Endocrine-disrupting activity of the fungicide mancozeb used in the Vhembe District of South Africa

MF Seshoka[1], MC van Zijl[2], NH Aneck-Hahn[2] and IEJ Barnhoorn[1]*

[1] Department of Zoology, University of Venda, Thohoyandou, South Africa
[2] Department of Urology, Steve Biko Academic Hospital and University of Pretoria, Arcadia, South Africa

*Correspondence: Irene.barnhoorn@univen.ac.za; irene.barnhoorn@gmail.com

Abstract

Many chemicals released into the environment are believed to disrupt normal endocrine functions in humans and animals. A major group of endocrine disrupting chemicals (EDCs) that could be responsible for reproductive defects are those that mimic natural oestrogens, known as xenoestrogens. A number of *in vivo* and *in vitro* screening strategies are being developed to identify and classify xenoestrogens, in order to determine whether they pose a health risk to humans and animals. Oestrogens and androgens mediate their activity via intracellular receptors, directly in muscular tissue, as well as indirectly via stimulation of growth hormones from the pituitary glands and other growth factors from liver plus several other organs. Mancozeb is a metal ethylenebisdithiocarbamate (EBDC) fungicide used to protect many fruits and vegetables and field crops against pathogenic fungal. The T47D-KBluc, GH3.TRE-Luc and MDA-kb2 reporter gene assays were used to determine the possible endocrine disrupting activity/potential of mancozeb. No activity was detected in any of the assays and no mancozeb was detected in any of the dams either. Oestrogenic activity was detected in Albasini Dam, Nandoni Dam and Xikundu weir, but all values were below 0.7 ng l−1 trigger value for oestrogenic activity in drinking water.

Keywords: endocrine disrupting chemicals, reporter gene assays, xenoestrogen

Introduction

Endocrine-disrupting chemicals (EDCs) and their possible effects in freshwater sources of South Africa is a well discussed topic (London et al. 2000; Bornman et al. 2007; Burger and Nel 2008; Aneck-Hahn et al. 2009; Barnhoorn et al. 2009; Fatoki et al. 2010; van Wyk et al. 2014; Bornman et al. 2017; Robson et al. 2017). Endocrine disruption is the result of hormone function interference by EDCs causing changes in development, growth, and reproduction in wildlife and humans (Toppari et al. 1996; Diamanti-Kandarakis et al. 2009). EDCs include various synthetic substances, such as pesticides, metals, additives or contaminants in food, and personal care products (Monneret 2017). A fungicide, such as mancozeb, is a pesticide and also considered to be an EDC (Rossi et al. 2006).

Humans, especially farmers or those who work at agricultural sites exposed to this fungicide, are mostly at risk imposed by this fungicide on their health. Mancozeb has been proven to show short-term toxicity to humans, by inducing toxic effects in cells of the

immune system and in other non-immune cells. Rossi et al. (2006) reported that the metabolite ethylenethiourea (ETU) of mancozeb may cause various toxic effects, including thyroid and hepatic neoplasms. Although mancozeb is considered an EDC, not much is known about the endocrine disrupting properties thereof. It is mainly used in the Vhembe district to protect fruits, such as guavas, bananas and mangos (D Alberts, pers. comm., Vhembe area pesticide specialist), but also on many other farms of South Africa to protect plants, crops and deciduous fruits and vegetables (Schutte et al. 1997; Vermeulen et al. 2001; Reinecke et al. 2002; Schutte et al. 2003; Rossouw et al. 2018; Rebel et al. 2020). The Albasini-, Nandoni-, and TateVondo dams, as well as the Xikundu Weir are dams situated in the Vhembe District and in the heart of big scale and self-sustained fruit farming practises. Although these dams may receive effluent from fruit farms water is also used for potable means, therefore, increasing the risk of exposure to humans and animals.

