaluation, and authorisation of chemicals
RXR	Retinoid X receptor
SA	South Africa
SANS	South African National Standards
SDGs	Sustainable Development Goals
SPE	Solid-phase extraction
T3	Triiodothyronine
TDS	Testicular dysgenesis syndrome
TG	Test guideline
TP	Transformation products
TR	Thyroid receptor
UNEP	United Nations Environment Programme
USA	United States of America
US EPA	United States Environmental Protection Agency
WHO	World Health Organisation
WRC	Water Research Commission
WSP	Water Safety Planning
WWTP	Wastewater treatment plant
XETA	Xenopus eleutheroembryo thyroid assay
YAES	Yeast anti-estrogen screen
YAS	Yeast androgen screen
YES	Yeast estrogen screen

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CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Water, sanitation and health has seen a number of countries, including South Africa, adopting a number of international policies and introducing new regulations in order to ensure sustainable access to these basic services. The Sustainable Development Goals (SDG) adopted in 2015, highlight the importance of water, sanitation and hygiene for health. The World Health Organisation (WHO) states that "access to safe drinking-water is essential to health, a basic human right and a component of effective policy for health protection" (WHO, 2017). Despite the sixth United Nations Sustainable Development Goal (SDG) that requires countries to provide safe and affordable drinking water to everyone by 2030 (UNSDG, 2015), the number of South Africans who have access to a source of drinking water in 2018 is only 89% (StatisticsSA, 2018). Many people in especially rural communities in SA still do not have access to piped water in their houses, let alone access to potable water of decent quality (Pearson et al., 2019). These communities use their nearest water source, often a river or dam, for urination, defecation and will use water from the same sources for household purposes (cooking, drinking and washing of clothes). Downstream users are at risk of various waterborne infections and diseases (Pearson et al., 2019).

Almost half of SA receives less than 400 mm rainfall per year (Schulze and Lynch, 2006) and the mean annual runoff is 40 mm (Silberbauer, 2020). Low rainfall results in freshwater being a scarce resource and with the population growing at a rapid rate, there is an increase in water use, which puts this resource under enormous pressure (du Plessis, 2019). The quality of fresh water is further decreasing due to anthropogenic activities such as mining, deforestation, urbanisation, agriculture, destruction of wetlands and river catchments (Pearson et al., 2019). Indicating that SA's water sources are at risk and that a comprehensive water quality and safety plan for monitoring needs to be developed.

Although risk management helps to ensure safe drinking water, this often fails and waterborne disease outbreaks and pollution occurs (Baum and Bartram, 2017). The WHO Guidelines published in 2017 recommend a Water Safety Plan (WSP) as a systematic, preventative risk management strategy that should be applied from catchment to consumer to ensure safe drinking water (Bartram, 2009; Baum and Bartram, 2017; Davison et al., 2005). Water safety plans have been implemented in a number of high income countries, like Australia, Denmark and France amongst others (Baum and Bartram, 2017). The objectives of a WSP is to essentially prevent contamination of raw water resources, treat water to remove contamination, prevent contamination during storage, distribution and handling (Summerill et al., 2010). They protect the public through system assessment, monitoring and management plans (Davison et al., 2005; Summerill et al., 2010).

The benefit from having a WSP is that it improves the control of hazards, assures regulatory compliance, water quality, asset management, communication, knowledge of water supplies and public health outcomes (Gunnarsdóttir et al., 2012; Loret et al., 2016; Rinehold et al., 2017; Setty et al., 2017; Baum and Bartram, 2017). In order for a WSP to be successfully implemented it is essential that there is "buy-in" from senior management and other staff within the organisation. Many high income countries that follow Organisation for Economic Co-operation and Development (OECD) protocols have had success in improving water safety. However, other countries like South Africa (SA) and particularly lower income countries have limited experience in implementation (Baum and Bartram, 2017; Summerill et al., 2010).

1.1.1 Project context and rationale

South Africa is located in a subtropical region, with warm temperate conditions and variable rainfall patterns (≈464 mm annually) rendering the country vulnerable with regards to water availability (Horak et al., 2021; South African Government, 2020). As a developing country, South Africa is faced with an increase in population and rapid urbanization which can be directly associated with an increased demand for formal housing, as well as access to water and sanitation services (Archer et al., 2017). Ensuring a sustained and safe supply of drinking water in South Africa is challenging for the water industry, as this water comes from sources of varying quality including freshwater sources, as well as alternative means, such as the reuse of wastewater and desalination. Thus, any form of raw water pollution results in added pressure on this limited resource (Horak et al., 2021; de Souza et al., 2020).

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. Owing to this, water service institutions in South Africa are constantly under pressure to provide safe water and improved sanitation services. Drinking water treatment plants (DWTPs) and wastewater treatment plants (WWTPs) need to function efficiently to ensure that the water is free of pollutants and pathogens. If they are not able to provide this service and adhere to water quality standards then this could have a negative impact on humans, animals and the environment (Archer et al., 2017). This challenge is not unique to South Africa but, all over the world studies have shown that wastewater effluent, surface water and even drinking water can contain a complex mixture of micropollutants such as pharmaceuticals, pesticides and industrial compounds (Neale and Escher, 2019), often at low concentrations, and targeted chemical analysis cannot detect all chemicals present.

In 2008, the Department of Water and Sanitation (DWS) in South Africa introduced the Blue and Green drop programmes. The Blue Drop Certification programme allows the DWS to measure all aspects that encompass a sustainable water services business and to provide safe water to citizens. This programme gives prominence to the World Health Organisation's (WHO) Water Safety Planning concept as the basis for a proactive, risk-based approach to drinking water quality management from catchment to consumer. The Green drop regulation, on the other hand, is set to improve the standard of wastewater management in SA and is recognised as an international best practice and has received both local and international praise as it also based on a risk management approach.

A combination of suspect screening, non-target analysis and individual chemical risk assessment can identify and provide insights on the risks associated with each of the compounds, but cannot provide any information about the potential toxic effects of the micropollutant mixture (Escher et al., 2020; GWRC, 2020a). Due to the large number of pollutants in the aquatic environment it is no longer possible to evaluate the elimination of a single compound in DWTPs or to guarantee the absence of their transformation products (TP)/ metabolites and disinfection by-products (DBP). It is also difficult to evaluate which mixtures may induce adverse health effects at a later date, given that very low concentrations may already cause adverse effects, e.g. endocrine disrupting effects. There is a scarcity of toxicity information on many of the chemicals currently in commercial use, and in most cases, it is impossible to conduct a proper risk assessment for all organic micro-pollutants. The possible health impacts of these substances are of major interest to water operators and public consumers alike. As these concerns are widely debated today, they require a scientific, objective and rigorous assessment of consumer exposures. 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.

Bioassays can account for mixture effects and are risk-scaled as more potent chemicals have a greater effect in the assay. Consequently, effect-based methods have been recommended to complement chemical analysis in water quality monitoring (Brack et al., 2019). Bioassays based on different stages of cellular toxicity pathways including induction of xenobiotic metabolism, receptor-mediated effects, adaptive stress responses

and apical effects have been widely applied to evaluate the effect of different water extracts (Escher et al., 2014; Rosenmai et al., 2018; Alygizakis et al., 2019). However, there are many different assays available, including multiple assays indicative of the same endpoint. This raises questions about which bioassays and how many should be applied for water quality assessment. Many of the bioassays included have also been applied to support health risk assessment of chemicals (GWRC, 2020a; Wetmore, 2015; Bell et al., 2018) and this information is useful to estimate how the *in vitro* endpoints are connected to health consequences. However, it must be stressed that the use of *in vitro* bioassays to water samples does not allow any prediction of health risk (GWRC, 2020a).

The key focus of this project was to use these innovative methods to assess water quality profiles potentially triggered by residual organic micro-pollutants at different parts of the DWTP, from resource to tap, through the whole water cycle, including WWTPs, conventional and alternative water treatment schemes and water reuse. The study explores the use of *in vitro* and *in vivo* screening assays for providing biologically relevant information in order to establish a toxicity paradigm or adverse outcome pathways (AOPs) that not only determines endocrine disruptive activity, but also for example, inflammation, genotoxicity and oxidative stress.

The University of Pretoria (lead organisation), with North West University (Potchefstroom campus), the University of Stellenbosch, UNISA, together with private partners in the water industry, Aquatox Kits and Solutions and BioLab have formed a consortium for the project. With the support of the WRC aim to bring the knowledge and solutions to South Africa from the different parts of the world for greater impact through smart international collaboration. The ultimate goal is to develop factsheets on the different applicable bioassays and a toolbox of effect-based methods (EBMs) that can be applied in conjunction with chemical analysis to assess risks associated with chemicals in water. This information will enable the relevant role players in the water industry to develop strategies, policies and regulations for the sound management of chemicals in water.

1.1.2 Project aims and objectives

The following were the aims of the project:

1. To develop a list of relevant stakeholders (particular reference to SA) and get an overview of ongoing pre-regulatory to fit in with protocols that will be developed for risk and effect-based monitoring plan for WP2 of the GWRC EBM in Water Safety Planning.
2. Based on the GWRC Toolbox to detect endocrine activity compile a recommended short list of appropriate and available bioassays in SA to develop a toolbox for the assessment of selected endocrine activity endpoints relevant to the South African scenario (De Jager et al., 2011a).
3. Select bioassays based on Mechanisms of Action (MOA) inspired by both Food safety approach & One-Health approach and elaborate innovative and smart combination of effect-based methods (EBM).
4. Provide effect-based trigger values (EBTV) specifically relevant to ecosystem as well as human health targets.

The objectives of this project were to:
1. Compile a list of appropriate bioassays for the assessment of selected MOA and relevant for WSP.
2. Review the Estrogenic activity Toolbox from 2011 (De Jager et al., 2011a).
3. Include *in vitro* assays with additional hormone receptor-mediated effects like androgenic and thyroid activity, as well other endpoints based on MOA for example, oxidative stress, and cell toxicity.
4. Include low complexity i*n vivo bioassays* for example the AMES test and FETAX

1.1.3 General approach and outputs

A comprehensive literature review was done by the GWRC (May 2020 report) to identify applicable bioassays for EBM. This report was used as a guideline to select the relevant bioassays applicable to the South African

scenario. The list of assays was dependent on capacity, infrastructure and which assays are currently available or under development within SA. The report addressing the aims and objectives stated in Section 1.1.2 is presented in two volumes:

- Volume 1 – covering literature review on the use of effect-based methods for water safety planning in South Africa and factsheets on potential bioassays for assessing different modes of action (MoAs) relevant for water safety planning in South Africa (this report)
- Volume 2 – contains a toolbox for sample processing for selected *in vitro* and *in vivo* bioassays

1.2 MANAGING ENDOCRINE DISRUPTING CHEMICALS IN WATER

Many natural and anthropogenic (synthetic) compounds produced and used daily in the home, industry, agriculture and health industry end up in the aquatic environment (van der Linden, 2013). These compounds, also referred to as micropollutants may include pharmaceutical, agricultural and industrial compounds (Neale and Escher, 2019). International studies found micropollutants in source water, WWTPs and DWTPs (Leusch et al., 2018b; Tröger et al., 2018; Glassmeyer et al., 2017; Machado et al., 2016; Neale and Escher, 2019). In the process of treating water for drinking purposes DBPs can form from the reaction of disinfectants, such as chlorine, chloramine and ozone, with organic and inorganic matter naturally present in source water (Neale and Escher, 2019; Richardson and Postigo, 2015) with DBPs (Jeong et al., 2015; Krasner et al., 2016; Neale and Escher, 2019). In addition to this, TP can also form during the water treatment process using disinfectants and during other advanced oxidation processes (Neale and Escher, 2019). In other countries, regulatory procedures have been similarly established to manage chemicals in water. For example, the US Environment Protection Agency (US EPA) has set up screening programs for chemicals for endocrine disrupter activity which have now been incorporated into the ToxCast Program (Braun et al., 2011; Street et al., 2021). In Europe the legislation for EDCs falls under the registration, evaluation, and authorisation of chemicals (REACH). Other international agreements include the Rotterdam Convention of the UNEP, the Stockholm Convention and from the OECD.

1.2.1 Categories of endocrine disrupting chemicals

Advances over the past years has led to the development of more than 80 000 chemicals, only a limited number of chemicals have been tested for their safety or toxicity concern (La Merrill et al., 2020; Futran Fuhrman et al., 2015). Humans and animals are exposed to these chemicals on a daily basis in the form of pesticides, insecticides, food additives and packaging materials; other consumer products like personal care products, pharmaceuticals and other industrial chemicals, for example flame retardants, plasticisers and insecticides (Karthikeyan et al., 2019; La Merrill et al., 2020; Meeker et al., 2010; Muncke, 2011; Bergman et al., 2013) (Table 1-1). Within this list of chemicals that scientists are referring to are a group of chemicals referred to as endocrine disrupting chemicals (EDCs) (Futran Fuhrman et al., 2015).

Table 1-1: Categories and subcategories of EDCs (adapted from Karthikeyan et al. (2019))

Category	Sub-category
Agricultural and farming	Bactericide
	Fertilizer
	Fungicide
	Herbicide
	Insecticide
	Pesticide
	Plant growth regulator
	Poultry feed
	Rodenticide

Category	Sub-category
Consumer products	Acaricide
	Algicide
	Electrical and electronics
	Flame retardant
	Food additives
	Household supplies
	Personal and Healthcare products
	Stationery
	Tobacco products
Industry	Analytical chemicals
	Automotive
	Bleaching agents
	Construction
	Coolant
	Fuel
	Fumigant
	Industrial additives
	Lubricants
	Minerals, metals, heavy metals
	Organic synthesis
	Paints
	Photography
	Plasticizer
	Solvent
Intermediates	Human metabolite
	Industrial intermediates
Medicine and health care	Antimicrobial
	Antiseptic and disinfectant
	Chemicals in diagnosis
	Drugs
Natural sources	Food
	Microorganisms
	Mycoestrogens
	Mycotoxin
	Plant
Pollution	Combustion
	Environmental Pollutant
	Explosives
	Industrial pollutant

1.2.2 Environmental endocrine disrupting chemical exposures and health impacts

Scientists were aware that certain chemicals were able to mimic endogenous hormones (estrogens and androgens) as early as 1946 (Schueler, 1946). One of the first reports indicating that wastewater contained steroid hormones was in 1965 by (Stumm-Zollinger and Fair) in (Snyder and Benotti, 2010). However the issue started gaining attention in the 1990s when Desbrow et al. (1998) and Routledge et al. (1998) found that natural and synthetic steroid hormones in wastewater was linked to reproductive effects on fish living downstream from the wastewater effluents. For example, they can alter the reproductive sexual characteristics of fish by masculinization or feminization (van der Linden et al., 2008). The first "Global Assessment of the State-of-the-science of Endocrine Disruptors" published in 2002 by the International Programme on Chemical Safety (IPCS) showed evidence that the health of some of the wild animal populations had been adversely affected by exposures to EDCs (Damstra et al., 2002). Studies showed abnormalities in male reproductive health and development in fish and other wildlife species exposed to contaminants (Bergman et al., 2013). However at the time the evidence regarding human health was limited due to an insufficient number of studies available.

Today our scientific understanding is far better. We know that there are periods of vulnerability during fetal and postnatal development when exposure to EDCs and particularly mixtures can have a strong and often irreversible effects on the developing organs (Figure 1-1). The effects of exposure on adults may cause fewer effects (Bergman et al., 2013). Maternal, fetal and childhood exposures to chemical pollutants seem to play a large role in the etiology of many endocrine diseases and disorder of the thyroid, immune and digestive, cardiovascular, reproductive and metabolic systems, that include diabetes and obesity (Bergman et al., 2013). Most of the research until recently has focused on the effects of EDCs on the estrogenic system (Freitas, 2012). However, the endocrine system is not limited to the reproductive axis, other hormone-driven signalling systems like progestogens, glucocorticoids, retinoids and thyroid hormones play a critical role in maintaining processes such as homeostasis (He et al., 2010), sexual development (Li and Kim, 2004), metabolism (Wiegratz and Kuhl, 2006), growth (Wightman et al., 2002) and behaviour (Mani and Oyola, 2012) throughout the different life-stages of a number of species (Freitas, 2012; Bergman et al., 2013).

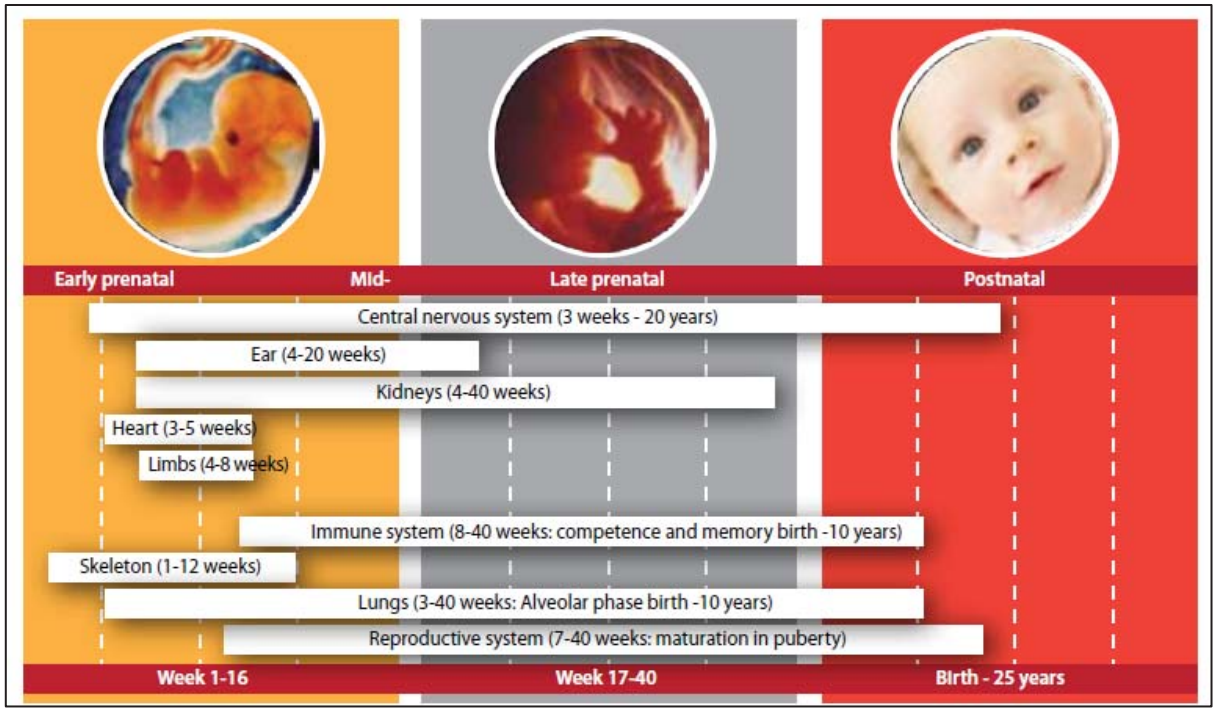

Figure 1-1: Hormones affect the timing of organ development in different ways throughout life, indicating that they are important (Bergman et al., 2013)

In 2013, the WHO and United Nations Environment Programme (UNEP) document presented the current state of the science of EDCs with much new information regarding which chemicals are emerging, adverse effects in humans and animals and importantly the knowledge gaps. Recently, the publication by La Merrill et al. (2020) on the “Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification”, described 5 key characteristics that can be used to identify chemicals with endocrine disruptive properties or characteristics. Hormones and EDCs exert specific actions that are programmed by the specific actions at cellular and tissue levels, this is also influenced by a number of factors (La Merrill et al., 2020; Zoeller et al., 2012), the way hormones are secreted may be an important factor in the signalling mechanism and EDCs can also interfere at this point (Kopp et al., 2017; La Merrill et al., 2020; Mimoto et al., 2017; Zoeller, 2007).

The risk of developing lifelong adverse health effects is increased when exposure to EDCs coincides with the formations and differentiation of organ systems during critical periods of early development (Bergman et al., 2013; La Merrill et al., 2020). The reviews by Karthikeyan et al. (2019) and (La Merrill et al., 2020) show that there is a plethora of literature on mechanistic studies showing the hazards associated with EDC exposure, crucially there is no widely accepted systematic method to integrate the data to identify EDC hazards. The key characteristics (KCs) of human carcinogens were developed and provided a uniform basis for searching, organizing and evaluating mechanistic evidence to support the identification of carcinogens (Smith et al., 2016). The group of authors of the consensus statement, all experts in endocrinology and EDCs, used this to develop ten key characteristics based on hormone actions and EDC effects (La Merrill et al., 2020) (Figure 1-2). Although these KCs express the current knowledge of EDCs, as new information is discovered it will continue to evolve and become more refined.

This approach has been endorsed by the International Agency for Research on Cancer (IARC) and the National Toxicology Program. The National Academies stated it “avoids a narrow focus on specific pathways and hypotheses and provides for a broad, holistic consideration of the mechanistic evidence”. They also indicated that KCs of other hazards not just carcinogens be developed (La Merrill et al., 2020; National Academies of Sciences et al., 2017). The authors developed the KCs of EDCs by looking at the common features of hormone regulation and action that are independent of the range of the effects of hormones during the life cycle (Table 1-2). Action of chemicals interfering with hormone regulation also have characteristics that interfere with hormone regulation (La Merrill et al., 2020).

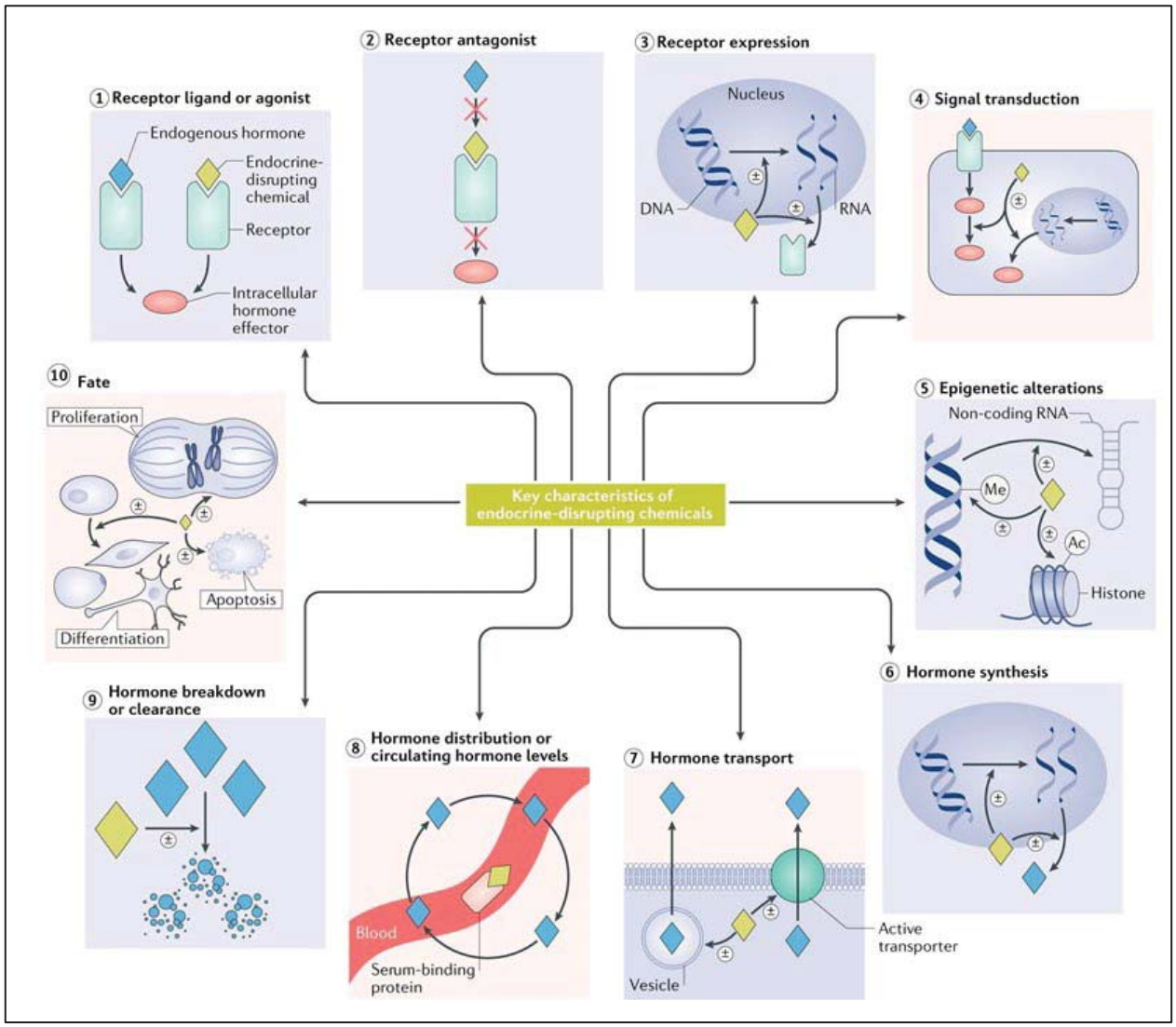

Figure 1-2: Ten key characteristics (KCs) exhibited by endocrine disrupting chemicals (EDCs) (La Merrill et al., 2020)

Arrows identify the ten specific KCs of EDCs. The ± symbol indicates that an EDC can increase or decrease processes and effects. KC1 states that an EDC can interact with or activate hormone receptors. KC2 states that an EDC can antagonize hormone receptors. KC3 states that an EDC can alter hormone receptor expression. KC4 states that an EDC can alter signal transduction (including changes in protein or RNA expression, post-translational modifications and/or ion flux) in hormone-responsive cells. KC5 states that an EDC can induce epigenetic modifications in hormone-producing or hormone-responsive cells. KC6 states that an EDC can alter hormone synthesis. KC7 states that an EDC can alter hormone transport across cell membranes. KC8 states that an EDC can alter hormone distribution or circulating hormone levels. KC9 states that an EDC can alter hormone metabolism or clearance. KC10 states that an EDC can alter the fate of hormone-producing or hormone-responsive cells. Depicted EDC actions include amplification and attenuation of effects. Ac, acetyl group; Me, methyl group.

Table 1-2: Key characteristics of EDCs, mechanisms of action and guideline description (taken from La Merrill et al. (2020))

Key characteristics	Examples of relevant streams of mechanistic evidence	Guideline description (species) [agency and guideline number][1]
KC1. Interacts with or activates hormone receptors	Binding or agonism of hormone receptors	Androgen Receptor Binding (rat) [US EPA 890.1150]; Estrogen Receptor Binding (rat) [US EPA 890.1250, OECD TG 493]; Estrogen Receptor Transcriptional Activation (human stable transfection) [US EPA 890.1300, OECD TG 455]; Androgen Receptor Binding (rat) [US EPA 890.1150]; Androgen Receptor Transcriptional Activation (human stable transfection) [OECD TG 458]; Uterotrophic (rat) [US EPA 890.1600, OECD TG 440]; Hershberger [US EPA 890.1400, OECD TG 441]
KC2. Antagonizes hormone receptors	Antagonism of nuclear or cell surface hormone receptors	Estrogen Receptor Transcriptional Activation (human) [OECD TG 455]; Androgen Receptor Transcriptional Activation (human) [OECD TG 458]; Hershberger [US EPA 890.1400, OECD TG 441]
KC3. Alters hormone receptor expression	Abundance, distribution and degradation of hormone receptors	None
KC4.Alters signal transduction in hormone-responsive cells	Abundance of post- translational modifications, cofactors, transcription factors and transcripts, and activity of associated enzymes	None
KC5.Induces epigenetic modifications in hormone-producing or hormone responsive cells	Chromatin modifications, DNA methylation and non- coding RNA expression	None
KC6. Alters hormone synthesis	Expression or activity of enzymes or substrates in hormone synthesis	Aromatase (human) [US EPA 890.1200]; Steroidogenesis (human) [US EPA 890.1550, OECD TG 456]
KC7. Alters hormone transport across cell membranes	Intracellular transport, vesicle dynamics or cellular secretion	None
KC8. Alters hormone distribution or circulating hormone levels	Blood protein expression and binding capacity, blood levels of pro-hormones and hormones	None
KC9. Alters hormone metabolism or clearance	Inactivation, breakdown, recycling, clearance, excretion or elimination of hormones	None
KC10. Alters fate of hormone-producing or hormone-responsive cells	Atrophy , hyperplasia, hypertrophy, differentiation, migration, proliferation or apoptosis	None
EPA , US Environmental Protection Agency. [1] Only assays that serve as the basis of regulatory decisions of the OECD and US EPA are provided		

1.2.3 EDCs and animal health

The potential adverse effects and the association between a complex mixture of endocrine disruptive pollutants and endocrine disruption of reproduction and development in animal studies was seen in fish and reptiles (like alligators), birds, and mammals living in the Laurentian Great Lakes of North America by Guillette's team of researchers (Guillette and Gunderson, 2001; Guillette and Edwards, 2008; Street et al., 2018). The effects suggested that the complex mixture consisted of estrogenic, androgenic, anti-androgenic and thyroid activity. The effects ranged from subtle changes to permanent effects, that included disturbed sex differentiation, changes in sexual behaviour to name a few (Guillette and Edwards, 2008; Street et al., 2018). Guillette's team found that male alligators exposed *in ovo* (as embryos) to various pesticides exhibited significantly reduced plasma testosterone concentrations, aberrant testicular morphology and small penis size. The female alligators were also affected exhibiting ovarian abnormalities associated with reduced fertility and high embryonic mortality (Guillette and Edwards, 2008; Guillette et al., 1994; Street et al., 2018). This evidence clearly indicated that the aquatic ecosystem health is negatively affected by exposures to these chemical mixtures. The majority of the knowledge on the harmful effects of EDCs stems from animal studies. Therefore it remains a critical to understand effects not only in human health but also environmental health (Street et al., 2018).

1.2.4 EDCs and human health

There has been much debate on whether the effects seen in animal models (including wildlife) can also cause a response in the human population when exposed to the same chemicals (Darbre, 2019). This also includes *in vitro* assays and whether they may be used to predict consequences *in vivo* for human health. One of the early chemicals associated with endocrine disruption is diethylstilbestrol (DES) (Darbre, 2019; Harris and Waring, 2012). Then in the 1990s human sperm quality started declining particularly in men living in Denmark compared to those of their less industrial counterparts in Finland (Darbre, 2019; Jensen et al., 2000). This decline was also associated with an increased incidence of hypospadias, cryptorchidism, undescended testes and testicular cancer, defining testicular dysgenesis syndrome (TDS) which is associated with exposure chemical pollutants, including EDCs (Darbre, 2019; Skakkebæk et al., 2001). In the "State of the Science on Endocrine Disrupting Chemicals" adverse effects as a result of exposure to environmental chemicals is discussed with regards to female, male, cancer, disorders of the thyroid, immune system and other systems are well reviewed (Darbre, 2019; Bergman et al., 2013).

The link between foetal development and adult onset of disease has become more concerning as evidence of transgenerational effects are being seen. *In utero* exposure to EDCs may lead to long-term effects in adult life without additional exposure in animals and humans (Darbre, 2019; Harris and Waring, 2012).

EDC exposures and the predisposition or programming for the development of EDC related cancers is associated with their ability to interfere at the epigenetic level during embryo-fetal programming of tissues and organs (Bergman et al., 2013; Street et al., 2018). This programming occurs during critical windows of development and occurs due to altering the regulation of genes involved in cell cycle, proliferation, apoptosis and other key signalling pathways. This results in consequences throughout the life stages and transgenerational effects (Figure 1-3).

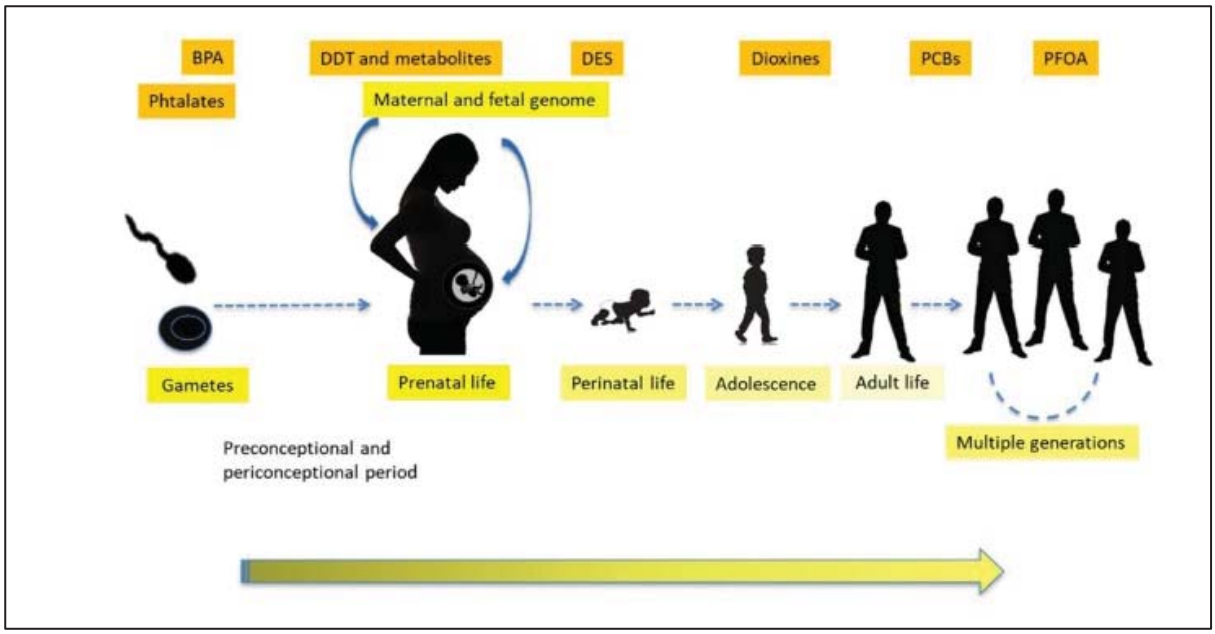

Figure 1-3: Importance of EDC driven epigenetic effects during life course and potential consequences across generations according to the Developmental Origins of Health and Disease (DOHaD) theory (Street et al., 2018).

1.2.5 EDCs and the environment

Present in the aquatic environment, these chemicals and their degradation products are difficult to assess for risk and impact on human and environmental health, as their toxicological data is limited or non-existent, even if their concentrations were known (van der Linden, 2013). Many of the compounds are found at low doses in the environment, but may have a high potency, however, there is no threshold for EDC effects due to the presence of active hormone pathways and EDCs are likely to have effects at low doses (Bergman et al., 2013; Neale et al., 2017a; van der Linden, 2013). 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).

1.3 USE OF THE ADVERSE OUTCOME PATHWAYS FRAMEWORK FOR EDC SCREENING AND TESTING

1.3.1 Overview

In 1991, the World Wildlife Fund hosted a group of expert scientists at the Wingspread Conference in Wisconsin, United States of America (USA). This is where the term “endocrine disruptor” was first coined (Hotchkiss et al., 2008; Street et al., 2018). The Weybridge meeting in 1996 had similar findings to those of the USA, similar meetings were held in Japan, Australia and Korea (Street et al., 2018). The WHO and UNEP released a comprehensive report on EDCs, highlighting the need for more studies into the association between EDC exposures and the risk to human and animal health (Bergman et al., 2013; Street et al., 2018).

These publications have led to discussions on how to introduce regulations to reduce or limit exposure to these harmful chemicals. In order for governments to be able to develop regulatory policies, they need data from comprehensive risk assessments and hazard identification of these environmental pollutants found in the water

systems (Archer et al., 2017). The actions of EDCs pose a number of challenges to traditional risk assessment strategies. Due to EDC action being receptor mediated their actions are specific and it therefore allows for effects at much lower concentrations and through targeted cellular actions (Archer et al., 2017; Darbre, 2019). Because their actions are tissue-specific, it is difficult to predict the exact effect the chemical may have in different tissue. The effects are dependent on the presence and or quantity of the receptors available in the target tissue or cells; they may differ due to the different life stage and critical windows during fetal or postnatal development. A further issue may be that the effects may only appear during adulthood and other late stages of life and include transgenerational effects (Darbre, 2019). The dose responses of EDCs challenge the traditional toxicological assumption of “dose make the poison” linear response that would allow the prediction of a safe low dose to be determined (Darbre, 2019; Vandenberg et al., 2012). The dose response can be nonmonotonic, similar to natural hormones EDCs may have different effects at low and high concentrations (Vandenberg et al., 2012). It has also been shown that EDCs that have similar mechanisms of action and are present in a complex mixture, can result in additive effects (Darbre, 2019; Street et al., 2021).

Work has been done by researchers on developing innovative methods for identifying chemical interactions with a molecular target (e.g. a hormone receptor or enzyme). These interactions can result in downstream biological reactions that can lead to adverse effects . The molecular effects and adverse responses are not usually looked at concurrently. In order to establish causal links between these events and adverse outcomes requires investigation to evaluate biologically plausible connections between responses at different levels and using different methods (La Merrill et al., 2020). In order to link key events in a theoretical biological sequence (for example carcinogenicity and endocrine effects), mode of action (MOA) analyses were developed (La Merrill et al., 2020).

The AOPs framework was developed as an extension to MOA analyses to provide a causal link to an adverse health effect after exposure to an EDC (Darbre, 2019; La Merrill et al., 2020). It works through a sequential or branching chain of events at different levels of biological organisation from molecular, cellular to whole body and population responses (Figure 1-4). It is a useful model to use to investigate environmental chemicals with endocrine disrupting properties, and will improve our understanding of toxicology and disease (Archer et al., 2017; Darbre, 2019, Biomed).

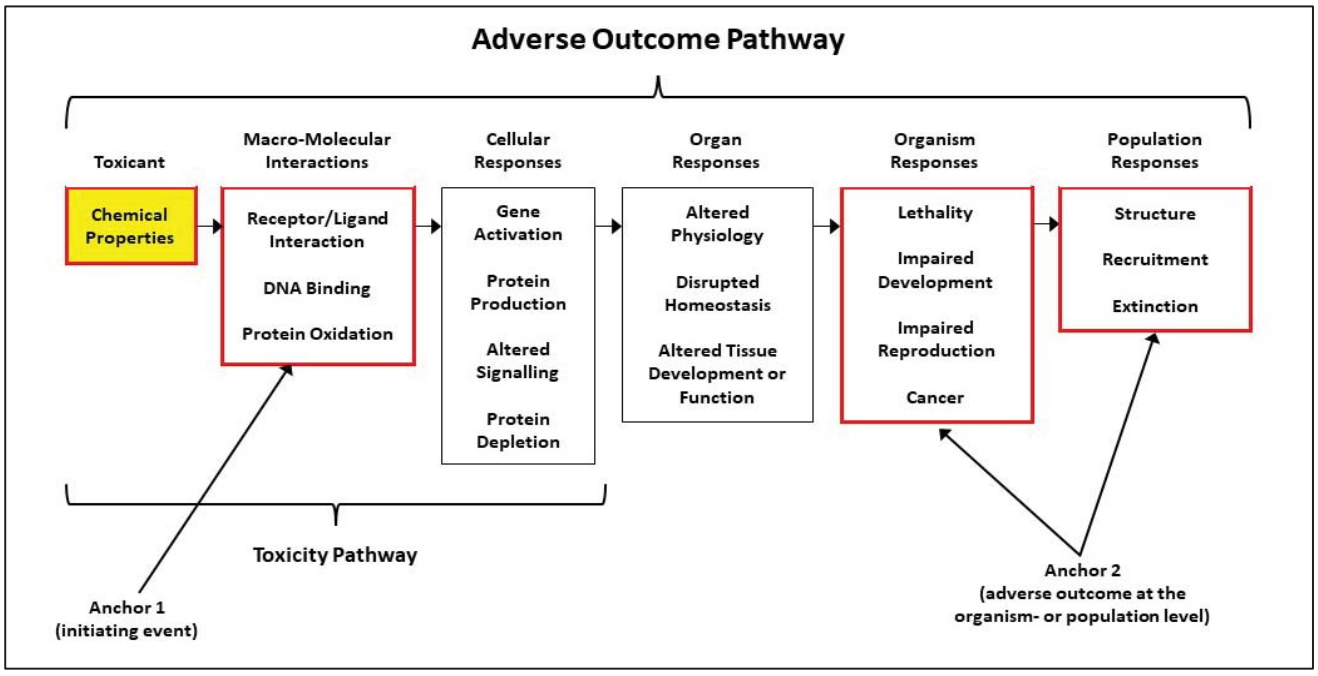

Figure 1-4: Adverse outcome pathways (AOPs) (Ankley et al., 2010; Snyder and Leusch, 2018)

1.3.2 Assessing endocrine disruptive activity in water

Most of the early work on assessing endocrine disrupting effects of exposures to environmental contaminants focussed on the estrogenic axis. Substantial effort has been put into the development of bioassays to assess the estrogenicity of various waters including drinking water, groundwater, surface water and wastewater. The Water Research Commission (WRC) has been involved in funding EDC research since 2001. An early collaboration with the Global Water Research Coalition (GWRC) in 2005, resulted in the publication, "Tools to Detect Estrogenic Activity in Environmental Waters" (GWRC, 2008). The WRC funded the development of a toolbox (WRC report number 1816/1/10) that was based on the global report, but focussed on bioassays with local applicability to the South African scenario (De Jager et al., 2011a). However, the endocrine system is not limited to estrogenic activity, there are several other hormonal pathways (such as androgens, progestagens, glucocorticoids, retinoids, thyroid, and peroxisome proliferator-activated receptor (PPAR) activity, etc.), that play a crucial role in the maintenance of homeostasis, sexual development, metabolism, growth and behaviour. Information on these endocrine endpoints is limited but it is evident that these pathways can also be disrupted by exposure to environmental contaminants (GWRC, 2012).

In 2017 the GWRC published their final report on "Bioanalytical Tools to Analyse Hormonal Activity in Environmental Waters" which investigated bioassays with other EDC endpoints and gave recommendations on the suitability of the various bioassays. However, a limited number of these assays are available in SA and the current toolbox for estrogenic activity (De Jager et al., 2011a) is outdated. The GWRC has embarked on a new study investigating the use of an EBM program for WSP. For water quality assessment and risk management, carcinogenesis, adverse effects on reproduction and development, effects on xenobiotic metabolism, modulation of hormone systems, DNA reactivity and adaptive stress responses are considered the most relevant toxicological endpoints.

1.4 EXPANSION OF THE TOOLBOX OF BIOASSAYS TO ASSESS OTHER MODES OF ACTION RELEVANT FOR WATER SAFETY PLANNING

1.4.1 Summary of GWRC work on the use of effect-based methods for water safety planning

The aquatic environment can contain a diverse range of micropollutants including pesticides, pharmaceuticals and industrial compounds, while water treatment processes, such as disinfection, can form DBPs or other micropollutant transformation products (Glassmeyer et al., 2017; Leusch et al., 2018b). A comprehensive literature review can be found in the GWRC (May 2020) report. The literature identified applicable bioassays using the following terms, as the "topic" in Web of Science and "title, abstract, keyword" in Scopus: *water AND "in vitro bioassay" OR "bioanalytical tool" OR "effect-based method" OR cell-based bioassay" OR "effect-based monitor"*. Additional terms *"in vitro assay" AND "wastewater" OR "sewage" OR "drinking water" OR "recycled water" OR "surface water"* were also searched in Web of Science and Scopus (GWRC, May 2020). The suitability of each collected paper was screened based on outlined criteria below (Table 1-3).

Table 1-3: The suitability of each paper was screened based on the following criteria (GWRC, 2020a)

No.	**Description of the criteria**
1	• Use of high-throughput *in vitro* bioassays (e.g. 96-well or 384-well plate) or well plate-based *in vivo* assays. • High-throughput assays are essential for routine water quality monitoring.
2	• Application to drinking water, surface water, wastewater, recycled water[1] or groundwater. • These water types were selected to cover the potential inputs and outputs of drinking water treatment plants (DWTPs), wastewater treatment plants (WWTPs) and advanced water treatment plants for water reuse.
3	• Water sample extracted by solid-phase extraction (SPE), passive sampling or liquid-liquid extraction (LLE), rather using whole or unextracted water. • Unextracted water may contain metals, salt and other inorganics, in addition to micropollutants, meaning that the response in an unextracted water sample cannot be attributed to micropollutants alone.
4	• Data presented as an effect concentration (EC) or equivalent concentration (EQ). • This information is essential to compare between studies that applied the same assay, so any studies that only reported positive/negative results, as was often the case for mutagenicity and genotoxicity assays, were excluded. • Note that more recently mutagenicity and genotoxicity tests such as the Ames and umuC assays have also gone beyond positive/negative results and provide EC values.

[1] For the purpose of this review, water recycling for direct or indirect drinking water augmentation is considered. This includes processes such as membrane filtration (e.g. reverse osmosis), advanced oxidation (ozonation, UV, hydrogen peroxide)

The initial search terms identified 623 papers, adding the additional terms to the search it brought the total papers to 760, another 24 papers were not identified in the original search databases making the final total 784. After applying the screening criteria, the number of papers were reduced to 124. The reviewed studies came from all continents excluding South America, 23 countries in total, approximately half the studies were done in Europe. The studies were published across a range of 27 journals, with the majority published in Water Research (20%; impact factor 9.1) and Science of the Total Environment (15%; impact factor 6.55). The majority of the studies have been published since 2016, indicating that these are recent studies (GWRC, 2020b). The most common water types found in the studies were surface water (65%) and wastewater (52%), the majority of studies applied solid phase extraction (SPE) (89%) or liquid liquid extraction (LLE) to extract water samples prior to bioanalysis. Some studies did apply passive sampling. A comprehensive list of the papers and the methods of extraction can be found in the GWRC WP3.2 report (GWRC, 2020a).

From the literature review the most commonly applied assay endpoints were indicative of xenobiotic metabolism, receptor-mediated effects, reactive toxicity, adaptive stress responses and apical effects. These were compared and their ability to detect effects in different water extracts was evaluated. The findings showed that after sufficient enrichment (or concentration of the original sample) the following bioanalytical assays showed activation of the **aryl hydrocarbon receptor (AhR)**, activation of the **pregnane X receptor (PXR)**, activation of the **estrogen receptor (ER)**, activation of the **androgen receptor (AR)**, **phytotoxicity**, **oxidative stress response** and **bacterial toxicity**. They were able to detect effects in wastewater, surface water and drinking water after sufficient enrichment. While the mammalian reporter gene assays indicative of activation of the thyroid receptor (TR) and activation of the mineralocorticoid receptor (MR) did not induce a response in any of the tested water extracts.

The GWRC WP3.2 reported on current assays indicative of different stages of cellular toxicity pathways and apical effects to detect effects in different water extracts. The reviewed assays are based on mammalian cell lines, while the assays measuring reactive toxicity are bacterial. Many of the assays indicative of apical effects used well plate-based *in vivo* assays. To allow comparison between different assays with similar endpoints the results of the assays were expressed in different dose-metrics and the units were standardised to allow comparison between different assays of similar endpoints. This is well explained in the GWRC (2020a) report.

1.4.2 Potential bioassays for assessing different modes of action (MoAs) relevant for water safety planning

Effect-based monitoring using bioanalytical tools (*in vitro* bioassays and well plate-based *in vivo* assays) are useful especially when applied in parallel to chemical analysis to detect the effect of all known and unknown chemicals that are active in a particular bioassay (Leusch et al., 2014). There is usually a selection of bioassays available for the similar endpoints. The bioanalytical endpoints listed below have been selected as potential EMB tools to complement or run in parallel to chemical analyses:

- Xenobiotic metabolism
- Hormone Receptor-mediated effects
- Other Receptor-mediated effects
- Reactive toxicity
- Adaptive stress responses
- Apical effects
- Multiplexed high-throughput assays

1.5 THE ROLE OF XENOBIOTIC METABOLISM IN CHEMICAL TOXICITY SCREENING IN WATER

Induction of xenobiotic metabolism may not lead to cytotoxicity, but it is an indicator of the presence of exposure to bioactive chemicals (Escher et al., 2014). Aryl hydrocarbon receptor (AhR), PPAR (α, β and γ) and PXR are important xenobiotic metabolism pathways (Escher et al., 2014; Omiecinski et al., 2010).

1.5.1 Activation of the Aryl Hydrocarbon Receptor (AhR)

Table 1-4 summarises the bioassays available for AhR receptor mediated activity in water extracts (GWRC, 2020a).

Table 1-4: Common cell-based reporter gene assays applied to evaluate aryl hydrocarbon receptor (AhR) activity in water extracts (GWRC, 2020a)

Assay	Cell line	Detection method	TCDD EC_{10} (M)	TCDD EC_{10} (ng/L)	EC Reference
AhR CAFLUX	H1.G1.1c3	Fluorescence	6.50×10^{-13}	0.21	(Jia et al., 2015)
AhR CAFLUX	H4.G1.1c2	Fluorescence	6.87×10^{-13}	0.22	(Konig et al., 2017, Neale et al., 2015)
AhR CALUX	H4L1.1c4	Luminescence	5.92×10^{-13}	0.19	(Nivala et al., 2018)
AhR reporter gene assay	HepG2	Luminescence	$6.22\times10^{-11*}$	20	(Rosenmai et al., 2018)
H4IIE-luc	H4IIE	Luminescence	1.60×10^{-13}	0.05	(Lee et al., 2015)

* Presented EC_{10} value converted from EC_{50} value assuming a slope of the log-logistic concentration response curve of 1.

1.5.2 Activation of the Peroxisome Proliferator-Activated Receptor γ (PPARγ)

The PPAR is also a transcription factor that belongs to the superfamily of nuclear receptors and is involved in the regulation of glucose and lipid metabolism and not so much in xenobiotic metabolism (van Raalte et al., 2004; Scarsi et al., 2007). As the name indicates, the main function of PPAR is the delivery of peroxisomes, which are important for fatty acid oxidation and thus relevant for lipid metabolism. The three isoforms, PPARα, PPARß (also called δ) and PPARγ, are encoded by different genes, show different tissue expression and perform slightly different functions. PPARα is expressed predominantly in metabolically active tissues like liver and kidney cells where its ligands include fatty acids, hypolipidemic drugs and xenobiotics (Seimandi et al., 2005). PPARα regulates the expression of genes involved in fatty acid-oxidation and is a major regulator of energy homeostasis (van Raalte et al., 2004). PPARγ is the key receptor in maintaining glucose and lipid homeostasis and its activation increases the insulin resistance of the cell (Scarsi et al., 2007; van Raalte et al., 2004). Environmental water extracts have been tested in the PPARγ assay and only a few studies to PPARα (Escher et al., 2014; Alygizakis et al., 2019). A summary of the PPAR assays that have been frequently applied to water extracts are listed in Table 1-5 (GWRC, 2020a).