In 1999, the Environmental Protection Agency in the United States (USEPA) established a testing strategy for screening chemicals for endocrine activity (O'Connor 1999). This strategy, using a battery of bioassays, is to detect endocrine-active compounds to elucidate potential mechanisms of action, as well as to determine the potency of the pesticides prior to *in vivo* testing (Ghisari et al. 2015). In South Africa the need for a battery of bioassays was initially started in 2000 and compiled in a report to the Water Research Commission (WRC) of SA (de Jager et al. 2011). The 2008 publication by the Global Water Research Coalition (GWRC) raised an awareness of the need to use a battery of assays. This led to the selection of oestrogen and anti-androgenic activity detection bioassays for the aquatic environment in South Africa (Dabrowski et al. 2015). Therefore, there seems to be an opportunity to use various bioassays to determine possible endocrine disrupting activity of a fungicide used in the agricultural setting of a rural area. Three biological assays T47D-KBluc reporter gene, GH3.TRE-Luc and MDA-kb2 have been used as part of a battery of assays to determine the endocrine disrupting activity of pesticides. These assays have been used effectively by various research groups. A study by Wilson et al. (2004) indicated that low concentrations of cadmium induced oestrogen-dependent gene expression, an effect that was completely inhibited by the potent anti-oestrogen ICI 182,780; showing how effective the T47D KBluc reporter gene assay is. The same group and others also indicated that T47D cells express a relatively high number of endogenous ER (67.6 ± 6.2 fmol mg^{-1}) cytosolic protein and these cells contain both the alpha and beta isoforms of the ER protein with very slightly higher levels of beta than alpha (Watanabe et al. 1990; Power and Thompson 2003). Another study by Wehmas et al. (2011) showed that the use of the T47D KBluc reporter gene assay to quantify total oestrogenicity of wastewater treatment plants effluents (WWTPs) and their outcomes illustrated that it was the most effective assay to estimate the availability of oestrogenicity of wastewater effluent. For the MDA-kb2 assay; Blake et al. (2010) and Ermler et al. (2010) tested combinations of androgens and compared the observed activity to expected androgenic activity, based on a concentration addition model. What they found was that their results demonstrated the potential utility of the androgen-responsive MDA-kb2 cell line for quantifying the activity of mixtures of endocrine-active chemicals in complex wastes, such as municipal effluents. Although recent studies have been trying to detect thyroid activity in environmental waters, Leusch et al. (2017) stated that more sensitive reviewed bioassays are still unlikely to detect this type of activity in environmental waters. This is also supported by Leusch et al. (2018).

The aim of this paper is to report on the possible levels of mancozeb and determine the (anti-) oestrogenic-, (anti-) androgenic- and (anti-) thyroid activity in the water of the Albasini, Nandoni, TateVondo dams and the Xikundu Weir; and to test for possible endocrine disrupting activity mancozeb may show using three different assays.

Materials and methods

Sample collection and extraction

Water samples were collected once off, in the autumn of 2017 from the Albasini (AD), Nandoni (ND), TateVondo (TD) dams and the Xikundu Weir (XW) in 1-litre glass bottles for the bioassays and mancozeb analysis. The criteria of collection included points where the community utilise the water source. Surface water samples were taken approximately 50 cm from the surface from the four sites (Table 1). Before collection, the bottles and lids were rinsed with HPLC grade methanol (Merk, Darmstadt, Germany) to avoid EDC contamination. The original pH of the water for both mancozeb and metal analyses and for bioassay samples were measured using a pH strip and was adjusted to between 2 and 3 by adding hydrochloric acid (Merk, Darmstadt, Germany). The samples were stored in 4 °C until extraction. Water samples were filtered using glass wool and 0.2 µm membrane disc filters and extracted by solid phase extraction (SPE) using Oasis HLB 200 mg cartridges (Water, Milford, Massachusetts. USA) (Rodríguez et al. 2006). The SPE cartridges were preconditioned with 5 ml double-distilled H2O followed by 5 ml methanol (HPLC grade) then equilibrated with 5 ml double-distilled water, before the samples (1 l) were loaded (1 l per cartridge) (de Jager et al. 2011). Throughout the extraction procedure care was taken not to let the cartridge run dry until the whole sample volume passed through the cartridge. The flow rate never exceeded 10 ml min^{-1}. Once all the sample volume passed through the cartridge, the cartridges were dried under vacuum. Five (5) ml methanol was added to each cartridge reservoir and the solvent was allowed to filter through the sorbent bed. Elution was allowed to happen through gravity alone into methanol rinsed glass test tubes.

Table 1: Oestrogenic activity of extraction control and water samples expressed as oestradiol equivalents (EEq) in ng l^{-1}

Sample site and control	EEq (ng l^{-1}) ± standard deviation
Extraction control	< dl
TateVondo Dam	< dl
Nandoni Dam	0.210 ± 0.059
Albasini Dam	0.226 ± 0.023
Xikundu Weir	0.237*

< dl: Below the detection limit of the assay
*EEq value could only be calculated for one of the triplicate plates

The test tubes containing eluent were placed in a heating block (37 °C) in a fume hood, then the needles of the sample concentrator unit were lowered into the tubes and the nitrogen flow was turned on. The needles were lowered every 30 min in order to keep a constant flow on the surface of the samples. After approximately 1 h 30 min, dried samples were reconstituted in 1 ml ethanol (1 000 × concentration factor). The samples were vortexed thoroughly for mixing, before the reconstituted sample was placed into methanol rinsed glass amber vials (4 ml volume) and stored at −20 °C for further analysis (de Jager et al. 2011).