Table 1-5: Common cell-based reporter gene assays applied to evaluate peroxisome proliferator-activated receptor (PPARγ) activity in water extracts (GWRC, 2020a)

Assay	Cell line	Detection method	Rosiglitazone EC_{10} (M)	Rosiglitazone EC_{10} (ng/L)	EC Reference
PPARγ CALUX	U2OS	Luminescence	1.00×10-8	3,600	(Gijsbers et al., 2011)
PPARγ GeneBLAzer	HEK 293	Fluorescence	3.30×10-10	118	(Jia et al., 2015)

1.5.3 Activation of the Pregnane X Receptor (PXR)

The PXR has a protective role of the body, it regulates proteins involved in the metabolism, the conjugation, and the transport of many exogenous and endogenous compounds, including many environmental molecules (Grimaldi et al., 2015). It is a promiscuous nuclear receptor with a large binding domain. Permanent activation of this receptor by xenobiotics may lead to premature drug metabolism, the formation, and accumulation of toxic metabolites and defects in hormones homeostasis (Orans et al., 2005; Grimaldi et al., 2015). Table 1-6 shows the summary of the common assays used to detect PXR activation in water samples (GWRC, 2020a)

Table 1-6: Common cell-based reporter gene assays used to evaluate activation of the pregnane X receptor (PXR) in water extracts (GWRC, 2020a)

Assay	Cell line	Detection method	di(2-ethylhexyl)-phthalate (DEHP) EC_{10}/PC_{10} (M)	di(2-ethylhexyl)-phthalate (DEHP) EC_{10}/PC_{10} (µg/L)	EC Reference
HG5LN hPXR	HG5LN (HeLa)	Luminescence	2.77×10^{-7}	108	(Escher et al., 2018)
PXR CALUX	U2OS	Luminescence	3.97×10^{-7}	155	(Escher et al., 2018)

1.6 USE OF HORMONE RECEPTOR-MEDIATED EFFECTS IN CHEMICAL TOXICITY SCREENING IN WATER

Several hormonal systems (such as androgens, progestagens, glucocorticoids, retinoids, thyroid, RXR and PPAR activity, etc.) are essential for the maintenance of homeostasis, sexual development, metabolism, growth and behaviour (GWRC, 2017). Endocrine disrupting chemicals, including synthetic hormones, industrial chemicals and pesticides, can interfere with hormonal systems by interacting with hormone receptors in an agonistic or antagonistic way (le Maire et al., 2010). The ER and AR mediated activity has been the most commonly studied receptors but other relevant nuclear receptors include the glucocorticoid receptor (GR), progesterone receptor (PR), TR, MR, retinoic acid receptor (RAR) and retinoid X receptor (RXR). A summary of the different nuclear receptors can be found in Le Maire et al. (2010). Leusch et al. (2017) addresses the sensitivity of these assays in his review.

1.6.1 Estrogen receptor

- Agonist

Many of the harmful effects of EDCs are attributed to their interference with hormonal signalling mediated by nuclear hormone receptors (NHRs). Nuclear receptors ERα and ERβ are important for the growth and homeostasis of the uterus and mammary glands, as well as bones and cardiovascular system (le Maire et al., 2010; GWRC, 2020a). The majority of assays applied to environmental water extracts focus on ERα (e.g. ERα CALUX, ERα GeneBLAzer), though the T47D-KBluc assay uses the T47D cell line, which expresses both ERα and ERβ (Wilson et al., 2004). In the literature estrogenic activity was by far the most commonly studied endpoint in water extracts, using a number of different assays. The GWRC report focused on assays that have been applied to water samples in four or more studies (GWRC, 2020a). In order to represent an *in vivo* assay the embryonic zebrafish assay (EASZY) was included (Brion et al., 2019). The GWRC report provides a summary of the included activation of ER assays with similar sensitivity for the mammalian reporter gene assays. The reference compound 17β-estradiol EC_{10} value varied between 0.13 ng/L for T47D-KBluc to 2.1 ng/L for HeLa-9903. The yeast estrogen screen (YES) and EASZY were less sensitive, additional information can be found in (Leusch et al., 2017). Table 1-7 summarises the various assays that have been used to detect estrogenic agonist activity.

Table 1-7: Common assays applied to evaluate estrogenic activity in water extracts (GWRC, 2020a)

Assay	Cell line/test system	Detection method	17β-estradiol EC_{10} (M)	17β-estradiol EC_{10} (ng/L)	EC Reference
Yeast reporter gene					
YES	Yeast	Absorbance	$3.75\times10^{-11*}$	10.2*	(Escher et al., 2008)
Mammalian reporter gene					
ERα CALUX	U2OS	Luminescence	7.13×10^{-13}	0.19	(Jia et al., 2015)
ERα GeneBLAzer	HEK 293	Fluorescence	9.87×10^{-12}	2.7	(Nivala et al., 2018)
HeLa-9903	HeLa	Luminescence	$7.78\times10^{-12*}$	2.1*	(Valcarcel et al., 2018)
MELN	MCF-7	Luminescence	2.42×10^{-12}	0.66	(Neale et al., 2015)
MVLN	MCF-7	Luminescence	$3.16\times10^{-12*}$	0.86*	(Shue et al., 2009)
T47D-KBluc	T47D	Luminescence	$4.63\times10^{-13*}$	0.13*	(Liu et al., 2018)
Cell proliferation					

Assay	Cell line/test system	Detection method	17β-estradiol EC_{10} (M)	17β-estradiol EC_{10} (ng/L)	EC Reference
E-Screen	MCF7	Absorbance (cell viability measured using CellTiter (MTS))	$8.18\times10^{-13*}$	0.22*	(Macova et al., 2010)
Whole organism					
EASZY	Embryonic zebrafish	Fluorescence	EC_{50} 6.20×10^{-10}	EC_{50} 168	(Brion et al., 2019)

*Presented EC10 value converted from EC50 value assuming a slope of the log-logistic concentration response curve of 1.

- Antagonist

Only 14% of the papers reviewed measured the anti-estrogenic activity in environmental water extracts (GWRC, 2020a). Three assays commonly applied to evaluate anti-estrogenic activity were the yeast anti-estrogen screen (YAES) and the mammalian reporter gene ERα CALUX and ERα GeneBLAzer. All used tamoxifen as the reference compound. Anti-estrogenic activity was either low or below detection in surface water, while no anti-estrogenic activity was detected in drinking water (GWRC, 2020a). Table 1-8 summarises the assays that measure anti-estrogenic activity in water extracts (GWRC, 2020a).

Table 1-8: Assays applied to evaluate anti-estrogenic activity in water extracts (GWRC, 2020a)

Assay	Cell line/test system	Detection method	Tamoxifen $EC_{SR0.2}$ (M)	Tamoxifen $EC_{SR0.2}$ (µg/L)	EC Reference
Yeast reporter gene					
YAES	Yeast	Absorbance	6.00×10^{-7}	223	(Conroy et al., 2007)
Mammalian reporter gene					
ERα CALUX	U2OS	Luminescence	1.50×10^{-9}	0.56	(Jia et al., 2015)
ERα GeneBLAzer	HEK 293	Fluorescence	5.86×10^{-6}	2177	(Neale et al., 2020b)

1.6.2 Androgen receptor

- Agonist

The AR is expressed in a range of tissues and has implications for the development and maintenance of a number of systems, including the reproductive, immune, musculoskeletal and cardiovascular systems (Wilson et al., 2002; Davey and Grossmann, 2016). According to the GWRC report 39% of the studies investigated agonistic androgen activity. Those assays that were used with regards to water extracts include the yeast androgen screen (YAS) and the mammalian reporter gene assays AR CALUX, AR GeneBLAzer and MDA-kb2. Based on the reference compound dihydrotestosterone (DHT), the mammalian reporter gene assays were more sensitive than YAS (GWRC, 2020a), summarised in Table 1-9. Further information about assay sensitivity can be found in Leusch et al. (2017).

Table 1-9: Common assays applied to evaluate androgenic activity in water extracts (GWRC, 2020a)

Assay	Cell line/test system	Detection method	DHT EC_{10} (M)	DHT EC_{10} (ng/L)	EC Reference
Yeast reporter gene					
YAS	Yeast	Absorbance	2.86×10^{-10}	83	(Sohoni and Sumpter, 1998)
Mammalian reporter gene					
AR CALUX	U2OS	Luminescence	1.00×10^{-10}	29	(Jia et al., 2015)
AR GeneBLAzer	HEK 293	Fluorescence	1.40×10^{-10}	41	(Leusch et al., 2017)
MDA-kb2*	MDA-MB-453	Luminescence	3.12×10^{-11}	9.1	(Neale et al., 2017c)

*MDA-kb2 assay is available in South Africa

- Antagonist

Anti-androgenic activity was assessed in 27 of the reviewed studies (22% of studies). Both the yeast and the mammalian reporter gene assay seemed to be similarly sensitive based on the reference compound flutamide $EC_{SR0.2}$ values in Table 1-10. Further information can be found in GWRC (2020a).

Table 1-10: Common assays applied to evaluate anti-androgenic activity in water extracts (GWRC, 2020a)

Assay	Cell line/test system	Detection method	Flutamide $EC_{SR0.2}$ (M)	Flutamide $EC_{SR0.2}$ (µg/L)	EC Reference
Yeast reporter gene					
YAAS	Yeast	Absorbance	$7.50\times10^{-7*}$	207*	(Stalter et al., 2011)
Mammalian reporter gene					
AR CALUX	U2OS	Luminescence	1.10×10^{-6}	304	(Jia et al., 2015)
AR GeneBLAzer	HEK 293	Fluorescence	$5.50\times10^{-7*}$	152*	(Leusch et al., 2017)
MDA-kb2	MDA-MB-453	Luminescence	2.07×10^{-7}	57	(Neale et al., 2017b)

*Presented $EC_{SR0.2}$ value converted from EC_{50} value assuming a slope of the log-logistic concentration response curve of 1.

1.6.3 Thyroid receptor

- Agonist

A comprehensive review was published by the GWRC indicating that there are a variety of assays with different endpoints and different reference compounds for thyroid activity. These include iodine uptake, thyroid peroxidase inhibition, thyroid hormone transport protein binding, thyroid receptor gene activation and cell proliferation assays (GWRC, 2012). This has been updated in the current report and the assays that have been used to investigate thyroid activity in environmental water extracts include yeast reporter gene assays, mammalian reporter gene assays, cell proliferation assays and a whole organism assay using the *xenopus eleutheroembryo* thyroid assay (XETA) are listed in Table 1-11. Further details can be found in the appendices section (Table A12) in the GWRC (2020a) report. Based on reference compound triiodothyronine (T3), the reporter gene assays were the most sensitive. It also suggests that the XETA assay, which incorporates toxicokinetic processes, may be more suitable to evaluate thyroid activity in water extracts than mammalian reporter gene assays (GWRC, 2020a).

Table 1-11: Common assays applied to evaluate thyroid activity in water extracts (GWRC, 2020a)

Assay	Cell line/test system	Detection method	Triiodothyronine EC_{10} (M)	Triiodothyronine EC_{10} (ng/L)	EC Reference
Yeast reporter gene					
Yeast two-hybrid	Yeast	Absorbance	2.60×10^{-8}	17,000	(Li et al., 2008)
Mammalian reporter gene					

Assay	Cell line/test system	Detection method	Triiodothyronine EC_{10} (M)	Triiodothyronine EC_{10} (ng/L)	EC Reference
TRβ CALUX	U2OS	Luminescence	8.60×10^{-12}	5.6	(Jia et al., 2015)
TRβ GeneBLAzer	HEK 293	Fluorescence	6.00×10^{-11}	41	(Leusch et al., 2017)
GH3.TRE-Luc	GH3	Luminescence	$6.67\times10^{-12*}$	4.3*	(Leusch et al., 2018a)
PC-DR-LUC	PC12	Luminescence	$2.00\times10^{-11*}$	13*	(Jugan et al., 2009)
Cell proliferation					
T-Screen	GH3	Fluorescence (cell viability measured using alamarBlue (Resazurin))	2.80×10^{-10}	182	(Jia et al., 2015)
Whole organism					
XETA	Embryonic Xenopus	Fluorescence	EC_{50} 4.50×10^{-9}	EC_{50} 3,000	(Leusch et al., 2018a)

*Presented EC10 value converted from EC50 value assuming a slope of the log-logistic concentration response curve of 1.

- Antagonist

Three assays have been applied to evaluate anti-thyroid activity in environmental water extracts, the yeast reporter gene yeast two-hybrid assay and the mammalian reporter gene assays GH3.TRE-Luc and TRβ GeneBLAzer (Table 1-12). The anti-thyroid activity assay reference compound is the pharmaceutical amiodarone hydrochloride, anti-thyroid activity was detected in the yeast two-hybrid assay and only the TRβ GeneBLAzer had a response in wastewater effluent. None of the samples having a response in GH3.TRE-Luc in antagonist mode (GWRC, 2020a).

Table 1-12: Assays applied to evaluate anti-thyroid activity in water extracts (GWRC, 2020a)

Assay	Cell line/test system	Detection method	Amiodarone hydrochloride EC_{50} (M)	Amiodarone hydrochloride EC_{50} (µg/L)	EC Reference
Yeast reporter gene					
Yeast two-hybrid	Yeast	Absorbance	3.10×10^{-5}	21,000	(Li et al., 2008)
Mammalian reporter gene					
TRβ GeneBLAzer	HEK 293	Fluorescence	7.30×10^{-6}	5,000	(Leusch et al., 2018a)
GH3.TRE-Luc	GH3	Luminescence	8.40×10^{-6}	5,700	(Leusch et al., 2018a)

The GH3.TRE-Luc assay is available in SA, but it still needs further refinement regarding the method.

1.6.4 Glucocorticoid receptor

- Agonist

Corticosteroid receptors, that includes the GR and MR, are widely expressed in organisms and are associated with numerous diseases and health outcomes (Zhang et al., 2019). The GR controls the actions of glucocorticoids, and a wide range of environmental contaminants can interfere with glucocorticoid activity (Zhang et al., 2019). Mammalian reporter gene assays have been applied to evaluate glucocorticoid activity in environmental water extracts summarised in Table 1-13. The GR CALUX and GR GeneBLAzer were the most commonly used assays to detect GR agonist activity. Pharmaceutical dexamethasone serves as the assay reference compound, with the lowest EC10 reported for GR GeneBLAzer (GWRC, 2020a).

Table 1-13: Assays applied to evaluate glucocorticoid activity in water extracts (GWRC, 2020a)

Assay	Cell line	Detection method	Dexamethasone EC_{10} (M)	Dexamethasone EC_{10} (ng/L)	EC Reference
GR CALUX	U2OS	Luminescence	8.00×10^{-10}	314	(Jia et al., 2015)
GR GeneBLAzer	HEK 293T	Fluorescence	2.08×10^{-10}	82	(Nivala et al., 2018)
GR Switchgear	HT1080	Luminescence	5.00×10^{-10}	196	(Jia et al., 2015)

- Antagonist

Literature only showed two assays, the GR CALUX and GR GeneBLAzer, were used to assess anti-glucocorticoid activity. The GR GeneBLAzer appeared much more sensitive than GR CALUX based on the reference compound mifepristone $EC_{SR0.2}$ values (Table 1-14) (GWRC, 2020a).

Table 1-14: Two assays were applied to evaluate anti-glucocorticoid activity in water extracts (GWRC, 2020a)

Assay	Cell line	Detection method	Mifepristone $EC_{SR0.2}$ (M)	Mifepristone EC_{10} (ng/L)	EC Reference
GR CALUX	U2OS	Luminescence	2.90×10^{-9}	1246	(Jia et al., 2015)
GR GeneBLAzer	HEK 293T	Fluorescence	1.00×10^{-10}	43	(Jia et al., 2015)

1.6.5 Mineralocorticoid receptor (MR)

The MR (NR3C2) classically mediates aldosterone effects on electrolyte balance and blood pressure. MR-expressing tissues include distal parts of the nephron, colon, salivary, and sweat glands (Caprio et al., 2007). The MR controls the action of mineralocorticoids (Zhang et al., 2019). There is one assay that is used to assess mineralocorticoid activity in water extracts, HG5LN-hMR, which can be run in both agonist, (reference compound aldosterone (Leusch et al., 2018b)) and antagonist (reference compound spironolactone (Bellet et al., 2012)) mode. Anti-mineralocorticoid activity has been detected in wastewater influent, wastewater effluent and surface water, but was below the limit of detection in drinking water (GWRC, 2020a).

1.6.6 Progesterone receptor

- Agonist

In the GWRC (2020a) report the PR CALUX and PR GeneBLAzer, are indicated as the tests that have been used to evaluate progestogenic activity in environmental water extracts. Both used the synthetic hormone levonorgestrel, as the agonist reference compound with both assays having similar EC10 values (Table1-15).

Table 1-15: Assays used to evaluate progestogenic activity in water extracts (GWRC, 2020a)

Assay	Cell line	Detection method	Levonorgestrel EC_{10} (M)	Levonorgestrel EC_{10} (ng/L)	EC Reference
PR CALUX	U2OS	Luminescence	$3.44\times10^{-11*}$	10.8	(Scott et al., 2014)
PR GeneBLAzer	HEK 293T	Fluorescence	$1.22\times10^{-11*}$	3.8	(Leusch et al., 2018b)

*Presented EC10 value converted from EC50 value assuming a slope of the log-logistic concentration response curve of 1.

- Antagonist

The PR CALUX and PR GeneBLAzer assays were also used to evaluate anti-progestogenic activity in environmental extracts, using the reference compound mifepristone the $EC_{SR0.2}$ value, was more sensitive in the PR CALUX than PR GeneBLAzer (GWRC, 2020a) Table 1-16.

Table 1-16: Assays applied to evaluate anti-progestogenic activity in water extracts (GWRC, 2020a)

Assay	Cell line	Detection method	Mifepristone $EC_{SR0.2}$ (M)	Mifepristone EC_{10} (ng/L)	EC Reference
PR CALUX	U2OS	Luminescence	2.00×10^{-11}	8.6	(Jia et al., 2015)
PR GeneBLAzer	HEK 293T	Fluorescence	3.00×10^{-10}	129	(Nivala et al., 2018)

1.6.7 Retinoic acid receptor (RAR) and Retinoid X receptor (RXR)

Limited studies have investigated the RAR and RXR. Contrasting results were obtained with the different studies (Leusch et al., 2018b; Konig et al., 2017). More information can be found in the GWRC (2020a) report.

1.7 OTHER RECEPTOR-MEDIATED EFFECTS

Other receptor-mediated specific modes of action include phytotoxicity, neurotoxicity and specifically for the mode of action relevant to pharmaceuticals. A summary can be found the GWRC (2020a) report.

1.8 USE OF REACTIVE TOXICITY IN CHEMICAL TOXICITY SCREENING IN WATER

Reactive toxicity occurs when chemicals form covalent bonds with DNA, proteins and membrane lipids. DBPs, which form due to the reaction of disinfectants, such as chlorine and chloramine, with organic matter in water, are reactive chemicals and are responsive in a number of assays indicative of reactive toxicity (Stalter et al., 2016). Two common bacterial assays used to assess reactive toxicity in environmental extracts include the umuC assay to detect genotoxicity and the Ames assay to detect mutagenicity. Both assays can be run either with or without rat liver S9 fraction, which is used to simulate metabolic activation. Suggested endpoints are listed below and further information can be found in the GWRC report (GWRC, 2020a).

1. Genotoxicity
2. Mutagenicity

1.9 USE OF ADAPTIVE STRESS RESPONSES IN CHEMICAL TOXICITY SCREENING

Adaptive stress responses pathways are activated to help restore cells back to homeostasis after damage from stressors, including organic chemicals, heat, ionizing radiation (Simmons et al., 2009). Three adaptive stress response pathways commonly applied to environmental water extracts (GWRC, 2020a):

- Oxidative stress response (Nrf2),
- Genotoxicity (p53)
- Inflammation (NF-κB response)

The cellular response to oxidative stress is an important part of the cellular defence against chemical insult (Escher et al., 2014). However, this mechanism has not yet been addressed in water quality assessment apart

from some attempts to directly quantify reactive oxygen species. Escher et al. (2012) demonstrated the applicability of the oxidative stress response of the AREc32 cells for water quality testing using water samples from sewage to drinking water. Table 1-17 summarises the current list of assays, further information can be found in the GWRC report (GWRC, 2020a).

Table 1-17: Common assays applied to evaluate oxidative stress response in water extracts (GWRC, 2020a)

Assay	Cell line	Detection method	tBHQ $EC_{IR1.5}$ (M)	tBHQ $EC_{IR1.5}$ (µg/L)	EC Reference
AREc32	MCF-7	Luminescence	1.32×10^{-6}	219	(Escher et al., 2012)
ARE GeneBLAzer	HepG2	Fluorescence	2.44×10^{-6}	406	(Neale et al., 2015)
Nrf2 CALUX	U2OS	Luminescence	$1.00\times10^{-6*}$	166	(van der Linden et al., 2014)
Nrf2 reporter gene assay	HepG2	Luminescence	2.00×10^{-6}	332	(Lundqvist et al., 2019)
Nrf2-MDA-MB	MDA-MB-231-745	Luminescence	3.30×10^{-5}	5490	(Jia et al., 2015)

*LOEC at an induction factor of 1.5

1.10 USE OF APICAL EFFECTS IN CHEMICAL TOXICITY SCREENING

In addition to assays indicative of different stages of the cellular toxicity pathway, whole organism assays indicative of apical effects are commonly applied to water quality monitoring. Further, some organisms, such as zebrafish, are used as a model species for human health risk assessment (Bambino and Chu, 2017). These assays can provide information about mortality, growth and development and capture effects from multiple toxicity pathways resulting in the same apical effect (Wernersson et al., 2015). While these assays are often used for direct toxicity assessment, we have focused on assays applied to water extracts, including:

1. Bacterial toxicity
2. Algal growth inhibition
3. Fish embryo toxicity

CHAPTER 2: TOWARDS THE USE OF EFFECT-BASED METHODS FOR WATER SAFETY PLANNING IN SOUTH AFRICA

2.1 WATER QUALITY MANAGEMENT IN SOUTH AFRICA

The two laws in South Africa that governs the quality of the country's water resources are the Water Services Act (WSA) (Act 108 of 1997) and the National Water Act (NWA) (Act 36 of 1998). The WSA has rules for municipalities to provide potable water and sanitation services to households and other municipal water users. The NWA is the domain of the national government and provides guidelines for how the water in streams, rivers, dams, and groundwater should be protected, used, developed, conserved, managed and controlled in an integrated manner (De la Harpe and Ramsden, 2017).

Under the NWA, the national government is responsible for the National Water Resource Strategy and should set targets for water quality for different water resources. This had been done in the form of the Water Quality Guidelines, but except for the drinking water guideline, had not been updated since 1996. The then Department of Water Affairs published eight volumes in a series of South African Water Quality Guidelines. 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 Annexure. The domestic use water guideline had been adopted 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 Annexure A. It differs from that of domestic use and was mainly derived from the World Health Organization's 'Guidelines for drinking-water quality' (SANS 241, 2015). The SANS 241 is currently (2022) under revision again.

Although the Water Quality Guideline documents also include guidelines for characteristics such as smell, turbidity and microbes, the focus of this report is mainly on the chemical compounds that may be toxicants. These guidelines for the different water uses have different levels for the same compounds, and sometimes the list of compounds are different (Annexure A): Barium is only listed in the SANS 241 document (drinking water). And aluminium (Al) appears in 'domestic use', three different aspects of agricultural use, aquatic ecology, and in the SANS 241 document but across these six categories there are five different acceptable concentrations. This situation is not conducive to effective monitoring: regular monitoring regarding toxicants is non-existent, let alone meeting guidelines with these variations.

The collection of data and interpretation of information on water and sanitation are critical for effective water and sanitation management. The Directorate Resource Quality Information Services (RQIS) in the National Department of Water and Sanitation (DWS) oversees six monitoring programmes on a national level: i) chemical, ii) microbial, iii) eutrophication, iv) toxicity and v) radioactivity monitoring programme along with vi) ecosystem monitoring programme (DWS, 2019a).

The National Toxicity Monitoring Programme (NTMP) is pertinent to this report because it is concerned with monitoring the concentrations of chemical pollutants in South African rivers and dams. The latest publication regarding the work done by RQIS on the NTMP itself that could be found on the RQIS website is a 'Draft phase 3: Pilot implementation and testing of design 2008-09' in which a case study was reported: 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 a number of organochlorine pesticides, some alkyl phenols, a few phthalates, and toxaphene. A final version of this has not yet been published on the site. A number of toxicity tests using *Danio rerio*, *Daphnia pulex*, *Poecilia reticulata*, *Selenastrum capricornutum* and

engineered *Alliivibrio fischeri* enzyme inhibition tests were also included (DWS, 2018). Research papers by Rimayi et al., are also listed but these studies were on a small number of sites and for selected targeted compounds only (Rimayi et al., 2015; Rimayi et al., 2016; Rimayi et al., 2017; Rimayi et al., 2018c; Rimayi et al., 2018a; Rimayi et al., 2018b; DWS, 2018). Some of the papers co-authored by Rimayi on similar topics have not been referenced on the RQIS website (Batayi et al., 2020; Rimayi and Chimuka, 2019; Rimayi et al., 2019).

The Blue Drop and the Green Drop Certification Programmes, based on the WHO's water safety planning approach were introduced in 2008 and implemented in 2009 by DWS (Burgess, 2016). The Blue and Green Drop programmes are incentive-based regulation programmes that are aimed to improve drinking water quality and management of wastewater treatment plants, respectively. One of the requirements of the Blue Drop programme is that a water safety planning approach must be followed and the limits stipulated on SANS 241 must be met. The South African National Standards for drinking water quality (SANS 241) are based on the WHO guidelines for drinking water quality (Kruger in preparation, 2021; (SANS 241, 2015)). A limited number of chemicals are listed for testing in the SANS 241 drinking water guidelines for SA, a summary of the guidelines can be found in Appendix A. There have been studies in SA that have investigated other target chemicals in various water sources (Archer et al., 2020; Archer and Genthe, 2021). Many of these can be found in various reports on the WRC website (www.wrc.org; Knowledge Hub). An example is the comprehensive report by Patterton (2013) who investigated the most important new substances in drinking water that could be a concern to human health in SA. Swartz et al. (2018) investigated the occurrence of chemicals of emerging concern in wastewater treated for direct potable reuse. Another report on chemicals of emerging concern in South African aquatic ecosystems by Archer et al. (2020) also provides information on chemicals that may present in our water sources. Wanda et al. (2017) reported on the occurrence of emerging micropollutants in water systems in Gauteng, Mpumalanga, and North West Provinces. They tested for carbamazepine (CBZ), galaxolide (HHCB), caffeine (CAF), tonalide (AHTN), 4-nonylphenol (NP), and bisphenol A (BPA) in water from Gauteng, Mpumalanga, and North West provinces using comprehensive two-dimensional gas chromatography coupled to high resolution time-of-flight mass spectrometry (GCxGC-HRTOFMS). Bisphenol A was detected in 62% of the water samples at a centration range from not detectable to 181 ± 8.3 ng/L.

A review by Odiyo and Makungo (2012) looking at water quality problems in vulnerable communities in Limpopo Province, showed that there are extensive and comprehensive policy, legal, technical and institutional frameworks to support water quality monitoring and management in South Africa. However, the issue is that there is ineffective implementation, a lack of technical capacity and financial problems at municipality level (Odiyo and Makungo, 2012). Sections in the NWS Act (No 108 of 1997) requires water service providers to monitor the performance of treatment systems and adhere to the compulsory standards in water from, or discharged into freshwater resources and to prevent "objectionable substances" from entering said water sources (Archer and Genthe, 2021). In other words the monitoring and treatment of chemicals of emerging concern (CECs) which includes EDCs should fall within the prescribed regulations (Archer and Genthe, 2021). This highlights the need for the National Toxicity Monitoring Program (NTMP) to be revised and updated to assist with environmental risk assessment (Odiyo and Makungo, 2012) and incorporating the use of effect-based monitoring (EBM) tools to improve water quality and safety. In his 2020 'State of the Nation' report, South African president Cyril Ramaphosa announced the revival of the Blue and Green Drop certification programme (Bega, 2021).

The requirements for the Green Drop programme is that at a minimum, the general effluent standard (Annexure A) should be met in the case of an unlicensed WWTP (Government Gazette, 2013). A licenced 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. However, in the period between 2012 and 2018, there was a break in implementing these programmes. 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 national water standards (AfriForum, 2020).

Despite South Africa globally being regarded for its progressive water legislation (Takacs, 2016), the implementation thereof, and specifically determining the quality of the water resources on a regular basis had been slipping to a point where it seems to be non-existent, except for a number of smaller research studies. In her opinion paper Schreiner (2013) ascribed 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 and prevent decision-making.

2.2 CURRENT PRACTICES ON USE OF EFFECT-BASED METHODS FOR SCREENING CHEMICAL TOXICITIES IN WATER

A literature review by Gani et al. (2021), looked at broad categories of CECs which included EDCs, pharmaceuticals, industrial chemicals, personal care products and pesticides. Different water matrices were used as terms for the review and included freshwater, drinking water, surface water, influent and effluent from drinking water treatment plants, tap water, groundwater and surface water (including rivers, lakes, dams, and oceans). The total number of publications from 1 January 2000 to 30 April 2020 was 41 and the majority focussed on CECs in wastewater, rather that surface and groundwater. The number of publications emanating from SA on these CECs in the aquatic environment is much lower than those from Europe and North America (Ademollo et al., 2012, Gani et al., 2021).

Many of the studies on CECs and EDCs have used one or possibly two effect-based methods (EBMs) to determine biological activity the of water source. Table 4-1 presents a summary of some of the studies that have been done in SA using *in vitro* and *in vivo* assays that include those testing for EDC activity using hormone receptor-mediated methods, but also includes other MOA based on AOPs. In a WRC report looking at CECs in the aquatic ecosystems by Archer et al. (2020) the fate, environmental health risk characterisation and substance use epidemiology in surrounding communities using chemical analyses and the yeast estrogen screen as an EBM to detect CECs in wastewater treatment plants but also in water from the surrounding communities. The study proposes a list of priority CECs that should receive attention based on their regular occurrence in surface waters, along with their established and/or proposed ability to act as stressors for various non-communicable health effects in wildlife and humans (Archer et al., 2020).

Table 2-1: *In vivo* and *in vitro* analyses performed in South Africa to test water quality

Target mode of action	No.	*In vivo/ in vitro*	Assay	Endpoint	Biological agent	Reference
Non-specific toxicity (baseline toxicity)						
Toxicity	1	*In vitro*	Biotox assay	Bioluminescence inhibition	*Aliivibrio fischeri* (=*Vibrio fischeri*)	Surujlal-Naicker et al., 2015; Tekere et al., 2016
	2	*In vitro*	Mammalian cell colony formation inhibition test	Colony formation	Buffalo Green monkey (BGM) kidney cells and Chinese hamster V79 cells	Slabbert, 1998
	3	*In vitro*	Bacterial growth inhibition assay	Growth inhibition	*Pseudomonas putida*	Slabbert et al., 1998
	4	*In vivo*	Biological diatom index, Generic diatom index, Specific pollution index	Abundance and species richness	Diatoms	Holmes and Taylor, 2015; Kock et al., 2019; Taylor et al., 2007
	5	*In vivo*	Fish diversity type indices, e.g. Fish Response Assessment Index (FRAI) & Fish Assemblage Integrity Index (FAII)	Abundance and species richness	Fish	Kleinhans, 1999; Malherbe et al., 2015; Malherbe et al., 2016
	6	*In vivo*	South African Scoring System version 5 (SASS5)	Abundance and species richness	Macroinvertebrates	De Necker et al., 2016; Malherbe, 2013; Malherbe et al., 2015; Malherbe et al., 2018; Van Deventer et al., 2021
	7	*In vivo*	Fish Health Assessment Index (FHAI)	Fish health	Fish	Erasmus et al., 2019; Malherbe, 2013; Nibamureke et al., 2016; Sara et al.,

						2014; Wagenaar and Barnhoorn 2018; Wepener et al., 2011
	8	*In vivo*	Ostracod toxkit-F	Growth inhibition	*Heterocypris incongruens*	Singh et al., 2017
	9	*In vivo*	Microplate-based *Hydra attenuata* assay	Growth rate	*Hydra vulgaris* (=*Hydra attenuata*)	Oberholster et al., 2008
	10	*In vivo*	Chronic acid tolerance bioassay	Growth rate (mass & length)	*Amietophrynus maculatus, Chiromantis xerampelina, Hildebrandtia ornata*, *Pyxicephalus edulis*	Farquharson et al., 2016
	11	*In vivo*		Hatching rate	*A. maculatus, C. xerampelina, H. ornata*, *P. edulis*	Farquharson et al., 2016
	12	*In vivo*		Hatching rate	*Pyxicephalus adspersus*	Oberholster et al., 2008
	13	*In vivo*	Diptera assay	Mortality	*Chironomus caffrarius*	Singh et al., 2017
	14	*In vivo*	Fish lethality test	Mortality	*Danio rerio, Labeobarbus aeneus, Oreochromis mossambicus, Poecilia reticulata, Pseudocrenilabrus philander, Tilapia sparrmanii*	Botha et al., 2015; Brand et al., 2020; Slabbert et al., 1998; Tekere et al., 2016
	15	*In vivo*	*Daphnia/Ceriodaphnia* lethality test	Mortality	*Daphnia magna Daphnia pulex Ceriodaphnia dubia*	Botha et al., 2015; Oberholster et al., 2008; Slabbert et al., 1998; Tekere et al., 2016
	16	*In vivo*		Swimming behaviour	*D. rerio*	Brand et al., 2020
Staining assays	17	*In vitro*	Resazurin cell proliferation assay	Mitochondrion activity	GH3 rat pituitary carcinoma cells	Simba, 2017

	18	*In vitro*	MTT viability assay	Mitochondrion activity	HuTu-80 human duodenum adenocarcinoma cell H4IIE-*luc* rat hepatoma cell MDA-kb2 human breast carcinoma cells	Prinsloo et al., 2013 Pheiffer et al., 2019; Vogt et al., 2019; Powrie, 2016
	19	*In vitro*	WST-1 viability assay	Mitochondrion activity	RAW264.7 mouse macrophage cells	Makene and Pool, 2015; Makene et al., 2016
	20	*In vitro*	XTT viability assay	Mitochondrion activity	RAW264.7 mouse macrophage cells	Makene and Pool, 2015
Specific toxicity						
ENDOCRINE DISRUPTION						
Androgen receptor (AR)						
AR(ant)-agonism	21	*In vitro*	Reporter gene assay	Receptor binding	MDA-kb2 human breast carcinoma cells	De Jager et al., 2011; Powrie, 2016
	22	*In vitro*	YAS	Receptor binding	*Saccharomyces cerevisiae*	Truter et al., 2016
Oestrogen receptor (ER)						
ER(ant)-agonism	23	*In vitro*	E-screen (modified)	Proliferation	MCF-7 human breast carcinoma cells	Swart et al., 2011
	24	*In vitro*	YES	Receptor binding	*S. cerevisiae*	Aneck-Hahn et al., 2005; Aneck-Hahn et al., 2008; Aneck-Hahn et al., 2009; Archer et al., 2020; Du Preez and Slabbert, 2008; Van Zijl et al., 2017; Patrick et al., 2020
	25	*In vitro*	Reporter gene assay	Receptor binding	T47D-kB*luc* human breast carcinoma cells	Van Zijl et al., 2017; Patrick et al., 2020
Alternative ER techniques	26	*In vitro*	MCF-7 ERα ELISA	ERα protein concentration	MCF-7 breast carcinoma cells	Swart and Pool, 2009, Swart et al., 2011
	27	*In vivo*	Gonadosomatic index Urogenital papilla length index	Gonad size; Urogenital papilla length	*Clarias gariepinus*	Kruger et al., 2013
	28	*In vivo*	Vtg ELISA	Vitellogenin production	*D. rerio, O. mosambicus*	Du Preez and Slabbert, 2008; Swart et al., 2011

	29	*In vivo*	Primary rainbow trout hepatocyte assay	Vitellogenin (Vtg) production	*Oncorhynchus mykiss*	Du Preez and Slabbert, 2008
	30	*In vivo*	Vtg ELISA	Vitellogenin production	*Xenopus laevis*	Pool, 2008
Thyroid receptor (TR)						
TR activity	31	*In vitro*	Reporter gene assay	Receptor binding	GH3.TRE.*luc* rat pituitary tumour cells	Simba, 2017
DIOXIN-LIKE ACTIVITY						
Aryl hydrocarbon receptor (AhR)						
AhR activity	32	*In vitro*	Reporter gene assay	Receptor binding	H4IIE-*luc* rat hepatoma cell	Pheiffer et al., 2019; Vogt et al., 2019
DEVELOPMENTAL TOXICITY						
	33	*In vivo*	Toad embryo teratogenicity test	Embryo development (size & length), pigmentation, head shape, form of spines and tails	*X. laevis*	Slabbert et al., 1998
	34	*In vivo*	Abalone embryo development test	Operculate veliger stage embryo	*Haliotis midae*	Shackleton et al., 2002
	35	*In vivo*	Invertebrate reproduction test	Reproduction	*D. magna, C. dubia*	Slabbert et al., 1998
HEPATOTOXICITY						
Cytochrome P450	36	*In vivo*	Demethylating fluorescent activity kit	Cytochrome P450 activity	*Caridina nilotica, C. gariepinus, Perna perna*	Coetzee, 2015; Pheiffer, 2017; Van Rensburg et al., 2020
METAL TOXICITY						
	37	*In vivo*	Metallothionein content	Metallothionein content	*Atractolytocestus huronensis, C. nilotica, C. gariepinus, Contracaecum sp., Cyprinus carpio, Dreissena polymorpha*	Brand et al., 2019; Erasmus et al., 2020; Van Rensburg et al., 2020
NEUROTOXICITY						

Acetylcholine sterase (AChE) activity	38	*In vivo*	AChE activity assay	AChE activity	*A. huronensis, C. nilotica, C. gariepinus, Contracaecum sp., C. carpio, P. perna, T. sparmanii*	Coetzee, 2015; Erasmus et al., 2020; Malherbe, 2013; Pheiffer, 2017; Van Rensburg et al., 2020
IMMUNOTOXICITY						
Cytotoxicity	39	*In vitro*	ELISA	LDH concentration	Whole blood culture	Pool and Magcwebeba, 2009
Immunity (cell mediated)	40	*In vitro*	ELISA	IFN-y concentration	Whole blood culture	Pool and Magcwebeba, 2009
Immunity (hormonal)	41	*In vitro*	ELISA	IL-10 concentration	Whole blood culture	Pool and Magcwebeba, 2009
Inflammatory activity	42	*In vitro*	ELISA	IL-6 concentration	RAW264.7 mouse macrophage cells Whole blood culture	Makene and Pool, 2015; Makene et al., 2016; Pool and Magcwebeba, 2009; Pool et al., 2000
	43	*In vitro*	Griess reaction	Nitric oxide concentration	RAW264.7 mouse macrophage cells	Makene and Pool, 2015; Makene et al., 2016
Reactive toxicity						
Mutagenicity						
	44	*In vitro*	Ames	Colony formation	*Salmonella typhimurium*	Slabbert et al., 1998
	45	*In vitro*	Cell transformation test	Evidence of malignancies	Hamster embryo cells	
Oxidative stress						
	46	*In vivo*	Cellular energy allocation	Energy consumption (energy needed to reduce oxygen)	*A. huronensis, C. nilotica, C. gariepinus, Contracaecum sp., C. carpio, T. sparmanii*	Erasmus et al., 2020; Malherbe, 2013; Pheiffer, 2017; Van Rensburg et al., 2019
	47	*In vivo*	Catalase	Enzyme activity	*A. huronensis, C. nilotica, C. gariepinus, Contracaecum sp., C. carpio, D. polymorpha, H. vittatus, P. perna, T. sparmanii*	Brand et al., 2019; Coetzee, 2015; Erasmus et al., 2020; Gerber et al., 2018; Malherbe, 2013; Pheiffer, 2017; Van Rensburg et al., 2019

	48	*In vivo*	Superoxide dismutase	Enzyme activity	*A. huronensis, C. nilotica, C. gariepinus, Contracaecum sp., C. carpio, H. vittatus, P. perna*	Coetzee, 2015; Erasmus et al., 2020; Gerber et al., 2018; Pheiffer, 2017; Van Rensburg et al., 2019
	49	*In vivo*	Glutathione-S-transferase	Enzyme activity	*D. polymorpha*	Brand et al., 2019
	50	*In vivo*	Reduced glutathione	Glutathione content	*A. huronensis, C. nilotica, C. gariepinus, Contracaecum sp., C. carpio, H. vittatus*	Erasmus et al., 2020; Gerber et al., 2018; Van Rensburg et al., 2019
	51	*In vivo*	Lipid peroxidation	Malondialdehyde production	*A. huronensis, C. nilotica, C. gariepinus, Contracaecum sp., C. carpio, D. polymorpha, H. vittatus, P. perna, T. sparmanii*	Brand et al., 2019; Coetzee, 2015; Erasmus et al., 2020; Gerber et al., 2018; Malherbe, 2013; Pheiffer, 2017; Van Rensburg et al., 2019
	52	*In vivo*	Protein Carbonyl levels	Protein concentration	*A. huronensis, C. nilotica, C. gariepinus, Contracaecum sp., C. carpio, H. vittatus, P. perna, T. sparmanii*	Coetzee, 2015; Erasmus et al., 2020; Gerber et al., 2018; Malherbe, 2013; Pheiffer, 2017; Van Rensburg et al., 2019
Low-complexity *in vivo* plant assays						
Algal growth and PSII inhibition	53	*In vivo*	Algal growth inhibition assay	Growth inhibition	*Selenastrum capricornutum*	Slabbert et al., 1998; Tekere et al., 2016
Cytotoxicity, growth inhibition	54	*In vivo*	Root growth	Root growth	*Allium cepa*	Oberholster et al., 2008
Seed germination and root growth	55	*In vivo*	Seed germination	Seed germination	*Lactuca sativa*	Oberholster et al., 2008
	56	*In vivo*	Phytotoxkit-F	Seed germination, root and shoot growth inhibition	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*	Singh et al., 2017
Low complexity *in vivo* protozoan assay						

Oxygen uptake	57	*In vivo*	Protozoan oxygen uptake assay	Oxygen consumption rate	*Tetrahymena pyriformis*	Bulannga and Schmidt 2022; Slabbert and Morgan 1982; Slabbert et al., 1998

2.3 CURRENT CAPACITY IN SOUTH AFRICA

Table 2-2: A comparison between all the assays proposed to be included in the SA EBM toolbox for water quality screening

	Skilled human capacity	Obtaining biological entities	Obtaining materials	Specialized equipment	Sample preparation	Interpretation of results	Type of water	Cost per sample
IN VITRO								
Xenobiotic metabolism								
H4IIE-*luc*	H		Easy	LUM	Extensive	Complex	DW, GW, SW, WW, TWW	R 2 800
Hormone receptor mediated effects								
Estrogenic activity								
YES/YAES	H	COM	Easy	LUM	Extensive	Complex	DW, GW, SW, WW, TWW	R 2 800
T47D-KB*luc*	H	COM	Easy	LUM	Extensive	Complex	DW, GW, SW, WW, TWW	R3 000
Androgenic activity								
YAS/YAAS	H	COM	Easy	LUM	Extensive	Complex	DW, GW, SW, WW, TWW	R 2 800
MDA-kB2	H	COM	Easy	LUM	Extensive	Complex	DW, GW, SW, WW, TWW	R 2 800
Thyroid activity								

GH_3.TRE-*luc*	H	COM	Easy	LUM	Extensive	Complex	DW, GW, SW, WW, TWW	R 2 800
Adaptive stress responses								
AREc32	H	COM	Easy	LUM	Extensive	Complex	DW, GW, SW, WW, TWW	R 2 800
ROS, GSH	H		Easy		Extensive	Complex	DW, GW, SW, WW, TWW	R 2 800
CAT, SOD, MDA, GPx	H		Easy		Extensive	Complex	DW, GW, SW, WW, TWW	R 2 800
Steroidogenesis								
H295R steroidogenesis	H	COM	Easy	SPEC	Extensive	Complex	DW, GW, SW, WW, TWW	R 2 800
Reactive toxicity								
MTT viability assay	H		Easy	SPEC*	Extensive	Complex	DW, GW, SW, WW, TWW	R 2 800
IN VIVO								
Reactive toxicity								
Salmonella fluctuation test (AMES)	M	COM	Easy		Minimal	Middle	GW, SW, WW, TWW	
Apical effects								
Allivibrio fisheri bioluminescence test	M	COM	Easy	LUM	Minimal	Easy	GW, SW, WW, TWW	R2 800
Pseudokirchneriella subcapitata growth inhibition test	M	COM	Easy	SPEC	Minimal	Easy	GW, SW, WW, TWW	R2 500
Daphnia magna/pulex acute toxicity test	M	COM	Easy		Minimal	Easy	GW, SW, WW, TWW	R2 500
Fish acute toxicity test	M	COM	Easy		Minimal	Easy	GW, SW, WW, TWW	R2 500
Heterocypris incongruens direct contact sediment test	M	COM	Easy	LM	Minimal	Middle	GW, SW, WW, TWW	R4 500
Frog Embryo Teratogenesis Assay Xenopus (FETAX)	M	COM	Easy	LM	Minimal		GW, SW, WW, TWW	

*Spectrophotometer to measure absorbance/optical density at specific wavelengths (use filters or adjustable wavelengths); H=high; M=middle; L=low, LUM=luminometer, SPEC=spectrophotometer; LM=light microscope

COM=commercially available; All assays listed require a dedicated laboratory for specific assays, i.e. *in vitro* or *in vivo*. Equipped with incubators/growth chambers, laminar flow cabinet, inverted microscope; bench space. Types of water: DW = drinking water; GW= groundwater; SW=surface water; WW= wastewater; TWW=treated wastewater

CHAPTER 3: FACTSHEETS ON THE USE OF EFFECT-BASED METHODS FOR THE ASSESSMENT OF CHEMICAL RISKS IN WATER

Compiled by: S Horn, R Pieters, MC Van Zijl and NH Aneck-Hahn

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, and 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).

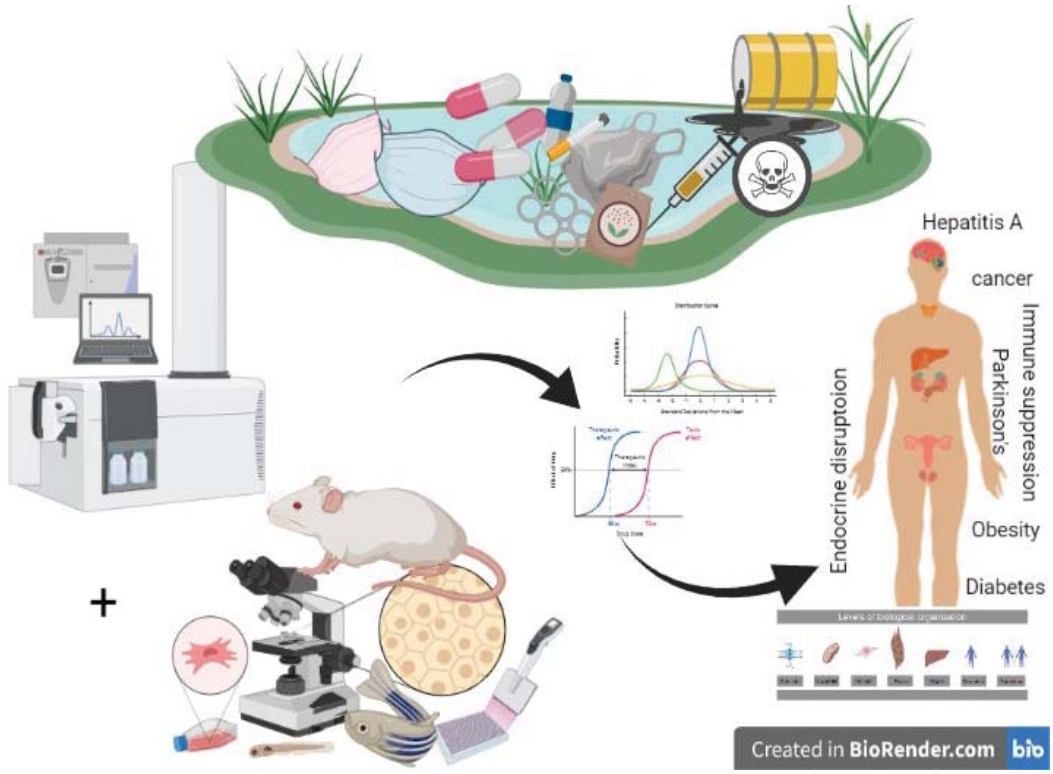

Figure 3-1: Schematic flow diagram on the use of bioassays for water quality monitoring. Figure created by A Kruger

3.2 BIOASSAYS TAILORED TO MEET DIFFERENT WATER QUALITY REQUIREMENTS

In South Africa guidelines for different water uses are specified, i.e. domestic use, recreational use, agricultural use, aquatic ecosystems, and drinking water quality (DWAF, 1996). Bioassays with outcomes capable of discerning between different quality guideline levels are the ideal.

3.2.1 *In vitro* bioassays

- Xenobiotic metabolism
  - H4IIE-luc (AhR)
- Hormone receptor-mediated effects
  Estrogenic
  - Yeast estrogen screen (YES)
  - T47D-KBluc

  Androgenic
  - Yeast androgen screen (YAS)
  - MDA-kb2

  Thyroid
  - GH3.TRE-Luc
- Other receptor-mediated effects
  - H295R *steroidogenesis*
- Adaptive stress response (Oxidative stress bioassays)
  - *AREc32*
  - *ROS, MDA, etc.*
- Reactive toxicity
  - *MTT viability assay*

3.2.2 Low complexity *in vivo* bioassays

A.1. Reactive toxicity
  - *Salmonella* fluctuation test (ISO 2010)

- Apical effects
  - *Allivibrio fisheri bioluminescence test (SANS 2013a)*
  - *Pseudokirchneriella subcapitata growth inhibition test (SANS 2015b)*
  - *Daphnia magna/pulex acute toxicity test (immobilisation test) (SANS 2015a)*
  - *Fish acute toxicity test (immobilisation test) (SANS 2013b)*
  - *Heterocypris incongruens direct contact sediment test (ISO, 2012)*
  - *Frog Embryo Teratogenesis Assay Xenopus (FETAX) test (ASTM, 1998)*

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* 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.

3.4 TYPES OF MATRICES THAT MAY BE EVALUATED

These assays may be used on all forms of water, i.e. wastewater or industrial effluents, sewage samples, aqueous extracts and leachates, freshwaters (surface and groundwater), eluates of sediment (freshwater, brackish and sea water), pore water, or potable water.

Depending on the nature of the assay, the whole water sample will be used but specifically for the *in vitro* assays, the water must be extracted first for targeted toxicants and the sample concentrated. Sediment might be collected in some instances depending on the type of water evaluated: ecological water might benefit from sediment evaluation, but potable water not because potable water should not contain sediment anymore. The ostracod test is the only *in viv*o test that can be applied for sediment, but all the *in vitro* tests can be applied, provided the appropriate extraction procedures are used.

The reason for the research or monitoring determines the volume of water or mass of sediment to be collected as well as whether grab or passive sampling should be employed.

3.5 EXTRACTION METHODS

Appropriate extraction methods should be used for the different targeted pollutants and matrices. 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). Sediment is extracted after freeze-drying first followed by accelerated solvent extraction or Soxhlet using appropriate solvents (USEPA, 2007a,b).

One single bioassay will not provide a true reflection of the quality of the water and therefore a battery of three to four assays with different modes of action (MOA) is recommended. At sites (e.g. rural water treatment plants) with no access to sophisticated laboratory facilities, it may be possible to apply very simple cytotoxicity assays, such as bacterial toxicity assays. Ideally such assays that only provide information about non-specific effects should be complemented with assays indicative of specific effects.

The suggested combination of bioassay test batteries for the different water sources for example, drinking water, wastewater and recycled water will be available in the diagnostic tool factsheet.

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USEPA 2007b (UNITED STATES ENVIRONMENT PROTECTION AGENCY) Method 3545A (SW-846): Pressurized fluid extraction (PFE), Revision 1. Washington, DC.

CHAPTER 4: FACTSHEETS FOR SELECTED *IN VITRO* BIOASSAYS

4.1 FACTSHEET: THE AHR REPORTER GENE ASSAY (H4IIE-*LUC* CELLS)

Compiled by: R Pieters

4.1.1 Purpose

The H4IIE-*luc* reporter gene assay is an *in vitro* assay that was developed by international research laboratories to measure nuclear receptor mediated metabolism of xenobiotics such as polychlorinated dibenzo-*p*-dioxins, dibenzofurans and dioxin-like polychlorinated biphenyls.

4.1.2 Application

This assay is used to assess the biological activity of chemicals, chemical mixtures, water (e.g. drinking water, groundwater, surface water, wastewater, water for reuse, etc.) and other environmental samples (e.g. sediment).