Bioassays for oestrogenic activity

T47D-KBluc reporter gene assay

The T47D-KBluc reporter gene bioassay was developed by the USEPA to test for environmental samples and chemicals for estrogenic and androgenic activities (Wilson et al. 2004). The assay was performed according to standard procedure (de Jager et al. 2011). The T47D KBluc cells were grown in Roswell Park Memorial Institute (RPMI) (Sigma-Aldrich, St. Louis, MO, USA) medium supplemented with 10% foetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g l^{-1} sodium bicarbonate (Gibco, Life Technologies Corporation, Paisley, UK). One week prior to the assay, cells were grown in RPMI supplemented with 10% charcoal/dextran treated FBS to withdraw the cells from steroids. The cells were seeded at 5×10^4 cells per well in 96-well luminometer plates (100 µl per well) and placed in the incubator to allow the cells to attach overnight. The dosing medium contained 5% dextran/charcoal treated FBS. Ethanol was used as vehicle control and it did not exceed 0.2%. The following stock concentrations were prepared in ethanol: 10 mM E2; 1 mM ICI 182, 780; 200 mg ml^{-1} mancozeb 1 000 × concentrated dosing solutions were prepared for controls and test chemicals in HPLC grade ethanol in 2 ml Eppendorf tubes and were vortexed. A dilution series was prepared for samples and controls in ethanol, of which 2 µl was transferred to 1 ml medium to prepare the dosing solutions. The range of concentrations tested for each sample and controls were as follows; oestradiol (E2) 100 pM to 0.03 pM (agonist control), ICI 10 nM to 0.003 nM (antagonist control) and mancozeb 200 mg l^{-1} to 2 pg l^{-1}.

The plates were incubated for 24 h in a 5% CO2 incubator at 37 °C. The plates were removed from the incubator and assessed under the microscope for any signs of cytotoxicity or any other abnormality. The cells were then rinsed with phosphate buffered saline (PBS) (Gibso Life Technologies Corporation, Paisley, UK). The PBS was discarded and 25 µl of lyses buffer (Promega, Madison, Wisconsin, USA) was added to each well followed by one freeze/thaw cycle to lyse the cells. The luciferase activity was determined using a LUMIstar OPTIMA luminometer (BMG Labtech, Offenburg, Germany) with two dispensers programmed to inject 25 µl reaction buffer (25 mM glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg ml^{-1} BSA, pH 7.8), followed by 25 µl 1 nM D-luciferin 5 s later. Luciferin activity was quantified as relative light units (RLU).

MDA-kb2 reporter gene assay

The MDA-kb2 reporter gene assay was used to determine androgenic activity in the samples, according to the method described by Wilson et al. (2002). The MDA-kb2 cells (cat no. CRL-2713, purchased from ATCC, Manassas, Virginia, USA) were grown in Leibovitz's L-15 media supplemented with 10% FBS. Cells were seeded at 5×10^4 cells per well in 96-well luminometer plates (100 µl per well) and placed in the incubator to allow cells to attach overnight.

Fifty (50) µl of the dosing solutions was added to the appropriate wells containing 50 µl vehicle control, 10 nM dihydrotestosterone (DHT) (agonist control) or 100 µM flutamide (F) (antagonist control).

Concentrations and dilutions were as follows; DHT 20 nM to 0.003 nM, F 100 µM to 0.03 µM and mancozeb at the same concentrations as in the T47D-KBluc assay. The plates were

incubated for 24 h in an incubator at 37 °C. After the 24 h exposure period, the cells were lysed and luciferase activity determined using the same method described for the T47D-KBluc assay above. The cells were terminated, and plates read using the same procedure as for the T47D-KBluc reporter gene assay.

GH3.TRE-Luc reporter gene assay
The GH3.TRE-Luc cell line was used to determine thyroid activity. Cells were maintained in regular growth medium (DMEM/F12 (1:1) with 15 mM HEPES supplemented with 10% FBS) in an incubator at 37 °C and 5% CO2. The GH3. TRE-Luc cells were received as a gift from Professor AJ Murk from the Wageningen University (The Netherlands) to the University of Pretoria.

Cells were seeded at 3×10^4 cells per well in clear bottom 96-well tissue culture plates (100 µl per well) and allowed to attach overnight. The growth medium was replaced with 100 µl serum-free PCM medium (DMEM/F12 (1:1) with 15 mM HEPES supplemented with 10 µg ml^{-1} bovine insulin, 10 µM ethanolamine, 10 µg ml^{-1} sodium selenite, 10 µg ml^{-1} human apotransferrin and 500 µg ml^{-1} BSA) to deplete the cells of thyroid hormones. The plates were incubated for 24 h. After removing old PCM, the cells were exposed to test chemicals, water extracts and controls, serially diluted in PCM (200 µl final volume). The tested concentration ranges were as follows; triiodothyronine (T3) (agonist control) 10 nM to 0.00001 nM, sodium arsenite (antagonist control) 200 µM to 0.1 µM. mancozebsame as in T47D-KBluc. Dilution of water extracts was done in the following series: 10 ×, 3 ×, 1 ×, 0.3 ×, 0.1 × the concentration of the original water sample.

Test chemicals were tested alone and in the presence of 1 nM T3 or 100 µM sodium arsenite. Each plate (of 3) had a T3 dose response curve and vehicle control and the triplicate plates were exposed for 24 h. The cells were lysed and luciferase activity determined using the same method described for the T47D-KBluc reporter gene assay above.

Cytotoxicity was determined on a separate plate, using the resazurine cell proliferation assay. After a 24 h exposure period, 8 µl of resazurine (400 µM in PBS, pH 7.4) was added to each well and the plates were incubated in the dark for 4 h (37 °C, 5% CO_2). The fluorescence was measured at 530 nm excitation and 590 nm emission. A sample was considered cytotoxic, if the fluorescence was less than the fluorescence of the vehicle control minus 3x the standard deviation.

Data analysis (T47D-KBluc, MDA-kb2 and GH3.TRE-Luc reporter gene assays)
The standard curves (sigmoidal function and variable slope) were fitted using Graphpad Prism (version 4).

Oestradiol, DHT and T3 equivalent concentrations were calculated for samples that induced dose dependent luciferase activity, which could be inhibited by the antagonist. Sample concentrations were calculated from the agonist standard curve as unpaired *y*-values and were corrected for the appropriate dilution factors to determine the equivalent value for the original sample. Equivalent concentrations were reported as the mean ± SD of triplicate

samples. The antagonist control curve (co-incubated with 0.1 nM agonist) was fitted to quantify antagonist activity. The relative induction efficiency (RIE) was calculated as RIE = (Max RLU chemical/max RLU E2) × 100, and relative potency (RP) as RP = (EC50 of E2/EC50 of test chemical) × 100.

Mancozeb analyses
A standard house method 039/2008 using gas chromatography electron capture detector spectrometry (GC-ECD) techniques were applied by a South African National Accreditation System (SANAS) and ISO 17025 accredited laboratory for mancozeb analyses.

Results

T47D-KBluc
Mancozeb did not show any sign of estrogenic activity (Figure 1), but cytotoxicity was observed under a microscope in the wells containing the two highest concentrations (100 mg l^{-1} and 200 mg l^{-1}). Cytotoxicity was considered when cells were shrivelled and shrunken, indicating death. All cells in the last two highest concentrations were affected.

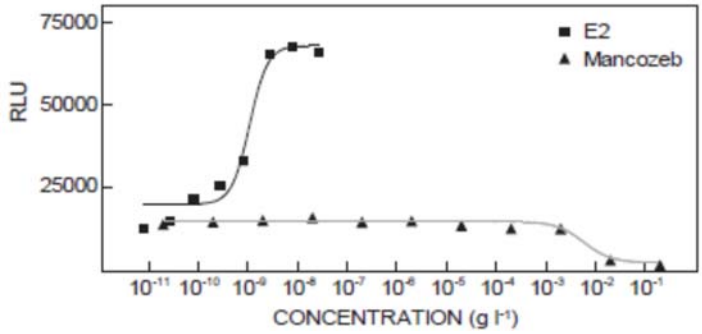

Figure 1: Dose-response curves for mancozeb and positive control 17β-oestradiol (E2) in T47D-KBluc cells

ICI (antagonist control) and mancozeb were co-incubated with 100 pM E2 to test for anti-oestrogenic activity (Figure 2). ICI was able to suppress the E2-induced luciferase activity from 1 nM (0.6 µg l^{-1}). The response of E2 curve co-incubated with mancozeb showed a decrease in E2-luciferase activity from 100 mg l^{-1}. However, microscopic evaluation of the cells before lysis revealed cytotoxicity at the two highest concentrations (100 mg l^{-1} and 200 mg l^{-1}). The decrease in E2-induced luciferase activity when co-incubated with mancozeb is therefore as a result of cytotoxicity, rather than anti-oestrogenic activity.