4.1.3 Test principle

The rat/mouse hepatoma cancer cells (rat: H4L1.1c4; mouse: H1L6.1c2), contain the aryl hydrocarbon receptor (AhR) (that activates the expression of the P450 enzymes) and have been genetically modified to contain a luciferase reporter gene. The presence of the luciferase enzyme can then be assessed by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. The amount of light produced is relative to the degree of AhR activity of the sample. When testing samples using the cells, an agonist is defined as a substance that induces dose dependent luciferase activity. Agonists stimulate luciferase expression and are compared to amount of light emitted by known concentrations of the reference compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) (Aarts et al., 1993).

4.1.4 Sampling and sample preparation

Sample collection (1 L) should be done in amber glass, polytetrafluoroethylene (PTFE), aluminium or uncoated stainless steel bottles rinsed with analytical grade acetone.
Samples can be extracted with liquid-liquid extraction using dichloromethane (DCM) or flocculation and a high-speed and high-pressure extractor using toluene. Sample enrichment can be done using solid phase extraction on C18 cartridges and eluting with DCM and toluene.

4.1.5 Method summary

The cells are maintained in a suitable growth medium such as MEM-alpha also containing streptomycin and penicillin (rat cells are also supplemented with 10% fetal bovine serum). On day one of the three-day assay, the cells are seeded into 96-well plates with growth medium (rat cells: supplemented with dextran-charcoal treated FBS). Cells are seeded at 8 x 10^5 cells/mL in 96-well luminometer plates and incubated for 22-26h to allow to attach. Dosing dilutions are prepared in growth media. Each plate should contain agonist positive

control (2,3,7,8-tetrachlorodibenzo-*para*-dioxin; TCDD, negative control (vehicle only), and procedural blank (growth medium). One 96-well plate offers ten triplicate positions which can be used for dilutions of a sample, a blank or negative control. Final concentrations of TCDD varies for different cell lines (mouse: 46.1-0.18 pg/mL; rat: 95.2-0.095 pg/mL). Cells are exposed for 24 h with 100 µL/well dosing solution at 37°C, 5% CO_2. After the incubation period, cells are washed with phosphate buffered saline at room temperature and lysed with 25 µL lysis buffer. Luciferase activity is determined using a luminometer and quantified as relative light units. Each well receives 50 µL substrate mixture containing each of the following in mmol/L: 20 Tricine; 1.07 $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$; 2.67 $MgSO_4 \cdot 7H_2O$; 0.1 EDTA, 1.5 DTT, 0.539 D-luciferin, 5.49 ATP. The luminescence is recorded. The stop reagent (0.2 mol/L NaOH) is added to quench the luminescence. The TCDD standard curve is fitted (sigmoidal function, variable slope) and the TCDD-equivalents (TCDD-Eq) of extracts are interpolated from the TCDD standard curve and corrected with the appropriate dilution factor for each sample. For chemicals, the relative potency (RP) and relative induction efficiency (RIE) compared to TCDD are reported (Escher et al., 2018).

4.1.6 Advantages and/or limitations

Advantages

- The assay is relatively rapid, eliminates the need for transfection, can be conducted in 96-well plates and consistent results are produced.
- This assay can be used in place of the DR-CALUX as it is less expensive, but equally sensitive.

Limitations of the bioassay

- Requires a dedicated cell culture laboratory.
- Extraction and sample preparation are specialised.
- Matrix interference in the form of cytotoxicity.

4.1.7 Specialized facilities and equipment required

Dedicated cell culture laboratory
Microplate luminometer with two dispensers

4.1.8 Acquisition of the cell line

The commercial cell line, DR-CALUX is available from BioDetection Systems in The Netherlands (https://biodetectionsystems.com). Prof. Michael Denison from University of California, Davis (https://etox.ucdavis.edu/denison-michael) gives away H4L1.1c2. An import permit will also be required from the Department of Agriculture, Land Reform and Rural Development, RSA.

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4.2 FACTSHEET: THE YEAST (ANTI-)ESTROGEN SCREEN BIOASSAYS

Compiled by: E Archer

4.2.1 Purpose

The yeast estrogen screen (YES) and yeast anti-estrogen screen (YAES) are two *in vitro* assays using the same cell line that was developed to quantify receptor-mediated estrogenic and anti-estrogenic activity respectively of test chemicals and environmental matrices.

4.2.2 Application

This assay is used to assess the either agonist or antagonist affinity of chemicals, mixtures and/or environmental sample matrices (aqueous and solids) against binding to the human estrogen receptor alpha (hERα).

4.2.3 Test principle

A recombinant *Saccharomyces cerevisiae* yeast strain was developed to identify compounds that can interact with the hERα. The hERα gene was stably transfected into its main genome, along with an expression plasmid containing an estrogen response element (ERE)-linked lac-Z gene complex that encode for the enzyme β-galactosidase. The hER in the cell line is expressed in a form capable of binding to the ERE within a hybrid promoter on the expression plasmid. Activation of the receptor by binding of a ligand (steroid hormone or EDC) causes expression of the reporter gene Lac-Z that promotes the transcription and translation for the enzyme β-galactosidase. This enzyme is secreted into the assay medium and result in the hydrolysis of the chromogenic substrate chlorophenol red-b-D-galactopyranoside (CPRG) in the assay medium from a yellow- to a red product that is measured by absorbance (Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998; Jobling et al., 2009). The concentration of ligands in the test sample will then either initiate or interfere with steroid hormone receptor-dependent β-galactosidase production in the bioassay (estrogenic or anti-estrogenic respectively). The subsequent level of CPRG hydrolysis in the assay media is thus directly correlated to the presence of natural and/or anthropogenic substances that interfere with hERα binding in a dose-dependent manner.

4.2.4 Sampling and sample preparation

Sample collection (1 L) should be done in glass bottles rinsed with HPLC grade methanol. Aqueous samples need to be processed through solid phase extraction (SPE) using Oasis HLB glass cartridges. Solid samples need to be processed using either ultrasonicated-assisted extraction coupled with solid phase extraction (UAE-SPE), microwave-assisted extraction coupled with solid phase extraction (MAE-SPE) or accelerated solvent extraction (ASE).

4.2.5 Method summary

The yeast cells are maintained in a culture media that contain a minimal growth medium, supplemented with 20% glucose, L-aspartic acid, a vitamin solution, L-threonine, and copper-(II)-sulphate (see in the latest Toolbox for EBM in South Africa for the comprehensive make-up of reagents). Assay medium containing the culture media and 0.5 mL of a 10 g/L CPRG solution is seeded with $4x10^7$ cells per 50 mL of the assay medium. A dilution series of the test chemicals/environmental samples and reference standard controls are prepared in a 96-well microtiter plate, whereby, 10 µL is transferred to a new sterile 96-well optically flat bottom microplate

with a low evaporation lid. For the YES bioassay, each assay plate should include an agonist positive control (consisting of a 12-point dilution of 200 nM 17β-estradiol), as well as negative control (vehicle solvent only).

For the YAES bioassay, the assay plate is coated with 10 µL of a submaximal concentration of E_2 (except for the negative control wells) prior to the addition of the antagonist positive control (consisting of a 12-point dilution of 100 µM hydroxy-tamoxifen), negative and positive blank controls (containing vehicle solvent only and vehicle + submaximal E_2 spike respectively), and the test chemicals/environmental samples. The transferred samples/controls/E_2 spikes are allowed to evaporate to dryness in the assay plate, before 200 µL of the assay medium, containing the yeast and CPRG, is added to each well. The assay plates are sealed with autoclave tape and incubated at 32°C in the dark for 48 to 72 hours. After the incubation period, the assay plates are measured spectrophotometrically at 620 nm for cell turbidity (cytotoxicity and corrected absorbance determination) and 570 nm (CPRG metabolism caused by hER-mediated β-galactosidase production).

The agonist (E_2) and antagonist (hydroxy-tamoxifen) standard curves are fitted (sigmoidal function, variable slope) and the E_2 equivalents (EEq) and tamoxifen equivalents (TAM-Eq) of extracts are interpolated from the estradiol and hydroxy-tamoxifen standard curves and corrected with the appropriate dilution factor for each sample. For individual chemical and mixture exposures, the relative potency (RP) and relative induction efficiency (RIE) compared to the agonist or antagonist positive controls are reported.

4.2.6 Advantages and/or limitations

Advantages

- The assay is relatively rapid (2-3 days) and can be performed in 96 well plates to produce consistent results.
- The cell line is less likely to fail in highly-polluted and/or concentrated sample matrices compared to mammalian cell lines.
- Potential to evaluate both cytotoxicity and hER-mediated (anti-)estrogenicity.

Limitations of the bioassay

- The yeast cells contain only ERα and may therefore not be as sensitive as other bioassays containing both ERα and ERβ.
- The yeast cell wall may impede active and passive transport of test chemicals and environmental samples into the intracellular space, resulting in false negatives compared to its mammalian cell line bioassays.
- Requires a dedicated cell culture laboratory.
- May be less sensitive than mammalian cell lines (higher limit of detection), depending on extraction procedures followed.
- Special care needs to be taken to prevent estrogenic contamination from external sources and leaching of volatile estrogenic products within the assay plate.
- Matrix interference in the form of cytotoxicity that can result in the masking of receptor binding responses in the bioassay.

4.2.7 Specialized facilities and equipment required

- Dedicated cell culture laboratory (Wigley, 2006) including the following:
    - Vacuum system for sample filtration and solid-phase extraction
    - Ultrasonicator, microwave extractor or accelerated solvent extractor (extraction in solids)
    - Laminar flow cabinet
    - Orbital shaker (holding 200 mL volumetric flasks at 150rpm).
    - 96-well microplate spectrophotometer (wavelength range between 400 to 700 nm).
- Software package (Microsoft Excel and Graphpad Prism)

4.2.8 Acquisition of the cell line

The yeast cell line can be obtained from Xenometrix, Switzerland (Cat. No. N05-230-E). The local distributor is ToxSolutions Kits and Services:
E-mail: hesmarie@toxsolutions.co.za

The Department of Agriculture, Land Reform and Rural Development is responsible for issuing a permit for importing genetically modified organisms:
Postal address: Directorate Genetic Resources, Private Bag X973, Pretoria, 0001
E-mail: GMO@dalrrd.gov.za Website: https://www.dalrrd.gov.za/Branches/Agricultural-Production-Health-Food-Safety/Genetic-Resources/Biosafety

Ms Refilwe Ngoepe
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Ms Bathobile Mahlangu
Tel: 012 319 6364
E-mail: BathobileM@daff.gov.za

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JOBLING S, BURN RW, THORPE K, WILLIAMS R and TYLER C (2009) Statistical modelling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. *Environmental Health Perspectives* **117** (5) 797-802.
LEUSCH FDL (2008) Tools to detect estrogenic activity in environmental waters. Global Water Research Coalition Report.
ROUTLEDGE EJ and SUMPTER JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry* **15** (3) 241-248.
SOHONI P and SUMPTER JP 1998 Several environmental oestrogens are also anti-androgens. *Journal of Endocrinology* **158**, 327-339.
WIGLEY CB (2006) The cell culture laboratory. In: DAVIS JM editor. Basic cell culture. 2nd edition. Oxford University Press New York, p1-27.

4.3 FACTSHEET: THE T47D-KBLUC REPORTER GENE ASSAY (ESTROGENIC ACTIVITY)

Compiled by: MC Van Zijl and NH Aneck-Hahn

4.3.1 Purpose

The T47D-KBluc reporter gene assay is an *in vitro* assay that was developed by the US EPA to measure nuclear receptor mediated estrogenic and anti-estrogenic activity.

4.3.2 Application

This assay is used to assess the biological activity of chemicals, chemical mixtures, water (e.g. drinking water, groundwater, surface water, wastewater, water for reuse, etc.) and other environmental samples (e.g. sediment).

4.3.3 Test principle

The T47D human breast cancer cells, contain both endogenous estrogen receptor (ER)-α and -β and have been modified to contain a luciferase reporter gene. The presence of the luciferase enzyme can then be assessed by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. The amount of light produced is relative to the degree of estrogenic activity of the sample. When testing samples using the T47D-KBluc cells, an agonist is defined as a substance that induces dose dependent luciferase activity, which could be specifically inhibited by the anti-estrogen ICI 182,780. Agonists stimulate luciferase expression and are compared to the vehicle control or to the 17β-estradiol (E_2) control. Anti-estrogens block the E_2-induced luciferase expression, which is compared to the E_2 control (Wilson et al., 2004).

4.3.4 Sampling and sample preparation

Sample collection (1L) should be done in glass bottles rinsed with high performance liquid chromatography (HPLC) grade methanol.

Samples need to be extracted using solid phase extraction and Oasis HLB glass cartridges.

4.3.5 Method summary

The T47D-KBluc cells are maintained in RPMI growth media supplemented with 2.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/L $NaHCO_3$, 10% fetal bovine serum (FBS), 100 µg/mL penicillin, 100 U/mL streptomycin and 0.25 µg/mL amphotericin B. The cells are incubated at 37°C, 5% CO_2. One week prior to the test assay, cells are placed in growth media supplemented with 10% dextran-charcoal treated FBS, without antibiotic supplements.

Cells are seeded at 5 x 10^4 cells per well in 96-well luminometer plates and allowed to attach overnight. Dosing dilutions are prepared in growth media containing 5% dextran-charcoal treated FBS. Each plate should contain agonist positive control (E_2), negative control (vehicle only), antagonist control (E_2 plus ICI) and background control (vehicle plus ICI). Each sample should be tested alone as well as in the presence of 0.1 nM E_2 (to test for anti-estrogenic activity) or 10 nM ICI. Cells are exposed for 24h with 100 µL/well dosing solution at 37°C, 5% CO_2.

After the incubation period, cells are washed with phosphate buffered saline at room temperature and lysed with 25 µL lysis buffer. Luciferase activity is determined using a luminometer and quantified as relative light units. Each well receives 25 µL reaction buffer (25 mM glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg/mL BSA, pH 7.8), followed by 25 µL 1 mM D-luciferin 5s later.

The E_2 standard curve is fitted (sigmoidal function, variable slope) and the estradiol equivalents (EEq) of extracts are interpolated from the estradiol standard curve and corrected with the appropriate dilution factor for each sample. For chemicals, the relative potency (RP) and relative induction efficiency (RIE) compared to E_2 are reported.

4.3.6 Advantages and/or limitations

Advantages

- The assay is relatively rapid, eliminates the need for transfection, can be conducted in 96 well plates and consistent results are produced.
- This assay can be used in place of the ER-CALUX as it is less expensive, but equally sensitive (Leusch, 2008).

Limitations of the bioassay

- Requires a dedicated cell culture laboratory.
- Special care needs to be taken in order to prevent estrogenic contamination.
- Matrix interference in the form of cytotoxicity.

4.3.7 Specialized facilities and equipment required

Dedicated cell culture laboratory
Microplate luminometer with two dispensers

4.3.8 Acquisition of the cell line

The cells can be obtained from LGC Standards South Africa (catalogue no ATCC-CRL-2865).
An import permit will also be required from the Department of Health, RSA.

REFERENCES

LEUSCH FDL (2008) Tools to detect estrogenic activity in environmental waters. Global Water Research Coalition, London, United Kingdom.
WILSON VS, BOBSEINE K and GRAY JR, LE (2004) Development and characterisation of a cell line that stably expresses and estrogen-responsive luciferase reporter for the detection of estrogen receptor agonists and antagonists *Toxicological Sciences* **81** 69-77.

4.4 FACTSHEET: THE YEAST (ANTI-)ANDROGEN SCREEN BIOASSAYS

Compiled by: E Archer

4.4.1 Purpose

The yeast androgen screen (YAS) and yeast anti-androgen screen (YAAS) are two *in vitro* assays using the same cell line that was developed to quantify receptor-mediated androgenic and anti-androgenic activity respectively of test chemicals and environmental matrices.

4.4.2 Application

This assay is used to assess the either agonist or antagonist affinity of chemicals, mixtures and/or environmental sample matrices (aqueous and solids) against binding to the human androgen receptor (hAR).

4.4.3 Test principle

A recombinant *Saccharomyces cerevisiae* yeast strain was developed to identify compounds that can interact with the androgen receptor. The hAR gene was stably transfected into its main genome, along with an expression plasmid containing an androgen response element (ARE)-linked lac-Z gene complex that encode for the enzyme β-galactosidase. The hAR in the cell line is expressed in a form capable of binding to the ARE within a hybrid promoter on the expression plasmid. Activation of the receptor by binding of a ligand (steroid hormone or EDC) causes expression of the reporter gene Lac-Z that promotes the transcription and translation for the enzyme β-galactosidase. This enzyme is secreted into the assay medium and result in the hydrolysis of the chromogenic substrate chlorophenol red-b-D-galactopyranoside (CPRG) in the assay medium from a yellow- to a red product that is measured by absorbance (Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998; Jobling et al., 2009; Urbatzka et al., 2007). The concentration of ligands in the test sample will then either initiate or interfere with steroid hormone receptor-dependent β-galactosidase production in the bioassay (androgenic or anti-androgenic respectively). The subsequent level of CPRG hydrolysis in the assay media is thus directly correlated to the presence of natural and/or anthropogenic substances that interfere with steroid hormone receptor binding in a dose-dependent manner.

4.4.4 Sampling and sample preparation

Sample collection (1L) should be done in glass bottles rinsed with HPLC grade methanol.

Aqueous samples need to be processed through solid phase extraction (SPE) using Oasis HLB glass cartridges. Solid samples need to be processed using either ultrasonicated-assisted extraction coupled with solid phase extraction (UAE-SPE), microwave-assisted extraction coupled with solid phase extraction (MAE-SPE) or accelerated solvent extraction (ASE).

4.4.5 Method summary

The PGKhAR cells are maintained in a culture media that contain a minimal growth medium, supplemented with 20% glucose, L-aspartic acid, a vitamin solution, L-threonine, and copper-(II)-sulphate (see Sohoni and Sumpter, 1998, for comprehensive make-up of reagents). Assay medium containing the culture media and 0.5 mL of a 10 g/L CPRG solution is seeded with $4x10^7$ cells per 50 mL of the assay medium. A dilution series of the test chemicals/environmental samples and reference standard controls are prepared in a 96-well microtiter plate, whereby, 10 µL is transferred to a new sterile 96-well optically flat bottom microplate with a low

evaporation lid. For the YAS bioassay, each assay plate should include an agonist positive control (consisting of a 12-point dilution of 100 µM dihydrotestosterone, DHT), as well as negative control (vehicle solvent only).

For the YAAS bioassay, the assay plate is coated with 10 µL of a submaximal concentration of DHT (except for the negative control wells) prior to the addition of the antagonist positive control (consisting of a 12-point dilution of 100 µM flutamide), negative and positive blank controls (containing vehicle solvent only and vehicle + submaximal DHT spike respectively), and the test chemicals/environmental samples. The transferred samples/controls/DHT spikes are allowed to evaporate to dryness in the assay plate, before 200 µL of the assay medium, containing the yeast and CPRG, is added to each well. The assay plates are sealed with autoclave tape and incubated at 32°C in the dark for 48 to 72 hours. After the incubation period the assay plate is measured spectrophotometrically at 620 nm for cell turbidity (cytotoxicity and corrected absorbance determination) and 570 nm (CPRG metabolism caused by hAR-mediated β-galactosidase production).

The agonist (DHT) and antagonist (flutamide) standard curves are fitted (sigmoidal function, variable slope) and the DHT equivalents (DHT-Eq) and flutamide equivalents (FLU-Eq) of extracts are interpolated from the DHT and flutamide standard curves and corrected with the appropriate dilution factor for each sample. For individual chemical and mixture exposures, the relative potency (RP) and relative induction efficiency (RIE) compared to the agonist or antagonist positive controls are reported.

4.4.6 Advantages and/or limitations

Advantages

- The assay is relatively rapid (2-3 days) and can be performed in 96 well plates to produce consistent results.
- The cell line is less likely to fail in highly-polluted and/or concentrated sample matrices compared to mammalian cell lines.
- Potential to evaluate both cytotoxicity and hAR-mediated (anti)androgenicity.

Limitations of the bioassay

- The yeast cell wall may impede active and passive transport of test chemicals and environmental samples into the intracellular space, resulting in false negatives compared to its mammalian cell line bioassays.
- Requires a dedicated cell culture laboratory.
- May be less sensitive than mammalian cell lines (higher limit of detection), depending on extraction procedures followed.
- Matrix interference in the form of cytotoxicity that can result in the masking of receptor binding responses in the bioassay.

4.4.7 Specialized facilities and equipment required

- Dedicated cell culture laboratory (Wigley, 2006) including the following:
    - Vacuum system for sample filtration and solid-phase extraction
    - Ultrasonicator, microwave extractor or accelerated solvent extractor (extraction in solids)
    - Laminar flow cabinet
    - Orbital shaker (holding 200 mL volumetric flasks at 150rpm).
    - 96-well microplate spectrophotometer (wavelength range between 400 to 700 nm).
- Software package (Microsoft Excel and Graphpad Prism)

4.4.8 Acquisition of the cell line

The yeast cell line can be obtained from Xenometrix, Switzerland (Cat. No. N05-230-A).

E-mail: hesmarie@toxsolutions.co.za

The Department of Agriculture, Land Reform and Rural Development is responsible for issuing a permit for importing genetically modified organisms:
Postal address: Directorate Genetic Resources, Private Bag X973, Pretoria, 0001
E-mail: GMO@dalrrd.gov.za
Website:https://www.dalrrd.gov.za/Branches/Agricultural-Production-Health-Food-Safety/Genetic-

Resources/Biosafety. Contact:

1. Ms Refilwe Ngoepe
   Tel: 012 3196364
   E-mail: RefilweN@daff.gov.za
2. Ms Bathobile Mahlangu
   Tel: 012 319 6364
   E-mail: BathobileM@daff.gov.za

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DE JAGER C, ANECK-HAHN NH, BARNHOORN IEJ, BORNMAN MS, PIETERS R, VAN WYK JH AND VAN ZIJL C (2011) The compilation of a toolbox of bio-assays for detection of androgenic activity in water. WRC Report No. 1816/1/10, ISBN 978-1-4312-0061-0.

JOBLING S, BURN RW, THORPE K, WILLIAMS R and TYLER C (2009) Statistical modelling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. *Environmental Health Perspectives* **117** (5) 797-802.

LEUSCH FDL (2008) Tools to detect androgenic activity in environmental waters. Global Water Research Coalition Report.

ROUTLEDGE EJ and SUMPTER JP (1996) Androgenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry* **15** (3) 241-248.

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4.5 FACTSHEET: THE MDA-KB2 REPORTER GENE ASSAY (ANDROGENIC ACTIVITY)

Compiled by: Suranie Horn, Annika Kruger and Rialet Pieters

4.5.1 Purpose

The MDA-kb2 reporter gene assay is an *in vitro* assay that was developed by the US EPA to measure nuclear receptor-mediated androgenic and anti-androgenic activity.

4.5.2 Application

This assay is used to assess the biological activity of chemicals, chemical mixtures, water (e.g. drinking water, groundwater, surface water, wastewater, water for reuse) and other environmental samples (e.g. sediment).

4.5.3 Test principle

The MDA-kb2 cell line was genetically modified with the MMTV.luciferase.neo reporter gene construct and both glucocorticoid (GR) and androgen (AR) receptors act through the MMTV promotor. The MDA-kb2 cell line can therefore be used to detect (ant-)agonists for both hormonal receptors (Wilson et al., 2002).

In the assay, the presence of agonists to the GR and AR are made known by the light emitted when exposed cells receive the luciferin substrate for the luciferase enzyme, whereas antagonists lead to a decline in light. The luciferase enzyme is expressed when agonists successfully bind to the hormonal receptors. The amount of light is directly correlated to the concentration of the agonists. The effect of antagonists is tested in slightly altered conditions when compared to the agonistic assays: cells are first treated to an agonist so that light is emitted upon receipt of the substrate. If antagonists are present, a decrease in light will be evident when compared to the control cells (cells that received agonists only) (Carter & Sheih, 2015).

When a response is seen during the activation assay, either one (or both) of the receptors (AR and GR) could have been activated. To distinguish between the two receptors, the activation assay needs to be repeated, but with one of the two receptors blocked (Wilson et al., 2002).

4.5.4 Sampling and sample preparation

Sample collection (1 L) should be done in glass bottles rinsed with HPLC grade methanol. Samples need to be extracted using solid phase extraction.

4.5.5 Method summary

The MDA-Kb2 cells are maintained in Leibovitz's L-15 (L-15) growth media supplemented with 10% fetal bovine serum (FBS), 100 µg/mL penicillin, 100 U/mL streptomycin and 0.25 µg/mL amphotericin B.

This assay is performed using L-15 medium supplemented with FBS that had been stripped from hormones using dextran-coated carbon. Cells are seeded at 1.2 x 10^5 cells/mL in 96-well plates and allowed to attach overnight. Sample extracts are prepared in methanol and serially diluted, before being dosed in triplicate. Each plate should contain a vehicle control and a concentration range of an agonist positive control (testosterone) (0.0022, 0.009, 0.0359, 0.1438, 0.575 and 2.3 mg/mL). Cells are exposed to 250 µL/well of the dosing solution for 48h at 37°C, 5% CO_2. The AR antagonist is flutamide (0.9765, 3.9063, 15.625, 62.5, 250 and 1000 mg/mL) and GR agonist is dexamethasone (0.12, 0.6, 3, 15, 75 and 375 mg/mL).

After the 48h incubation period, cells are washed with phosphate-buffered saline at room temperature and lysed with 25 µL lysis buffer. Luciferase activity is determined using a luminometer and quantified as relative light units. Each well receives 25 µL reaction buffer (20 mM tricine, 2.67 mM $MgSO_4 \cdot 7H_2O$, 33.3 mM dithiothreitol (DTT), 470 µM beetle luciferin, 270 µM coenzyme A, 530 µM adenosine triphosphate (ATP), 0.1 mM EDTA-disodium salt and 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2 \cdot 5H_2O$.

The positive control standard curve is fitted (sigmoidal function, variable slope) and the bioassay equivalents (BEq) of extracts are interpolated from the positive control standard curve and corrected with the appropriate dilution factor for each sample. For chemicals, the relative potency (RP) and relative induction efficiency (RIE) compared to positive control are reported.

4.5.6 Advantages and/or limitations

Advantages

- The assay is relatively rapid, eliminates the need for transfection, can be conducted in 96 well plates and consistent results are produced.
- This assay can be used in place of the AR-CALUX and GR-CALUX as it is less expensive, but equally sensitive.

Limitations of the bioassay

- Requires a dedicated cell culture laboratory.
- Special care needs to be taken in order to prevent androgenic contamination.
- Matrix interference in the form of cytotoxicity.

4.5.7 Specialized facilities and equipment required

Dedicated cell culture laboratory
Microplate luminometer with two dispensers

4.5.8 Acquisition of the cell line

The cells can be obtained from LGC Standards South Africa (catalogue no ATCC-CRL-2713).
An import permit will also be required from the Department of Health, RSA.

REFERENCES

CARTER M and SHIEH JC (2015) *Guide to research techniques in neuroscience*. 2nd edition. Academic Press: London.

WILSON VS, BOBSEINE K, LAMBRIGHT CR and GRAY JR, LE (2002) A novel cell line, MDA-kb2, that stably expresses an androgen-and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. *Toxicological Sciences* **66** (1), 69-81.

4.6 FACTSHEET: THE GH3.TRE-LUC REPORTER GENE ASSAY (THYROID ACTIVITY)

Compiled by: MC Van Zijl and NH Aneck-Hahn

4.6.1 Purpose

The GH3.TRE-Luc assay is an *in vitro* luciferase reporter gene assay that was developed to measure receptor mediated thyroid hormone activity.

4.6.2 Application

This assay is used to assess the biological activity of chemicals, chemical mixtures, water (e.g. drinking water, groundwater, surface water, wastewater, water for reuse, etc.) and other environmental samples (e.g. sediment).

4.6.3 Test principle

The rat pituitary tumor GH3 cell line constitutively expresses both thyroid hormone receptor (THR) isoforms and has been modified to contain a luciferase reporter gene (Freitas et al., 2011). The presence of the luciferase enzyme can then be assessed by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. The amount of light produced is relative to the degree of thyroid hormone activity of the test chemical. Agonists stimulate luciferase expression and are compared to the vehicle control or to the triiodothyronine (T3) control. The antagonist control is sodium arsenite. Cytotoxicity is assessed using resazurine.

4.6.4 Sampling and sample preparation

Sample collection (1 L) should be done in glass bottles rinsed with HPLC grade methanol.
Samples need to be extracted using solid phase extraction and Oasis HLB glass cartridges.

4.6.5 Method summary

The assay is performed according to Freitas et al. (2011), with modifications from Mengeling and Furlow (2015). The cells are maintained in DMEM/F12 (1:1) supplemented with 10% fetal calf serum and incubated at 37°C, 5% CO_2. Cells are seeded at 5 x 10^4 cells per well in 96-well luminometer plates and allowed to attach overnight. After 24h incubation the medium is replaced with serum free medium (DMEM/F12 (1:1) supplemented with 10 µg/mL bovine insulin, 10 µM ethanolamine, 10 ng/mL sodium selenite, 10 µg/mL human apotransferrin and 500 µg/mL bovine serum albumin) and incubated for a further 24h before exposing.

Dosing dilutions are prepared in the serum free medium and each plate should contain agonist positive control (T3), negative control (vehicle only), antagonist control (T3 plus sodium arsenite) and background control (vehicle plus sodium arsenite). Each sample should be tested alone as well as in the presence of 0.25 nM T3 (to test for antagonist activity). Cells are exposed for 24h with 100 µL/well dosing solution at 37°C, 5% CO_2.

After the incubation period, cytotoxicity is assessed by adding 8 µL resazurine (400 µM) to each well, incubating in the dark for 4h (37°C, 5% CO_2) and measuring fluorescence at 530 nm excitation and 590 nm emission. Luciferase activity is determined using a luminometer and quantified as relative light units. The cells are washed with phosphate buffered saline at room temperature and lysed with 25 µL lysis buffer. Each well

receives 25 µL reaction buffer (25 mM glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg/mL BSA, pH 7.8), followed by 25 µL 1 mM D-luciferin 5s later.

The T3 standard curve is fitted (sigmoidal function, variable slope) and the T3 equivalents (T3Eq) of extracts are interpolated from the estradiol standard curve and corrected with the appropriate dilution factor for each sample. For chemicals, the relative potency (RP) and relative induction efficiency (RIE) compared to T3 are reported.

4.6.6 Advantages and/or limitations

Advantages
The assay is relatively rapid, eliminates the need for transfection and can be conducted in 96 well plates.

Limitations of the bioassay
- Requires a dedicated cell culture laboratory.
- Matrix interference in the form of cytotoxicity.

4.6.7 Specialized facilities and equipment

Dedicated cell culture laboratory
Microplate fluorometer
Microplate luminometer with two dispensers

4.6.8 Acquisition of the cell line

The cells can be obtained from Wageningen University (The Netherlands) and will require a material transfer agreement (MTA). An import permit from the Department of Health, RSA will also be required.

REFERENCES

FREITAS J, CANO P, CRAIG-VEIT C, GOODSON M, FURLOW JD and MURK AJ (2011) Detection of thyroid hormone receptor disruptors by a novel stable in vitro reporter gene assay. *Toxicology in vitro* **25** 257-66.
MENGELING BJ and FURLOW JD (2015) Pituitary specific retinoid-X receptor ligand interactions with thyroid hormone receptor signalling revealed by high throughput reporter and endogenous gene responses. *Toxicology in Vitro* **29** (7):1609-1618.

4.7 FACTSHEET: THE AREC32 OXIDATIVE STRESS ASSAY

Compiled by: MC Van Zijl and NH Aneck-Hahn

4.7.1 Purpose

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

4.7.2 Application

This assay is used to assess the induction of oxidative stress and cytotoxicity of chemicals, chemical mixtures, water (e.g. drinking water, groundwater, surface water, wastewater, water for reuse, etc.) and other environmental samples (e.g. sediment).

4.7.3 Test principle

The AREc32 cell line was generated by Wang et al. (2006). It is derived from the MCF-7 human breast cancer cell line, with the addition of a luciferase gene construct attached to the Antioxidant Response Element (ARE). ARE is activated by Nrf2, which activates the cellular defense mechanism against oxidative stress. In this system, the induction of Nrf2 is proportional to the amount of luciferase produced by the cells, which can be assessed by a bioluminescence assay (Esher et al., 2021).

4.7.4 Sampling and sample preparation

Sample collection (1 L) should be done in glass bottles rinsed with high performance liquid chromatography (HPLC) grade methanol.
Samples need to be extracted using solid phase extraction and Oasis HLB cartridges.

4.7.5 Method summary

The assay is done according to the method described by Esher et al. (2012). The AREc32 cells are maintained in DMEM with sodium pyruvate and L-glutamine, high glucose, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% glutamax, 1.6% geneticin (G418), incubated at 37°C, 5% CO_2. Cells are seeded in 96 well luminometer plates at 1.2 x 10^4 cells/well and allowed to attach overnight. Cells are exposed for 24h to controls and chemicals or extracts before cytotoxicity or luciferase activity is assessed. Each plate must include one serial dilution of the tert-Butylhydroquinone (tBHQ) positive control (1-7.5 µM) and one row of medium blank.

Cell viability is assessed with the MTS assay. After 24h of incubation the medium in each plate is replaced by 120 µL MTS in Hyclone DMEM without phenol red and the absorbance is read at 492 nm after 2h incubation. Cell viability is calculated by dividing the absorbance of the sample with the absorbance of the controls.

Lucuferase activity is assessed using the Luciferase Assay System (Promega E1 500) according to the Promega protocol and luminescence is quantified as relative light units using a luminometer. The induction ratio (IR) is calculated by dividing the RLU of the sample by the average RLU of the controls. The IR values are then plotted against the concentration (up to an IR of 5) to derive a linear concentration-effect curve. The tBHQ equivalent concentration (tBHQ-Eq) is calculated as the ratio of the concentration of the reference compound that induces an IR of 1.5 to the concentration of the sample that induces an IR of 1.5.

4.7.6 Advantages and/or limitations

Advantages

- The assay is relatively rapid, eliminates the need for transfection, can be conducted in 96 well plates and consistent results are produced.

Limitations of the bioassay

- Requires a dedicated cell culture laboratory.
- Matrix interference in the form of cytotoxicity.

4.7.7 Specialized facilities and equipment required

Dedicated cell culture laboratory
Microplate luminometer
Microplate fluorometer

4.7.8 Acquisition of the cell line

The cells can be obtained from the European Collection of Authenticated Cell Cultures (ECACC), cat no 16071902.

An import permit will also be required from the Department of Health, RSA.

REFERENCES

ESCHER BI, DUTT M, MAYLIN E, TANG JYM, TOZE S, WOLF CR and LANG M (2012) Water quality assessment using the AREc32 reporter gene assay indicative of the oxidative stress response pathway. *Journal of Environmental Monitoring* **14** 2877-2885.

WANG XJ, HAYES JD AND WOLF CR (2006) Generation of a Stable Antioxidant Response Element-Driven Reporter Gene Cell Line and Its Use to Show Redox-Dependent Activation of Nrf2 by Cancer Chemotherapeutic Agents. *Cancer Research* **66** 22 10983-10994.

4.8 FACTSHEET: OXIDATIVE STRESS BIOMARKER – REACTIVE OXYGEN SPECIES (ROS) DETERMINATION

Compiled by: Suranie Horn

4.8.1 Purpose

Pollutants have the potential to increase the level of reactive oxygen species (ROS) and decrease antioxidants within the vertebrate body. This imbalance in ROS levels and cellular antioxidant endogenous mechanisms are known as oxidative stress.

4.8.2 Application

This assay is used to assess the biological activity of chemicals, chemical mixtures, water (e.g. drinking water, groundwater, surface water, wastewater, water for reuse, etc.) and other environmental samples (e.g. sediment).

4.8.3 Test principle

The level of ROS, including hydrogen peroxide (H_2O_2), can be measured in cells using a fluorogenic dye such as 2'-7'dichlorodihydrofluorescein diacetate (H_2DCFDA) (Katerji et al., 2019). The H_2DCFDA diffuses into the cells and is hydrolysed to 2'-7'dichlorodihydrofluorescein (DCFH) where it remains trapped within the cells. If H_2O_2 is produced by the cells as a result of increased oxidative stress, the DCFH will react with the H_2O_2 and generate fluorescent 2'-7'dichlorofluorescein (DCF) (Katerji et al., 2019) which can be measured at excitation and emission wavelengths of 480 nm and 535 nm using a fluorescence plate reader. The relative fluorescence unit (RFU) of ROS produced by the cells can then be calculated as the difference between experimental and blank measurements. The negative control will consist of untreated cells stimulated with 0.03% H_2O_2, one hour prior to the assay.

4.8.4 Sampling and sample preparation

Sample collection (1 L) should be done in glass bottles rinsed with HPLC grade methanol.
Samples need to be extracted using solid-phase extraction.

4.8.5 Method summary

Cells are maintained according to each cell line's specifications. Cells are seeded in 24-well plates, after a 24 0.03% hydrogen peroxide for 1 h. The 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (10 µM) dye is added to all the wells and incubated for 30 min @37°C in the dark. Subsequently, that plates are washed and cells are trypsinised. The cell suspension is centrifuged and the supernatant discarded. The pellet is re-suspended in PBS and loaded in a black-walled 96-well to measure the fluorescence. Measure fluorescence with excitation at 480 nm and emission at 535 nm.

The relative fluorescence units (RFU) of the intracellular ROS are calculated as the difference between experimental and blank measurements (Yao et al., 2015 and Wu et al., 2011).

The oxidative stress biomarker responses should be expressed in terms of per milligram protein content of the exposed cells. The protein contents of each sample of each batch were determined using the Bradford (1976)

method. This method is based on the binding of Coomassie brilliant blue (active ingredient in Bradford reagent) dye to proteins which can then be quantified using a spectrophotometer. The protein content is determined by the use of a protein standard (bovine serum albumin (BSA0) calibration curve.

4.8.6 Advantages and/or limitations

Advantages

- The assay is relatively rapid and can be conducted in 24-well plates and consistent results are produced.

Limitations of the bioassay

- Requires a dedicated cell culture laboratory.
- Some cell lines for, e.g. liver tissue cells, rapidly produce catalase in response to oxidative stress. The level of ROS is therefore too low (already converted) to be compared to control cells, and does not accurately reflect the stress response due to ROS.

4.8.7 Specialized facilities and equipment required

Dedicated cell culture laboratory
Microplate luminometer with two dispensers

4.8.8 Acquisition of the cell line

The cells can be obtained from LGC Standards South Africa (ATCC), Sigma.
An import permit will also be required depending on the nature of the cell line to be used.

REFERENCES

BRADFORD MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 1-2 248-254.
KATERJI M, FILIPPOVA M & DUERKSEN-HUGHES P (2019) Approaches and methods to measure oxidative stress in clinical samples: Research applications in the cancer field. *Oxidative Medicine and Cellular Longevity* 1279250.
WU D and YOTNDA P (2011) Production and detection of reactive oxygen species (ROS) in cancers. *Journal of visualized experiments: JoVE*, **57**.
YAO Q, LIN M, WANG Y, LAI Y, HU J, FU T, WANG L, LIN S, CHEN L and GUO Y (2015) Curcumin induces the apoptosis of A549 cells via oxidative stress and MAPK signalling pathways. *International Journal of Molecular Medicine* **36** 4 1118-1126.

4.9 FACTSHEET: OXIDATIVE STRESS BIOMARKER – CATALASE DETERMINATION

Compiled by: Suranie Horn

4.9.1 Purpose

Pollutants have the potential to increase the level of enzymes responsible to neutralize and oxidative stress response and decrease antioxidants within the vertebrate body. This imbalance in catalase (CAT) levels and cellular antioxidant endogenous mechanisms are known as oxidative stress.

4.9.2 Application

This assay is used to assess the biological activity of chemicals, chemical mixtures, water (e.g. drinking water, groundwater, surface water, wastewater, water for reuse, etc.) and other environmental samples (e.g. sediment).

4.9.3 Test principle

Catalase is the enzyme responsible for the degradation of H_2O_2 to water (H_2O) and oxygen (O_2) (Katerji et al., 2019) and therefore CAT determination is based on the principle of measuring the enzyme-catalysed decomposition of H_2O_2 (Cohen et al., 1970). H_2O_2 is added to the cells and after incubation, H_2SO_4 is used to stop the reaction. The amount of H_2O_2 remaining after catalase action in the cells will be determined by titration with potassium permanganate ($KMnO_4$), a very strong oxidizing reagent. The potassium permanganate which did not react (residual) with the H_2O_2 is measured spectrophotometrically at a wavelength of 490 nm and the amount remaining is inversely proportional to the activity of the catalase enzyme. This is followed by the addition of excess potassium permanganate ($KMnO_4$) to react with H_2O_2. Catalase activity is determined by measuring absorbance at a wavelength of 490 nm. The response is compared to the control and a change in catalase activity indicates oxidative stress.

4.9.4 Sampling and sample preparation

Sample collection (1 L) should be done in glass bottles rinsed with HPLC grade methanol. Samples need to be extracted using solid-phase extraction.

4.9.5 Method summary

Cells are maintained according to each cell line's specifications. Cells are seeded in 24-well plates, and after 24 h of exposure to the water extracts, the plates are washed and cells are trypsinised. The cell suspension is centrifuged and the supernatant discarded. The pellet is re-suspended in ice-cold phosphate buffer, sonicated and centrifuged again. The supernatant is added to a white-walled 96-well together with H_2O_2. The reaction is stopped with H_2SO_4 where after $KMnO_4$ was added to each well and absorbance measured within 30 to 60 seconds at 490 nm. All tests were done in triplicate and results were reported as a mean of three readings and expressed as µmol H_2O_2/min/mg protein (Cohen et al., 1970).

The oxidative stress biomarker responses should be expressed in terms of per milligram protein content of the exposed cells. The protein contents of each sample of each batch were determined using the Bradford (1976) method. This method is based on the binding of Coomassie brilliant blue (active ingredient in Bradford reagent) dye to proteins which can then be quantified using a spectrophotometer. The protein content is determined by the use of a protein standard (BSA) calibration curve.

4.9.6 Advantages and/or limitations

Advantages

- The assay is relatively rapid and can be conducted in 24-well plates and consistent results are produced.

Limitations of the bioassay

- Requires a dedicated cell culture laboratory.

4.9.7 Specialized facilities and equipment required

Dedicated cell culture laboratory
Microplate luminometer with two dispensers

4.9.8 Acquisition of the cell line

The cells can be obtained from LGC Standards South Africa (ATCC), Sigma.
An import permit will also be required depending on the nature of the cell line to be used.

REFERENCES

BRADFORD MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 1-2 248-254.
COHEN G, DEMBIEC D, & MARCUS J (1970). Measurement of catalase activity in tissue extracts. *Analytical Biochemistry*, **34** 1 30-38.
KATERJI M, FILIPPOVA M & DUERKSEN-HUGHES P (2019). Approaches and methods to measure oxidative stress in clinical samples: Research applications in the cancer field. *Oxidative Medicine and Cellular Longevity*, 1279250.

4.10 FACTSHEET: OXIDATIVE STRESS BIOMARKER SUPEROXIDE DISMUTASE (SOD) DETERMINATION

Compiled by: Suranie Horn

4.10.1 Purpose

Pollutants have the potential to increase the level of enzymes responsible to neutralize and oxidative stress response and decrease antioxidants within the vertebrate body. This imbalance in superoxide dismutase (SOD) levels and cellular antioxidant endogenous mechanisms are known as oxidative stress.

4.10.2 Application

This assay is used to assess the biological activity of chemicals, chemical mixtures, water (e.g. drinking water, groundwater, surface water, wastewater, water for reuse, etc.) and other environmental samples (e.g. sediment).

4.10.3 Test principle

Superoxide dismutase catalyses the partitioning of superoxide radicals (O_2^-) into ordinary O_2 and H_2O_2 and pyrogallol is an organic compound that auto-oxidises rapidly. Superoxide dismutase activity can therefore be quantified in a kinetic reaction using pyrogallol auto-oxidation. The pyrogallol assay for SOD activity investigates the ability of SOD to inhibit the auto-oxidation of pyrogallol into a yellow solution (Katerji et al., 2019), which can be determined by measuring the optical density at 560 nm. A yellow-brown colour indicates autoxidation and thus no SOD activity, while a white colour indicates no autoxidation and SOD activity. The response is compared to the control and a change in catalase activity indicates oxidative stress.

4.10.4 Sampling and sample preparation

Sample collection (1 L) should be done in glass bottles rinsed with HPLC grade methanol.
Samples need to be extracted using solid-phase extraction.

4.10.5 Method summary

Cells are maintained according to each cell line's specifications. Cells are seeded in 24-well plates, and after 24 h of exposure to the water extracts, the plates are washed and cells are trypsinised. The cell suspension is centrifuged and the supernatant discarded. The pellet is re-suspended in ice-cold phosphate buffer, sonicated and centrifuged again. The supernatant and tris buffer is added to a white-walled 96-well together with diethylene triamine penta-acetic acid (DTPA)/Tris buffer. The reaction is initiated by adding pyrogallol to each well and absorbance measured for 560 nm every 30 seconds for 5 minutes 10 readings. All tests were done in triplicate and results were reported as a mean of three readings and expressed as µmol H_2O_2/min/mg protein (Cohen et al., 1970).

The oxidative stress biomarker responses should be expressed in terms of per milligram protein content of the exposed cells. The protein contents of each sample of each batch were determined using the Bradford (1976) method. This method is based on the binding of Coomassie brilliant blue (active ingredient in Bradford reagent) dye to proteins which can then be quantified using a spectrophotometer. The protein content is determined by the use of a protein standard (BSA) calibration curve.

4.10.6 Advantages and/or limitations

Advantages

- The assay is relatively rapid and can be conducted in 24-well plates and consistent results are produced.

Limitations of the bioassay

- Requires a dedicated cell culture laboratory.

4.10.7 Specialized facilities and equipment required

Dedicated cell culture laboratory
Microplate luminometer with two dispensers

4.10.8 Acquisition of the cell line

The cells can be obtained from LGC Standards South Africa (ATCC), Sigma.
An import permit will also be required depending on the nature of the cell line to be used.

REFERENCES

BRADFORD MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72** 1-2 248-254.
COHEN G, DEMBIEC D, & MARCUS J (1970) Measurement of catalase activity in tissue extracts. *Analytical Biochemistry*, **34** 1 30-38.
KATERJI M, FILIPPOVA M and DUERKSEN-HUGHES P (2019) Approaches and methods to measure oxidative stress in clinical samples: Research applications in the cancer field. *Oxidative Medicine and Cellular Longevity*,1279250.

CHAPTER 5: FACTSHEETS FOR SELECTED *IN VIVO* BIOASSAYS

5.1 FACTSHEET: SALMONELLA /MICROSOME FLUCTUATION TEST (AMES FLUCTUATION TEST)

Compiled by: Lizet Swart and Hesmarie Pearson

5.1.1 Purpose

This International Standard (ISO/DIS 11350, 2011 "Water quality – Determination of the genotoxicity of water and wastewater – *Salmonella*/microsome fluctuation test (Ames fluctuation test))" specifies a method for the determination of the genotoxic potential of water and wastewater using the bacterial strains *Salmonella typhimurium* TA98 and TA100 in a fluctuation assay. This combination of strains is able to measure genotoxicity of chemicals that induce point mutations (base pair substitutions and frameshift mutations) in genes coding for enzymes that are involved in the biosynthesis of the amino acid histidine.

NOTE 1 For measuring genotoxicity of samples containing DNA crosslinking agents ISO 13829 (2000) should be applied.

5.1.2 Application

This method is applicable to:

- Freshwater
- Wastewater
- Aqueous extracts and leachates
- Eluates of sediments (freshwater)
- Porewater
- Aqueous solutions of single substances or of chemical mixtures
- Drinking water

NOTE 1 When testing drinking water, extraction and pre-concentration of water samples may be necessary.

5.1.3 Method principle

The bacteria are exposed under defined conditions to various concentrations of the test sample and incubated for 100 minutes at 37 ± 1°C in 24-well plates. Due to this exposure, genotoxic agents enclosed in the test sample may be able to induce mutations in one or both marker genes of the bacterial strains used (hisG46 for TA100 and hisD3052 for TA98) in correlation to the applied concentrations. Induction of mutations will cause a concentration-related increase in the number of mutant colonies. After exposure of the bacteria, reversion

indicator medium, containing the pH indicator dye bromocresol purple, is added to the wells. Subsequently, the batches are distributed to 384 wellplates (48 wells for each parallel) and incubated for 48 hours to 72 hours. Mutagenic activity of the test sample is determined by counting the number of purple to yellow shifted wells (per 48 wells of each parallel), treated with the undiluted or the diluted test sample, compared to the negative control. The lowest dilution (1 : N) of the test sample which induces no mutagenic effect under all experimental conditions (if any mutagenic effect is induced by the test sample) is the criterion for evaluating the mutagenic potential. Sample dilutions above this (1 : A, A < N) shall induce a mutagenic effect according to the criteria of this standard in at least one strain under at least one activation condition (with or without addition of S9 mix). The respective D_{min}-value is N. If no mutagenic effect is observed under all experimental conditions, this dilution is 1 : 1 and the respective D_{min}-value is 1.

5.1.4 Sampling and sample preparation

Take samples as specified in ISO 5667-1 (2020), ISO 5667-3 (2018), ISO 5667-14 (2014), and ISO 5667-16 (2017).

5.1.5 Method summary

- Prepare overnight culture
- Prepare S9 mix
- Preparation of tester strains
- Test culture without S9 mix
- Test culture with S9 mix
- Measurement of revertant growth
- Calculation of cytotoxicity

5.1.6 Advantages and/or limitations

Advantages
The Ames test has several key advantages: It is an easy and inexpensive bacterial assay for determining the mutagenicity of any chemical. Results are robust, and the Ames test can detect suitable mutants in large populations of bacteria with high sensitivity. It does not require any special equipment or instrumentation.

Limitations
Bacterio-toxic effects of the test sample may lead to a reduction of viable bacteria and to a reduction of wells with revertants due to a repression of revertant growth.

This method includes sterile filtration of water and wastewater prior to the test. Due to this filtration, solid particles are separated from the test sample. Thus, genotoxic substances adsorbed on particles might not be detected.

5.1.7 Specialized facilities and equipment

Temperature- and time-controlled incubator, 37 ± 1°C, pH meter, Analytical balance, Steam steriliser, Dry steriliser, Magnetic stirrer, Rotary mixer, Freezer, at least ≤ -18°C and at ≤ -70°C, Pipettes, 0.1 mL, 0.5 mL, 1 mL, 2 mL, 5 mL, 10 mL and 25 mL, glassware or plastics, Sterile filters, 0.2 µm and 0.45 µm, 8-channel multi-stepper pipette (repeater pipette), 8-channel pipettes, 5 µL to 50 µL and 50 µL to 300 µL, Spectrophotometer, Transparent sterile polystyrene 24-well and 384-well plates with flat bottom and lid, Microplate photometer for 24 well plates and optionally for 384 well plates, filters: 420 nm ± 15 nm and 595 nm ± 10 nm.

Use mutant strains of *Salmonella typhimurium* LT2, which enable detection of point mutations, to determine the mutagenic potential of a test sample. Since point mutations can be subdivided into two classes (frameshift mutations and base pair substitutions), the two tester strains TA98 and TA100 are used. TA98 contains as a marker the frameshift mutation (+2 type) hisD3052, whereas TA100 bears the base pair substitution hisG46.

REFERENCES

Note: For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO (International Standards Organisation) 3696 (1987) Water for analytical laboratory use – Specification and test methods ISO/TC 47 Chemistry pp5.

ISO (International Standards Organisation) 5667-1 (2020) Water quality – Sampling – Part 1: Guidance on the design of sampling programmes and sampling techniques ISO/TC 147/SC 6 Sampling (general methods) pp39.