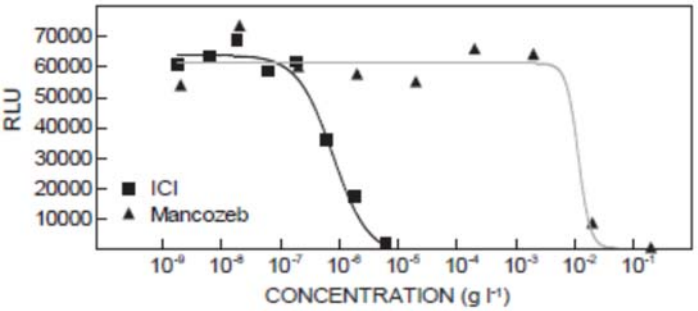

Figure 2: Dose-response curves for mancozeb and anti-oestrogen (ICI), co-incubated with 100 pM E2, in T47D-KBluc cells

The level of quantification (loq) for the assay was defined as the EC10 of the E2 dose-response curve. No oestrogenic activity was detected in the extraction control and the TD.

Oestrogenic activity was detected in the ND, AD and XW (Table 1). However, all values were below the average trigger value of drinking water (0.7 ng l^{-1}), which is the estrogenic equivalency factor used by the WHO for risk assessment. If a value is >1, it would be considered not safe (Genthe et al. 2013; Manickum and John 2015; Ngcobo 2017; Van Zijl et al. 2017; Archer 2018). No anti-oestrogenic activity was detected in any of the samples.

MDA-kb2

Mancozeb did not show any androgenic activity (Figure 3). Cytotoxicity in cells was observed as shrivelled and shrunken cells under a microscope in the wells with the two highest concentrations.

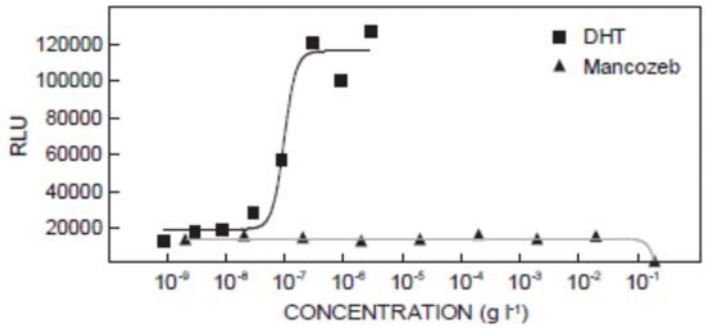

Figure 3: Dose-response curves for the mancozeb and dihydrotestosterone (DHT) in the MDA-kb2 cells

The F and mancozeb were co-incubated with DHT and both suppressed the DHT-induced luciferase activity (Figure 4). F was able to suppress the DHT curve at lower concentrations. However, cytotoxicity (shrivelled and shrunken cells) was observed in the well with the highest concentration of mancozeb (200 mg l^{-1}). The decrease in DHT-induced luciferase activity when co-incubated with mancozeb is therefore the result of cytotoxicity, rather than anti-androgenic activity.

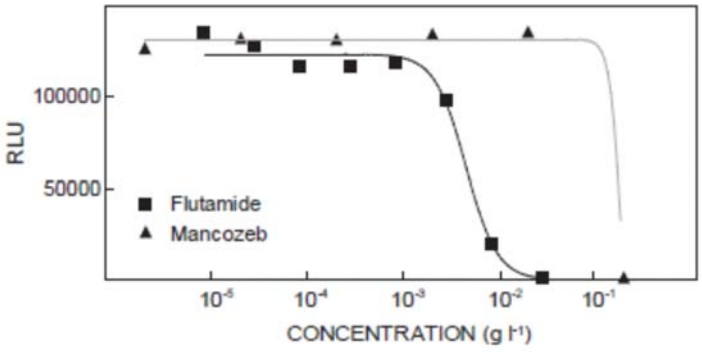

Figure 4: Dose-response curves for the mancozeb and flutamide (F), co-incubated with 10 nM DHT, in the MDA-kb2 cells

There was no androgenic activity detected in the extraction control.