ISO (International Standards Organisation) 5667-3 (2018) Water quality – Sampling – Part 3: Preservation and handling of water samples ISO/TC 147/SC 6 Sampling (general methods) pp52.

ISO (International Standards Organisation) 5667-10 (2020) Water quality – Sampling – Part 10: Guidance on sampling of wastewaters ISO/TC 147/SC 6 Sampling (general methods.

ISO (International Standards Organisation) 5667-14 (2014) Water quality – Sampling – Part 14: Guidance on quality assurance of environmental water sampling and handling ISO/TC 147/SC 6 Sampling (general methods) pp34.

ISO (International Standards Organisation) 5667-16 (2017) Water quality – Sampling – Part 16: Guidance on biotesting of samples ISO/TC 147/SC 6 Sampling (general methods) pp24.

ISO (International Standards Organisation) 7027 (2016) Water quality – Determination of turbidity – Part 1: Quantitative methods ISO/TC 147/SC 2 Physical, chemical and biochemical pp9.

ISO (International Standards Organisation /Draft International Standard) 11350 (2011) Water quality – Determination of the genotoxicity of water and wastewater – Salmonella/microsome fluctuation test (Ames fluctuation test) ISO/TC 147/SC 5 Biological methods pp37.

ISO (International Standards Organisation) 13829 (2000) Water quality – Determination of the genotoxicity of water and wastewater using the umu-test ISO/TC 147/SC 5 Biological methods pp18.

ISO/TS (International Standards Organisation /Technical Standard) 20281 (2006) Water quality – Guidance on statistical interpretation of ecotoxicity data ISO/TC 147/SC 5 Biological methods pp252.

ISO (International Standards Organisation) 21427-2 (2006) Water quality– Evaluation of the genotoxicity by measurement of the induction of micronuclei – Part 2: Mixed population method using the cell line V79 ISO/TC 147/SC 5 Biological methods pp20.

5.2 FACTSHEET: A*LIIVIBRIO FISCHERI* BIOLUMINESCENCE TEST

Compiled by: Lizet Swart and Hesmarie Pearson

5.2.1 Purpose

This method is used to determine the inhibition of the luminescence emitted by the marine bacteria *Aliivibrio fischeri* (NRRL B-11177) when exposed under controlled laboratory conditions. This method provides an easy-to-use bioassay for measuring acute toxicity of substances/pollution to bacteria, such as *Aliivibrio fischeri*. The significance of the *Aliivibrio fischeri* acute toxicity test is to assist in the assessment of possible risks to bacteria in the natural environment. It also aids in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other bacterial species for comparative purposes. The *Aliivibrio fischeri* test should form part of a battery of at least three tests representing different trophic levels in the aquatic environment (others include but are not limited to bacteria, algae, invertebrates, vertebrates, and protozoa amongst others). The reason for this is the variation between the sensitivity of the different species to different substances, and therefore using several different trophic levels, increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

5.2.2 Application

The *Aliivibrio fischeri acute toxicity* test is applicable to the following sample types:

- Wastewater or industrial effluents
- Sewage samples
- Aqueous extracts and leachates
- Freshwaters (surface and groundwater)
- Eluates of sediment (freshwater, brackish and sea water)
- Porewater
- Single substances diluted in water
- Products/chemicals

5.2.3 Test principle

The test measures the acute toxicity of water samples to the bacteria *Aliivibrio fischeri* and is based on the SANS11348-3 (2007) method, titled “Water Quality: Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) – Part 3 for the method using freeze-dried bacteria and describes the method for determining the inhibition of the luminescence emitted by the marine bacterium *Vibrio fischeri* (NRRL B-11177). Second edition”. The method measures the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Freshwater bioassays are simple, rapid, sensitive, and reproducible toxicity tests at low cost. These bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments.

5.2.4 Sampling and sample preparation

Collect samples in chemically inert, clean containers as specified in ISO 5667-16 (1998) and where necessary, store samples at 2°C to 5°C in the dark in the containers for no longer than 48 hours. Perform the necessary pH-adjustment and salt addition immediately before testing.

5.2.5 Method summary

The inhibition of light emission by cultures of *Aliivibrio fischeri* is determined by means of a batch test. This is accomplished by combining specific volumes of the test sample or the diluted sample with the luminescent bacterial suspension in a test tube. The test criteria are the luminescence, measured after a contact time of 15 or 30 minutes and optionally 5 minutes, considering a correction factor (f_{kt}), which is a measure of intensity changes of control samples during the exposure time. The inhibitory effect of the water sample is determined as the EC_{20} and/or EC_{50} values by means of a dilution series.

5.2.6 Advantages and/or limitations

Advantages

Freshwater bioassays are low cost, simple, rapid, sensitive, and reproducible toxicity tests. These bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments as well as monitoring of water quality. The *Aliivibrio fischeri* test is commonly used to represent the bacterial trophic level. A further advantage of using the test is to comply with the "Management of complex industrial wastewater discharges by introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach" (DWAF, 2003).

Limitations

Living organisms used during these tests, have species specific requirements for culture conditions, which may necessitate adjustment of, e.g. the sample pH, salinity or oxygen concentration. These actions may have an effect on the bioavailability or solubility of certain hazardous substances. Loss of luminescence caused by light absorption or light scattering may occur in the case of strongly coloured or turbid samples. Since oxygen is required for the bioluminescence, samples with a high oxygen demand (and/or a low oxygen concentration) may cause a deficiency of oxygen and lead to inhibition.

Readily biodegradable nutrients in the sample may cause a pollutant-independent reduction in bioluminescence. Samples with a pH outside the pH range 6.0-8.5 affect the luminescence of the bacteria. As the test organism *Aliivibrio fischeri* is a marine bacterium, testing salt-water samples with the standard procedure often leads to stimulation effects of bioluminescence, which may mask inhibition effects.

5.2.7 Specialized facilities and equipment

Temperature controlled room, chiller block and luminometer.

5.2.8 Acquisition of the test kits

The test kits can be obtained from Environmental Bio-detection Products Incorporated

735 Griffith Court L7L 5R9 Ontario. Tel: 001 905 826 8378

Email: Sales@biotoxicity.com

Webpage: https://www.biotoxicity.com/

REFERENCES

DWAF (Department of Water Affairs and Forestry) (2003) The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effects Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.

ISO (International Organization for Standardization) 11348-3 (2007) 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. ISO/TC 147/SC 5 Biological methods pp21.

ISO (International Organization for Standardization) 5667-16 (1998) Water quality – Sampling – Part 16: Guidance on biotesting of samples. ISO/TC 147/SC 6 Sampling (general methods) pp24.

SANS (South African National Standards) 11348-3 (2007) Water quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test) – Part 3 for the method using freeze-dried bacteria.

5.3 FACTSHEET: *PSEUDOKIRCHNERIELLA SUBCAPITATA* GROWTH INHIBITION TEST

Compiled by: Lizet Swart and Hesmarie Pearson

5.3.1 Purpose

This method measures the toxicity of effluents, receiving waters or chemicals/products and leachates to the green algae *Pseudokirchneriella subcapitata*. This method provides an easy-to-use bioassay for measuring toxicity of substances /pollution to aquatic algae, such as *Pseudokirchneriella subcapitata*. The significance of the *Pseudokirchneriella subcapitata* growth inhibition test is to assist in the assessment of possible risks to algae/plants in the natural environment. It also aids in determination of possible water quality criteria for regulatory purposes and for use in correlation with toxicity testing of other algal species for comparative purposes.

The *Pseudokirchneriella subcapitata* growth inhibition test should form part of a battery of at least three tests representing different trophic levels in the aquatic environment (others include but are not limited to bacteria, algae, invertebrates, vertebrates and protozoans amongst others). The reason for this is the variation between the sensitivity of the different species to different substances, and therefore using several different trophic levels increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

5.3.2 Application

The *Pseudokirchneriella subcapitata* growth inhibition test is applicable to the following samples:

- Wastewater or industrial effluents
- Sewage samples
- Aqueous extracts and leachates
- Freshwaters (surface and groundwater)
- Eluates of sediment (freshwater, brackish and sea water)
- Porewater
- Single substances diluted in water
- Products/chemicals

5.3.3 Sampling and sample preparation

Specific sample collection and preparation applies as specified in the relevant standard.

5.3.4 Test principle

The test measures the short-term toxicity of water samples to the algae *Pseudokirchneriella subcapitata* and is based on the SANS 8692 (2015) method, titled “Water Quality: fresh water algal growth inhibition test with unicellular green algae” and describes the method for determining the growth inhibition of algae exposed to potential pollutants. The method measures the toxicity of effluents and receiving waters to freshwater and marine organisms. Freshwater bioassays are simple, rapid, sensitive, and reproducible toxicity tests at low cost. These bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments.

5.3.5 Sampling and sample preparation

Specific sample collection and preparation applies as specified in the relevant standard.

5.3.6 Method summary

Pseudokirchneriella subcapitata, over several generations, are exposed to test samples for a period of 72 hours using defined conditions. Growth inhibition is measured as a reduction in growth rate relative to a control carried out under identical conditions. Growth is determined in terms of optical density (OD). Definitive tests (testing serial dilutions) are carried out on samples to determine toxicity endpoints EC20 (endpoint concentration causing 20% growth inhibition – minimum effect concentration) and EC50 (endpoint concentration causing 50% growth inhibition).

5.3.7 Advantages and/or limitations

Advantages
Freshwater bioassays are simple, rapid, sensitive, and reproducible toxicity tests at low cost. These bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments as well as monitoring of water qualities. The *Pseudokirchneriella subcapitata* test is commonly used representing the algal trophic level. A further advantage of using the test is to comply with the "The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach" (DWAF, 2003).

Limitations
Living organisms as used during these tests, also have species specific requirements for culture conditions, which may necessitate adjustment of, e.g. the sample pH or salinity or oxygen concentration and these actions may have an effect on the bioavailability or solubility of certain hazardous substances. High salt concentrations in samples can lead to precipitation upon algal medium addition, which can interfere with OD measurements. If a precipitate forms during testing, note this on the data sheet – such results are omitted from the battery of tests during the hazard classification process.

Volatile substances might inhibit growth of algae in other wells, including that of the control. Pathogenic and/or predatory organisms in the samples may affect survival. Coloured natural samples may interfere with OD readings of the algal suspensions, especially when the colour shows absorption at the (670 nm) wavelength which is used to measure algal density. It is important to note that the determination of the toxicity of highly coloured samples to microalgae is automatically biased by interference of the colour with light penetration in the medium containing the algae.

5.3.8 Specialized facilities and equipment

Temperature controlled room, incubator with lights, centrifuge, spectrophotometer.

5.3.9 Acquisition of the test kits

The test kits can be obtained from MICROBIOTESTS Inc.

Kleimoer 15, 9030 Gent, Belgium

Tel: 0032 9 380 8545

mailto:info@microbiotests.com

https://www.microbiotests.com

REFERENCES

DWAF (Department of Water Affairs and Forestry) (2003) The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effects Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.

ISO (International Organization for Standardization) 5667-16 (1998) Water quality – Sampling – Part 16: Guidance on biotesting of samples. ISO/TC 147/SC 6 Sampling (general methods) pp24.

ISO (International Organization for Standardization) 8692 (2012) Water Quality – fresh water algal growth inhibition test with unicellular green algae. Third edition ISO/TC 147/SC 5 Biological methods pp21.

SANS ISO (South African National Standards / International Organization for Standardization) 8692 (2015) Edition 1. Water Quality – fresh water algal growth inhibition test with unicellular green algae. SABS/TC 147/SC 05.

5.4 FACTSHEET: *DAPHNIA MAGNA/PULEX* ACUTE TOXICITY TEST

Compiled by: Lizet Swart and Hesmarie Pearson

5.4.1 Purpose

This method provides an easy-to-use bioassay for measuring acute toxicity of substances /pollution to aquatic invertebrates, such as various *Daphnia sp*. The significance of the *Daphnia magna/pulex* acute toxicity test is to assist in the assessment of possible risks to invertebrates in the natural environment. It also aids in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other invertebrate species for comparative purposes.

The *Daphnia magna/pulex* acute toxicity test should form part of a battery of at least three tests representing different trophic levels in the aquatic environment others include but are not limited to bacteria, algae, invertebrates, vertebrates, and protozoa amongst others). The reason for this is the variation between the sensitivity of the different species, and therefore using several species increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

5.4.2 Application

The *Daphnia magna/pulex* acute toxicity test is used for the determination of toxicity of water samples, and is applicable to the following water samples:

- Wastewater or industrial effluents
- Sewage samples
- Aqueous extracts and leachates
- Freshwaters (surface and groundwater)
- Eluates of sediment (freshwater, brackish and sea water)
- Porewater
- Single substances, diluted in water
- Products/chemicals

5.4.3 Method principle

The method measures the short-term acute toxicity of freshwaters to the Cladocera *Daphnia magna/pulex* and is based on the SANS 6341 (2015) method, titled “Water Quality: Determination of the inhibition of the mobility of *Daphnia magna/pulex* Straus (Cladocera, Crustacea) – acute toxicity test” measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms”. Freshwater bioassays are simple, rapid, sensitive, and reproducible toxicity tests at low cost. These bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments.

5.4.4 Sampling and sample preparation

Specific sample collection and preparation applies as specified in the relevant standard.

5.4.5 Method summary

The 24 to 48 hour bioassays are performed in disposable multi-well test plates containing neonates, uniform in size and in age, hatched from dormant eggs (ephippia). *Daphnia magna/pulex* ephippia, less than 24 hours old, are exposed to test samples for a period of 48 hours in a static test. Mortalities are recorded after 24 and 48 hour exposure. The test is applied directly (as a screening test) to receiving water and/or wastewater discharges (as definitive test) to determine the percentage mortality. Definitive tests (testing dilutions) are carried out on toxic samples to determine the LC_{10} (concentration causing more than 10% mortality, minimum effect concentration) and LC_{50} (concentration causing 50% mortality) values.

5.4.6 Advantages and/or limitations

Advantages

This freshwater bioassay is simple, rapid, sensitive and a reproducible toxicity tests at low cost. Furthermore, these bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments as well as monitoring of water qualities. The *Daphnia magna/pulex* test is commonly used representing the invertebrate trophic level. A further advantage of using the test is to comply with the "The Management of Complex Industrial Wastewater Discharges". Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach" (DWAF, 2003).

Limitations

Living organisms as used during these tests, also have species specific requirements for culture conditions, which may necessitate adjustment of, e.g. the sample pH or salinity or oxygen concentration and these actions may have an effect on the bioavailability or solubility of certain hazardous substances. Dark coloured samples and samples containing high loads of suspended solids may impede the observation of the test organisms. Samples containing oils and surface tension altering compounds may cause test organisms to float. Pathogenic and /or predatory organisms in the samples may affect survival.

5.4.7 Specialized facilities and equipment

Temperature controlled room, incubator, light box (bottom illumination).

5.4.8 Acquisition of the test kits

The test kits can be obtained from MICROBIOTESTS Inc.
Kleimoer 15, 9030 Gent, Belgium
Tel: 0032 9 380 8545
info@microbiotests.com
https://www.microbiotests.com

REFERENCES

DWAF (Department of Water Affairs and Forestry) (2003) The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effects Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.

ISO (International Organization for Standardization) 5667-16 (1998) Water quality – Sampling – Part 16: Guidance on biotesting of samples. ISO/TC 147/SC 6 Sampling (general methods) pp24.

ISO (International Organization for Standardization) 6341 (2012) Water Quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – acute toxicity test Edition 4. ISO/TC 147/SC 5 Biological methods pp22.

SANS (South African National Standards) 6341 (2015) Edition 1. Water Quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – acute toxicity test.

US EPA (United States Environmental Protection Agency) (2002) Method for measuring the acute toxicity of effluents and receiving water to freshwater and marine organisms. EPA-821-R-02-012. U.S. Environmental Protection Agency, Office of Water (4303T), 1200 Pennsylvania Avenue, NW, Washington DC, 20460. Fifth Edition.

5.5 FACTSHEET: *POECILIA RETICULATA* ACUTE TOXICITY TEST

Compiled by: Lizet Swart and Hesmarie Pearson

5.5.1 Purpose

This method provides an easy-to-use bioassay for measuring acute toxicity of substances/pollution to aquatic vertebrates, such as *Poecilia reticulata*. The significance of the *Poecilia reticulata* acute toxicity test is to assist in the assessment of possible risks to vertebrates in the natural environment. It also aids in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other vertebrate species for comparative purposes.

The *Poecilia reticulata* acute toxicity test should form part of a battery of at least three tests representing different trophic levels in the aquatic environment (others include but are not limited to bacteria, algae, invertebrates, vertebrates, and protozoans amongst others). The reason for this is the variation between the sensitivity of the different species, and therefore using several different trophic levels increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

5.5.2 Application

The *Poecilia reticulata* acute toxicity test is applicable to the following water samples:

- Wastewater or industrial effluents
- Sewage samples
- Aqueous extracts and leachates
- Freshwaters (surface and groundwater)
- Eluates of sediment (freshwater, brackish and seawater)
- Porewater
- Single substances diluted in water
- Products/chemicals

5.5.3 Method principle

The method measures the acute toxicity of freshwaters to the vertebrate *Poecilia reticulata* and is based on the SANS 7346-1 (2013) method, titled “Water Quality: Determination of the acute lethal toxicity of substances to a freshwater fish, Static method measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms”. Freshwater bioassays are simple, rapid, sensitive, and reproducible toxicity tests at low cost. These bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments.

5.5.4 Sampling and sample preparation

Specific sample collection and preparation applies as specified in the relevant standard.

5.5.5 Method summary

Poecilia reticulata fry (younger than 14 days) are exposed to the test substance for a period of 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours and the concentrations at which 50% fish mortalities occur (LC_{50}) are determined where possible. The test is applied directly (as a screening test) to the samples to determine the percentage mortality. Definitive tests (range of testing dilutions) are carried out on toxic samples to determine the LC_{10} (concentration causing 10% mortality, minimum effect concentration) and LC_{50} (concentration causing 50% mortality) values.

5.5.6 Advantages and/or limitations

Advantages

Freshwater bioassays are simple, rapid, sensitive, and reproducible toxicity tests at low cost. These bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments as well as monitoring of water qualities. The *Poecilia reticulata* test is commonly used to represent the vertebrate trophic level. A further advantage of using the test is to comply with the "The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach" (DWAF, 2003).

Limitations

Living organisms as used during these tests, also have species specific requirements for culture conditions, which may necessitate adjustment of, e.g. the sample pH or salinity or oxygen concentration and these actions may have an effect on the bioavailability or solubility of certain hazardous substances. Pathogenic and/or predatory organisms in the samples may affect survival of the test organisms. Dark coloured samples and samples containing high loads of suspended solids may impede the observation of the test organisms as well as clog the organism's gills leading to respiratory distress.

5.5.7 Specialized facilities and equipment

Temperature controlled room, organism culturing and holding facility.

REFERENCES

DWAF (Department of Water Affairs and Forestry) (2003) The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effects Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.

ISO (International Organization for Standardization) 5667-16 (1998) Water quality – Sampling – Part 16: Guidance on biotesting of samples. ISO/TC 147/SC 6 Sampling (general methods) pp24.

ISO (International Organization for Standardization) 7346-1 (1996) Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)] – Part 1: Static method. ISO/TC 147/SC 5 Biological methods pp11.

SANS (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) Part1: Static method.

US EPA (United States Environmental Protection Agency) (2002) Method for measuring the acute toxicity of effluents and receiving water to freshwater and marine organisms. EPA-821-R-02-012. U.S. Environmental Protection Agency, Office of Water (4303T), 1200 Pennsylvania Avenue, NW, Washington DC, 20460. Fifth Edition.

5.6 FACTSHEET: *SPIRODELA POLYRHIZA* GROWTH INHIBITION TEST

Compiled by: Lizet Swart and Hesmarie Pearson

5.6.1 Purpose

This method provides an easy-to-use bioassay for measuring toxicity of substances/pollution to aquatic plants, such as *Spirodela polyrhiza*. The significance of the *Spirodela polyrhiza* growth inhibition test is to assist in the assessment of possible risks to aquatic plants in the natural environment. It also aids in determination of possible water quality criteria for regulatory purposes and for use in correlation with toxicity testing of other aquatic plant species for comparative purposes.

The *Spirodela polyrhiza* growth inhibition test should form part of a battery of at least three tests representing different trophic levels in the aquatic environment others include but are not limited to bacteria, algae, invertebrates, vertebrates, and protozoans amongst others. The reason for this is the variation between the sensitivity of the different species, and therefore using several species increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

5.6.2 Application

The *Spirodela polyrhiza* growth inhibition test, specifies a method for the determination of the inhibition of the growth of the first fronds of *Spirodela polyrhiza* germinated from turions, by substances and mixtures contained in water or wastewater, including treated municipal wastewater and industrial effluents. The test is also applicable to pure chemicals and in particular plant protection products and pesticides.

5.6.3 Method principle

The method measures the toxicity of samples to *Spirodela polyrhiza* and is based on the ISO 20227 (2017) standard, titled*: "*Water quality – Determination of the growth inhibition effects of wastewaters, natural waters and chemicals on the duckweed *Spirodela polyrhiza* – Method using a stock culture independent microtest".

Freshwater bioassays are simple, rapid, sensitive, and reproducible toxicity tests at low cost. These bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments.

5.6.4 Sampling and sample preparation

Specific sample collection and preparation applies as specified in the relevant standard.

5.6.5 Method summary

Turions produced by culturing *Spirodela polyrhiza* or taken from test tubes in which they are stored are transferred to a Petri dish containing growth medium and incubated for 3 days at 25 ± 1°C with continuous illumination of at least 6 000 lux (corresponding approximately to 85 µE m^{-2} s^{-1}). During this time the turions germinate and produce a small (first) frond. One germinated, turion with its first frond is then taken from the Petri dish and inoculated into each cup of a 6 × 8 multi-well test plate which contains the toxicant dilutions and the negative control (each of which is prepared in growth medium).

On completion of the inoculations, a photo of the multi-well is taken (at t_0 hour) with a digital camera and transferred to a computer file. The multi-well is subsequently incubated for 3 days at (25 ± 1°C) with continuous illumination of minimum 6 000 lux, after which a photo is again taken (at t_{72} hours) and transferred to a computer file. The area of the first frond in each test cup is measured with the aid of an image analysis programme, on the two photos of the multi-well (i.e. taken at t_0 hour and at t_{72} hours). The growth of the first fronds in the controls and in the test concentrations or dilutions is calculated as the difference between the t_{72} hours areas and the t_0 hour areas, after which the growth inhibition and the 72 hour EC_{50} or EC_x values are determined.

5.6.6 Advantages and/or limitations

Advantages

This freshwater bioassay is simple, rapid, sensitive and a reproducible toxicity tests at low cost. Furthermore, these bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments as well as monitoring of water qualities.

The *Spirodela polyrhiza* growth inhibition test is commonly used representing the aquatic plants. A further advantage of using the test is to comply with the “The Management of Complex Industrial Wastewater Discharges”. Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach” (DWAF, 2003).

The Spirodela polyrhiza TOXKIT TM^F has multiple advantages over conventional *Lemna* duckweed tests:

- The assay is totally independent of the culturing/maintenance of live stocks of the test species
- The germination of the turions and their transfer to the test plate are very simple operations
- The test plates for the toxicity test are small, require little bench space and incubation space, and allow to set up multiple tests concurrently
- The test duration (after the germination of the turions) is only 3 days (instead of 7 days for the conventional *Lemna* tests)
- The selected effect parameter (growth inhibition) is the measurement of the area of the first fronds of the germinated turions at the start and at the end of the test, which is simple and rapid with the aid of image analysis
- The photos of the test plates with the grown duckweeds are stored on a computer which allows to postpone the area measurements

- The test procedure is highly standardized, and its precision has been evaluated in an extensive "International Interlaboratory comparisons"
- Validity criteria have been selected for the assay and a methodology has been worked out for a reference test (quality control test) with potassium chloride (KCl)
  The sensitivity of the Spirodela TOXKIT TM^{F} has been determined on a substantial number of inorganic and organic compounds and was found to be very similar to that of conventional *Lemna* tests

Limitations

- Living organisms as used during these tests, also have species specific requirements for culture conditions, which may necessitate adjustment of, e.g. the sample pH or salinity or oxygen concentration and these actions may have an effect on the bioavailability or solubility of certain hazardous substances.

- Dark coloured samples and samples containing high loads of suspended solids may impede the observation of the test organisms.

- Pathogenic and /or predatory organisms in the samples may affect survival.

5.6.7 Specialized facilities and equipment

Temperature controlled room, incubator, lux meter, image analysis system, digital camera.

5.6.8 Acquisition of the test kits

The test kits can be obtained from MICROBIOTESTS Inc.
Kleimoer 15, 9030 Gent, Belgium
Tel: 0032 9 380 8545
info@microbiotests.com
https://www.microbiotests.com

REFERENCES

DWAF (Department of Water Affairs and Forestry) (2003) The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effects Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.

ISO (International Organization for Standardization) 5667-16 (1998) Water quality – Sampling – Part 16: Guidance on biotesting of samples. ISO/TC 147/SC 6 Sampling (general methods) pp24.

ISO (International Organization for Standardization) 20227 (2017) Water quality – Determination of the growth inhibition effects of wastewaters, natural waters and chemicals on the duckweed *Spirodela polyrhiza* – Method using a stock culture independent Microbiotest.

5.7 FACTSHEET: *THAMNOCEPHALUS PLATYURUS* ACUTE TOXICITY TEST

Compiled by: Lizet Swart and Hesmarie Pearson

5.7.1 Purpose

The *Thamnocephalus platyurus* acute toxicity test, is applied to assess water pollution and are primarily used to screen for toxic substances in the aquatic environment and to some extend to predict the toxic effect of environmental impacts on aquatic invertebrates. The toxicity test is used in the assessment of possible risk to similar invertebrate species in the natural environment, as an aid in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other species for comparative purposes (US EPA, 2002).

The *Thamnocephalus platyurus* test should form part of a battery of at least three tests representing different trophic levels in the aquatic environment (for example invertebrates, bacteria, algae, vertebrates, protozoans amongst others). The reason for this is the variation between the sensitivity of the different species, and therefore using several species increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

5.7.2 Application

The *Thamnocephalus platyurus* acute toxicity test is used for the determination of toxicity of water samples, such as effluents, receiving water, products and chemicals. According to ISO 14380 (2011) this method is applicable to following water samples:

- Chemical substances which are soluble under the conditions of the test, or can be maintained as a stable suspension or dispersion under the conditions of the test
- Industrial or sewage effluents
- Treated or untreated wastewater
- Aqueous extracts and leachates
- Freshwater (surface and groundwater)
- Toxins of blue green algae

5.7.3 Method principle

The method measures the short-term acute toxicity of samples to the crustacean *Thamnocephalus platyurus* and is based on the SANS 14380 (2014) method, titled Water quality – Determination of the acute toxicity to *Thamnocephalus platyurus* (Crustacea, Anostraca)"

Freshwater bioassays are simple, rapid, sensitive and reproducible toxicity tests at low cost. These bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments.

5.7.4 Sampling and sample preparation

Specific sample collection and preparation applies as specified in the relevant standard.

5.7.5 Method summary

The 24-hour bioassays are performed in disposable multi-well test plates containing larvae, uniform in size and in age, hatched from dormant cysts. *Thamnocephalus platyurus* larvae*,* less than 22 hours old, are exposed to test samples for a period of 24 hours in a static test. Mortalities are recorded after 24 hour exposure. The test is applied directly (as a screening test) to receiving water and /or wastewater discharges (as definitive test) to determine the percentage mortality. Definitive tests (testing dilutions) are carried out on toxic samples to determine the LC_{50} (concentration causing more than 50% mortality, minimum effect concentration) values.

5.7.6 Advantages and/or limitations

Advantages

This freshwater bioassay is simple, rapid, sensitive and a reproducible toxicity tests at low cost. Furthermore, these bioassays are particularly suited for routine toxicity testing of chemicals and wastes released in the aquatic environments as well as monitoring of water qualities A further advantage of using the test is to comply with the “The Management of Complex Industrial Wastewater Discharges”. Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach” (DWAF, 2003).

Limitations

Living organisms as used during these tests, also have species specific requirements for culture conditions, which may necessitate adjustment of, e.g. the sample pH or salinity or oxygen concentration and these actions may have an effect on the bioavailability or solubility of certain hazardous substances. Dark coloured samples and samples containing high loads of suspended solids may impede the observation of the test organisms. Samples containing oils and surface tension altering compounds may cause test organisms to float. Pathogenic and /or predatory organisms in the samples may affect survival.

5.7.7 Specialized facilities and equipment

Temperature controlled cabinet/room, incubator, stereomicroscope, lightbox.

5.7.8 Acquisition of the test kits

The test kits can be obtained from MICROBIOTESTS Inc.
Kleimoer 15, 9030 Gent, Belgium
Tel: 0032 9 380 8545
info@microbiotests.com
https://www.microbiotests.com

REFERENCES

DWAF (Department of Water Affairs and Forestry) (2003) The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effects Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.

ISO (International Organization for Standardization) 14380 (2011) “Water Quality – Determination of the acute toxicity *Thamnocephalus platyurus* (Crustacea, Anostraca). ISO/TC 147/SC 5 Biological methods pp21.

SANS (South African National Standards) 14380 (2011) Water quality – Determination of the acute toxicity to *Thamnocephalus platyurus* (Crustacea, Anostraca) pp 24.

US EPA (United States Environmental Protection Agency) (2002) Method for measuring the acute toxicity of effluents and receiving water to freshwater and marine organisms. EPA-821-R-02-012. U.S. Environmental Protection Agency, Office of Water (4303T), 1200 Pennsylvania Avenue, NW, Washington DC, 20460. Fifth Edition.

5.8 FACTSHEET: THE FROG EMBRYO TERATOGENESIS ASSAY OF *XENOPUS* (FETAX)

Compiled by: Edward Archer and Christoff Truter

5.8.1 Purpose

The Frog Teratogenesis Assay of Xenopus (FETAX) is an acute, *in vitro* whole-embryo bioassay to determine teratogenesis and developmental toxicity of test chemicals, complex mixtures and/or environmental samples using the African clawed frog (*Xenopus laevis*) as sentinel organism.

5.8.2 Application

This 96-hour toxicity assay is well described in guidelines from the American Society of Testing and Materials (ASTM, 1991/1998) to serve as an alternative toxicity screen using mammalian organisms to evaluate developmental toxicity *in vivo*. Moreover, the target endpoints that include mortality and the degree of embryonic malformations are valuable to assess the developmental toxicity in test environmental samples such as treated surface- or wastewater and even drinking water quality.

5.8.3 Test principle

Developmental toxicity serves as a sensitive *in vivo* bioassay to show toxicity in test samples at lower concentrations than where toxicity is observed for adult organisms. Embryos of *X. laevis* thus serve as good sentinel organisms due to their direct nature of contact with environmental matrices, their ease of in-house breeding, and relatively quick and well-documented developmental phases (Nieuwkoop and Faber, 1975). Test environmental samples or test chemicals/mixtures can be prepared at varying concentrations and exposed to the embryos at the onset of a specific developmental stage, upon which mortality is continuously monitored and developmental malformations recorded upon completion of the 96-hour exposure period using an established *X. laevis* embryo malformation index (Bantle et al., 1999).

5.8.4 Sampling and sample preparation

Refer to the American Society of Testing and Materials Guidelines for FETAX (ASTM, 1991; section 10) for a detailed description of sample preparation.

5.8.4.1 Environmental samples

Test environmental samples should be collected in clean glass bottles (washed with detergent, followed by two ethanol rinses and two Milli-Q water rinses) and transported to the testing facility/laboratory on ice. Same-day processing of aqueous sample extracts or freeze-dried sediment/soil samples should be done to preserve the chemical integrity of the test sample.

5.8.4.2 Aqueous samples

Environmental aqueous samples may be exposed directly to the test organisms or concentrated using SPE to generate a dilution series of the test water sample using FETAX Solution (ASTM, 1998) as solvent. All samples and sample concentrations should be performed in triplicate.

5.8.4.3 Whole soil/sediment

Soil or sediment testing can be performed by replacing the petri dishes with glass tubes that can accommodate a Teflon mesh insert that serves as an exposure vessel. Up to 35 g of sediment (dry weight) can be placed in the bottom of the vessel, the Teflon mesh insert added, and filled with 140 mL of FETAX Solution (ASTM, 1998). If the soil will be diluted, it should be verified that the dilution material (such as laboratory reference soil) is non-toxic and as chemically/physically similar to the test matrix as possible. All samples and sample concentrations should be performed in triplicate.

5.8.4.4 Test chemicals and/or mixtures

All test material should be reagent- or analytical-grade unless a specific test mixture/formulation/commercial product is being tested. In the case of the latter, information of the active ingredients and impurities should be recorded, along with information regarding its biohazardous toxicity, solubility and stability in water, recommended handling procedures and other physico-chemical properties (pH, hardness, alkalinity, conductivity, etc.). Test chemicals can mostly be added directly to the test petri dishes containing the fertilized eggs using freshly made stock solutions on the day of exposure. The preferred FETAX Solution (ASTM, 1998) should be used as solvent for chemical and/or mixture stock solutions where possible. Volatile solvents such as acetone, ethanol, and methanol should be avoided as this may interfere with embryonic development. All samples and sample concentrations should be performed in triplicate.

5.8.5 Cleaning of glassware

Ensure that all glassware is cleaned as referred to in section 7.4.1. of the ASTM (1998) guideline.

5.8.6 Method summary

The 1998 ASTM FETAX guideline also includes appendices on concentration steps for range-finding tests, microsome isolation reagents, and nicotinamide adenine dinucleotide phosphate (NADPH)-generating system components (ASTM, 1998). Refer to the ASTM guidelines (ASTM, 1991/1998) for a comprehensive guideline on performing the FETAX. A brief method summary is described below:

Breeding pairs of *X. laevis* are primed with human chorionic gonadotropin and bred using an in-house breeding protocol. Fertilised eggs are then harvested, and the jelly coat removed from the eggs using 2% l-cysteine (see Dawson and Bantle, 1987). Normal cleaving embryos are then carefully selected using a stereo microscope and staged using the Nieuwkoop and Faber (1975) developmental atlas. Twenty haphazardly selected stage 8-11 (mid-blastula to early gastrula) embryos are then introduced to glass petri dishes (500 mL volume) and the experiment run in a controlled climate room (water temp. 24 ± 1°C; pH 6.5-7.4; DO > 6.5 mg/L; 12 h light:dark photoperiod) (ASTM, 1998; OECD 2008; Babalola et al., 2021).

Test chemicals and/or both aqueous and soil/sediment solutions should be changed every 24 hours during the 96-day exposure period. All test conditions should be done in triplicate, including a positive control containing FETAX Solution only and the experiment should be repeated twice. Physico-chemical parameters such as dissolved oxygen (DO), pH, conductivity, hardness, alkalinity, ammonia-nitrogen, and residual chlorine should be measured upon changing of the test solution each day. Mortality should be recorded daily, and deceased embryos removed immediately from the test solution. At the completion of the 96-hour exposure period, the experiment is terminated by euthanizing all embryos using benzocaine, upon which the test organisms are fixed in formalin, morphometric measurements (snout-to-vent length, body length, tail length, body mass) taken, along with developmental malformations recorded under a stereo microscope using the *X. laevis* embryo malformation index (Bantle et al., 1999).

5.8.7 Advantages and/or limitations

Advantages

- The assay is relatively rapid for an *in vivo* bioassay (4 days).
- The assay provides a variety of developmental malformation endpoints that can be evaluated through an established malformation index and can be extrapolated to higher vertebrates.
- The use of an ecologically relevant sentinel aquatic organism (*X. laevis* embryos) provides concrete evidence of direct teratogenicity of test material to higher trophic level aquatic organisms.
- The FETAX can be applied on treated effluents, surface/groundwaters, leachates, and solid-phase samples (soils, sediments, and particulate matter), allowing for a high-tier ecological risk characterisation tool.

Limitations

- Access to animal housing, breeding aquaria and incubation rooms may be less available.
- Breeding success of *X. laevis* breeding pairs may vary between seasons.
- High potential loss of organisms during the bioassay procedure if test conditions are not regularly monitored.
- The FETAX protocol as provided in the ASTM guidelines (ASTM, 1991/1998) has strict ranges of acceptable mortality and malformation outcomes in the control sample subset, which may require for the bioassay to be repeated several times.

5.8.8 Animal research ethics

Acquisition of institutional animal research ethics is needed prior to performing the bioassay.

5.8.9 Specialized facilities and equipment required

Refer to ASTM (1991) for a detailed description of specialised apparatus needed for the bioassay. A brief outline will be provided below:

- Aquaria for maintaining and breeding *X. laevis*
    - Climate room with photoperiod of 12-h day/12-h night
    - Breeding tanks and aeration apparatus
    - Glass holding tanks and aquaria equipment for handling fertilized eggs
- Climate room for experimentation
- Binocular dissection microscope capable of magnifications up to 30x
- Ultrapure water system (Milli-Q or similar)
- Covered 60-mm glass Petri dishes

5.8.10 Facilities available for the bioassay

Ecophysiology Laboratory,
Department of Botany and Zoology,
Stellenbosch University, Stellenbosch, 7600

Contact person/s:
Prof. Johannes H van Wyk
Tel: 021 808 3236 (current time of print)
Email: jhvw@sun.ac.za (current time of print)

Dr. Christoff Truter
Tel: 021 808 5805

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OECD, (2008) Series on testing and assessment. No. 91. Report of the validation of the amphibian metamorphosis assay (PHASE 3). Organisation for Economic Co-operation and Development. (ENV/JM/MONO(2008)18.

5.9 FACTSHEET: BIOASSAY INTERPRETATION AND EFFECT-BASED TRIGGER (EBT) VALUES

Compiled by: Catherina Van Zijl, Naledi Mmekwa and Natalie Aneck-Hahn

5.9.1 Purpose

To summarise the evaluation of the bioassay results and the use of the associated trigger values for water sources.

5.9.2 Calculating the bioanalytical equivalent concentration

Bioassay results used in effect-based monitoring (EBM) are expressed as bioanalytical equivalent concentrations (BEqs). This makes the measured effect comparable between different bioassays targeting the same mode of action (MOA), e.g. estrogenic, androgenic, thyroid activity, etc. (Escher and Leusch, 2012). 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.

5.9.3 Computer software programs required

- Microsoft Excel or similar
- Graphing and statistics software, e.g. GraphPad Prism®

5.9.4 Application of BEqs

Effect-based monitoring is often applied as a screening tool, but BEqs can be used as input for risk-based monitoring programs (Neale et al. 2020). BEqs can be used to quantify treatment efficacy at water treatment plants. Measuring a detectable effect does not necessarily imply adverse human and environmental effects. However, in surveillance and monitoring applications it is useful to have thresholds to determine the quality of the water source. The use of effect-based trigger values (EBT) can assist with differentiating between acceptable and poor water quality. For a sample that exceeds an EBT, further testing is recommended (Escher and Leusch, 2012).

5.9.5 Effect-based trigger (EBT) values

EBT values will differ depending on the sample type for example the EBT for drinking water will differ from surface water. Trigger values are associated with the specific type of bioassay (see Table 5-1). The EBT values in Table 5-1 are preliminary as limited data is available, but this will be addressed by future targeted bioassay research.

REFERENCES

ESCHER B and LEUSCH F (2012) Bioanalytical tools in water quality assessment. London, UK, IWA Publishing.

NEALE P, LEUSCH F and ESCHER E (2020) Factsheet: Use of effect-based monitoring for the assessment of risks of low-level mixtures of chemicals in water on man and the environment. *In:* GWRC (ed). Global Water Research Coalition.

Table 5-1: Summary of proposed effect-based trigger (EBT) values for both human health and ecological health expressed as bioanalytical equivalent concentrations (BEq) that are currently available in the literature.

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 [#]
	ERα GeneBLAzer	1.8 ng/L EEQ [1]	0.34 ng/L EEQ [3] 0.24 ng/L EEQ [10]

Endpoint	Assay name	Human EBT (Drinking and recycled water for indirect potable reuse)	Ecological EBT (Surface water)
	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#
	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]	

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY AND CONCLUSIONS

Chemical analysis and the use of individual chemical risk models for water safety planning comes with limitations and challenges, related to capacity, cost and infrastructure, but also with the vast number of chemicals that may be present in the environment particularly in SA, their application is limited. The approach of effect-based monitoring using *in vitro* bioassays and well plate-based *in vivo* assays has been recommended for water quality assessment (Brack et al., 2019). These assays are most often run in 96-well or 384-well plate format, making them useful for high-throughput, which is essential for routine water quality monitoring. There are a number of *in vitro* bioassays that are available that can be used to investigate the different stages of cellular toxicity pathways, including induction of xenobiotic metabolism, receptor-mediated effects, adaptive stress responses and cytotoxicity. Many of them have been used to assess the water quality in water from various sources. These assays can be used to develop a toolbox of EBMs not just estrogenic activity for water quality and safety in SA. One of the aims of developing a toolbox of assays is to allow water quality laboratories to build capacity and use these assays to test water quality from different water sources, from treated water to surface and groundwater on a regular basis. It will also enable water stakeholders to design a suitable fit-for-purpose bioassay test battery for a particular water type or source. The following criteria can be used:

1. What are the expected effects from chemicals detected in source and treated waters
2. Compliance requirements
3. Applied treatment technologies.

6.2 RECOMMENDATIONS

The recommended way to build a battery would consider bioassays that can target relevant MOAs (e.g. endocrine disruption, oxidative stress, genotoxicity, etc.), this will include overall cytotoxicity of a water sample that can be quantified. The measurement of cytotoxicity is important as it may mask other specific effects for example, estrogenic activity or androgenic activity. While a battery of three to four bioassays is recommended, there are some situations or constraints at specific locations that may not allow for this. Therefore, even a simple cytotoxicity assay can be considered. Figure 6-1 is an example of potential batteries of assays for different water sources like drinking water, wastewater and recycled water (GWRC Factsheet). There may also be more than one assay suitable for the same endpoint for example the YES and the T47D-KBluc assays for estrogenic activity. The YES being a stable robust cell line is particularly suitable for wastewater and treated wastewater, while the T47D-KBluc is a more sensitive assay and is recommended for example drinking water, surface and groundwater. At the same time other assay endpoints for example androgenic activity, thyroid activity and other receptor-mediated endpoints should also be included in the battery of assays when looking at the context of the water sample.

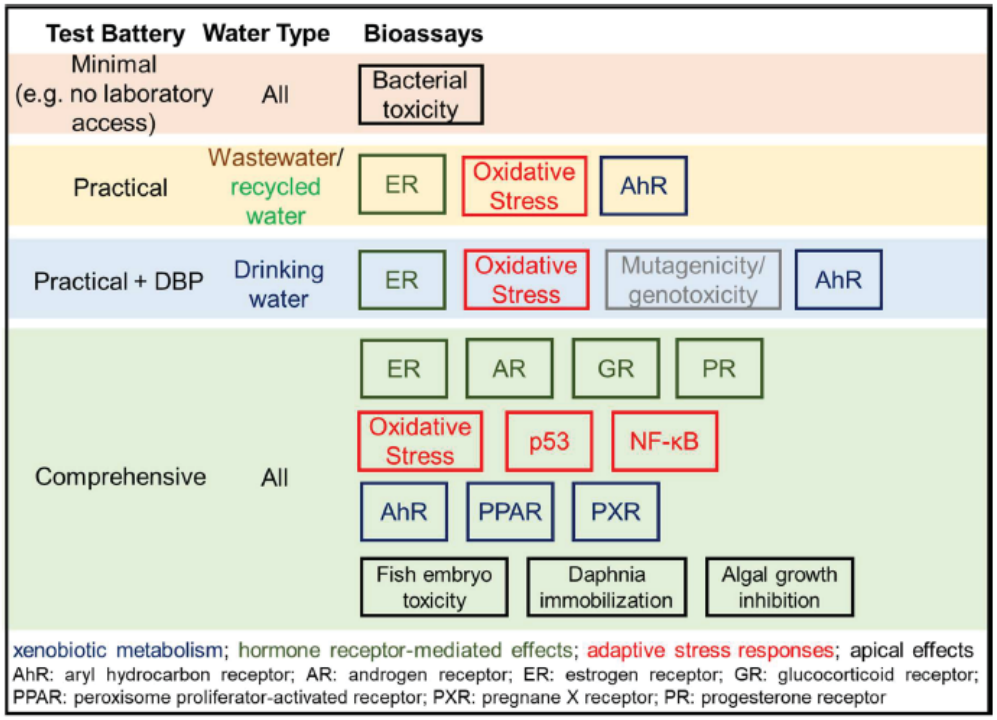

Figure 6-1: Options for bioassay test batteries for different water sources (Neale et al., 2020a)

The literature review and the collaboration with the GWRC has highlighted that EBM use in SA is limited. This may be for a number of reasons for example the cost of the bioassays and the infrastructure required like cost and capacity. The reality is that in SA water is a scarce resource which needs to be protected and implementing EBMs for water quality monitoring is becoming necessary. The current SANS 241 that is currently under revision should include EBMs and the accompanying EBTVs for drinking water. This stands true for other water quality guidelines and WSPs.

It is clear from the literature that SA's water is contaminated with CECs, pharmaceutical and personal care products (PPCP) and EDCs, using EBMs in monitoring programmes is important. Case studies looking at using the toolbox in parallel with chemical analysis will facilitate the development of these tools to assess the health risk of these compounds to humans and animals. The factsheets together with the updated toolbox should be used by water stakeholders in order to improve and increase the sustainability of water quality and use in SA.

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APPENDIX A

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) $0\text{-}5\times10^4$ ii) $0\text{-}1.2\times10^5$ iii) $0\text{-}3\times10^5$ iv) $0\text{-}10^6$			$2\times10^4\text{-}10^5$				
Aluminium	0-150			$0\text{-}5\times10^3$	$0\text{-}5\times10^3$	< 30	5-10	≤ 300		
Ammonia	$0\text{-}1\times10^3$					0-25	$0\text{-}7\times10^{-9}$	$\leq 1.5\times10^3$	6×10^3	2×10^3
Antimony								≤ 20		
Arsenic	$0\text{-}1\times10^{-8}$			0-100	$0\text{-}10^3$	0-50	10	≤ 10	20	0
Atrazine	$0\text{-}2\times10^{-9}$					$0\text{-}1.8\times10^{-8}$	10			
Barium								≤ 700		
Beryllium				0-100						
Boron				0-500	$0\text{-}5\times10^3$			$\leq 2.4\times10^3$	10^3	500
Cadmium	0-5			0-10	0-10		0-0.4	≤ 3	5	1
Calcium	$0\text{-}3.2\times10^4$				$0\text{-}10^6$					
Chemical oxygen demand			i) $0\text{-}10^4$ ii) $0\text{-}1.5\times10^4$ iii) $0\text{-}3\times10^4$ iv) $0\text{-}7.5\times10^4$						7.5×10^4	3×10^4
Chloride			i) $0\text{-}2\times10^4$ ii) $0\text{-}4.5\times10^4$ iii) $0\text{-}10^5$ iv) $0\text{-}10^5$	$0\text{-}10^5$	$0\text{-}1.5\times10^6$	$< 6\times10^5$		$\leq 3\times10^5$		
Chlorine	$0\text{-}10^5$						0.2	$\leq 5\times10^3$	250	0
Chromium (VI)				0-100	$0\text{-}10^3$	$< 0\text{-}2\times10^{-8}$	7		50	20
Cobalt				0-50	$0\text{-}10^3$		0-1.4			
Copper	$0\text{-}10^3$			0-200	0-500	< 5		$\leq 2\times10^3$	10	2
Cyanide						< 20	1	≤ 200	20	10
DOC	$0\text{-}5\times10^3$									

DO						6-9	80-120% of saturation			
Endosulfan							0.01			
Fluoride	$0\text{-}10^3$			$0\text{-}2\times10^3$	$0\text{-}2\times10^3$		750	$\leq 2.5\times10^5$	10^3	10^3
Iron	0-100		i) 0-100 ii) 0-200 iii) 0-300 iv) $0\text{-}10^4$	$0\text{-}5\times10^3$	$0\text{-}10^4$	< 10	< 10% of background dissolved iron concentration	$\leq 2\times10^3$	300	300
Lead	$0\text{-}10^{-8}$			0-200	0-100	< 10	0-1.2	≤ 10	10	6
Lithium				$0\text{-}2.5\times10^3$						
Magnesium	0-30 000				$0\text{-}5\times10^5$					
Manganese	0-50		i) 0-50 ii) 0-100 iii) 0-200 iv) $0\text{-}1\times10^4$	0-20	$0\text{-}10^4$	< 100	180	≤ 400	100	100
Mercury	$0\text{-}10^{-9}$				$0\text{-}10^3$	$< 10^3$	0.04×10^{-9}	≤ 6	5	1
Molybdenum				0-10	0-10					
Monochloramine								$\leq 3\times10^3$		
Nickel				0-200	$0\text{-}10^3$			≤ 70		
Nitrate	$0\text{-}6\times10^3$				$0\text{-}10^5$	$< 3\times10^5$		$\leq 1.1\times10^4$	1.5×10^4	1.5×10^3
Nitrite						< 50		≤ 900		
Nitrogen (inorganic)				$0\text{-}5\times10^3$			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	$0\text{-}10^{-9}$					$< 10^3$		≤ 10		
Phosphorus (inorganic)						100	v) $< 5\times10^{-9}$ vi) 5×10^{-9}-$2\ 5\times10^{-9}$ vii) $2\ 5\times10^{-9}$ - 250×10^{-9} viii) $>250\times10^{-9}$		10^4	10^3 (median); 2.5×10^3 (max)
Potassium	$0\text{-}5\times10^4$									

Selenium	$0\text{-}2\times10^{-8}$			0-20	$0\text{-}5\times10^{-8}$	< 300	2	≤ 40	20	20
Silica			i) $0\text{-}5\times10^{3}$ ii) $0\text{-}10^{4}$ iii) $0\text{-}2\times10^{4}$ iv) 0-1.5×10^{5}							
Sodium adsorption ratio				2						
Sodium	$0\text{-}10^{5}$			$0\text{-}7\times10^{4}$	$0\text{-}2\times10^{6}$			≤ 2×10^{5}		
Sulphate	$0\text{-}2\times10^{5}$		i) $0\text{-}3\times10^{4}$ ii) $0\text{-}8\times10^{4}$ iii) $0\text{-}2\times10^{5}$ iv) $0\text{-}5\times10^{5}$		$0\text{-}10^{6}$			≤ 5×10^{5}		
Sulphide						< 1				
Suspended solids			i) $0\text{-}3\times10^{3}$ ii) $0\text{-}5\times10^{3}$ iii) $0\text{-}5\times10^{3}$ iv) 0-2.5×10^{4}	$0\text{-}5\times10^{4}$					2.5×10^{4}	10^{4}
Total chromium								≤ 50		
Total dissolved solids	$0\text{-}4.5\times10^{5}$		i) $0\text{-}10^{5}$ ii) $0\text{-}10^{5}$ iii) $0\text{-}4.5\times10^{5}$ iv) 0-1.6×10^{6}	0-256	$0\text{-}10^{6}$	< 2×10^{3}				
Total Hardness			i) $0\text{-}5\times10^{4}$ ii) $0\text{-}10^{5}$ iii) $0\text{-}2.5\times10^{5}$ iv) $0\text{-}10^{6}$			20-100				
TOC								≤10		
Trihalomethanes	$0\text{-}10^{-7}$							≤ 1		
Uranium	0-70			0-10				≤ 10		
Vanadium	0-100			0-100	$0\text{-}10^{3}$					
Zinc	$0\text{-}3\times10^{3}$			$0\text{-}10^{3}$	$0\text{-}2\times10^{4}$		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	