Androgenic activity was below detection limit in all four dams using the MDA-kb2 bioassay. After samples were exposed to DHT in triplicates plates, none of the four samples showed any sign of anti-androgenic activity.

GH3.TRE-Luc

Mancozeb did not show any sign of thyroid activity at the tested concentrations (Figure 5). At higher concentrations, mancozeb showed cytotoxicity. This was confirmed using the resazurine assay, which indicated cytotoxicity at 20 mg l^{-1} and higher concentrations.

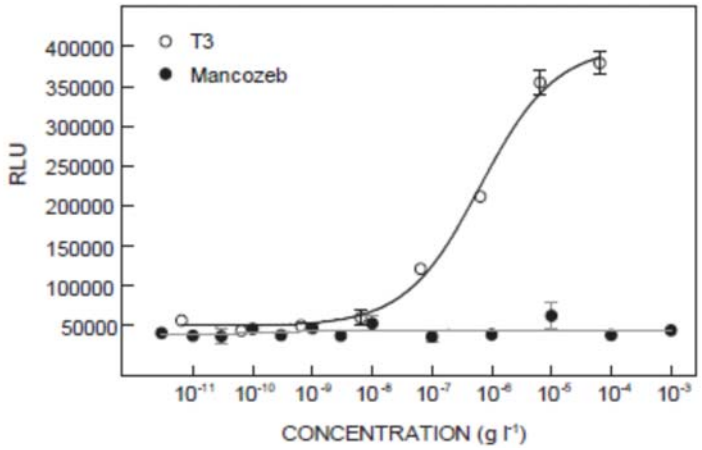

Figure 5: Dose-response curves for the mancozeb and positive control triiodothyronine (T3) in GH3.TRE-Luc assay cells

Mancozeb did not show any sign of anti-thyroid activity (Figure 6).

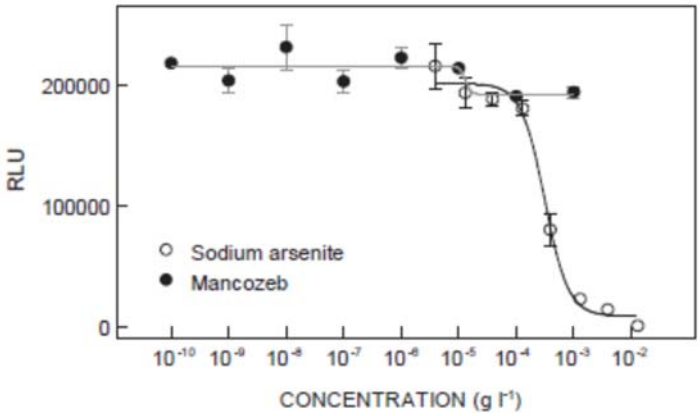

Figure 6: Dose-response curves and standard deviations for the mancozeb and antagonist control, co-incubated with 1 nM T3 in GH3.TRE-Luc cells

Thyroid activity was below the level of quantification in all the water samples.

Response curve for extracts

The level of quantification (loq) for the assay was defined as the EC10 of the E2 dose-response curve. No oestrogenic activity was detected in the extraction control, because all the values were well below the loq (Figure 7).

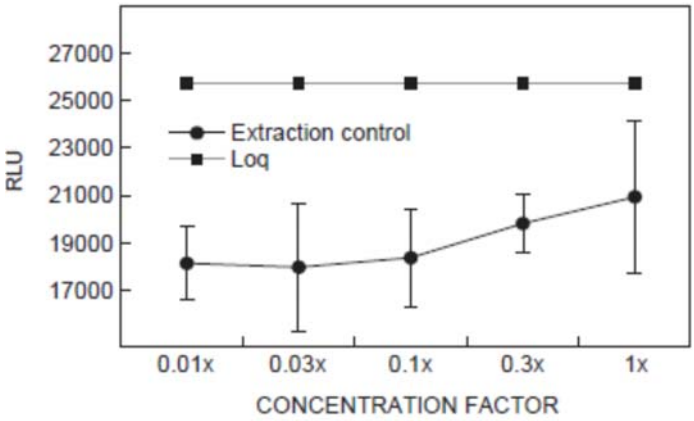

Figure 7: The response of the extraction control in the T47D-KBluc cells

There was no androgenic activity detected in the extraction control (Figure 8).

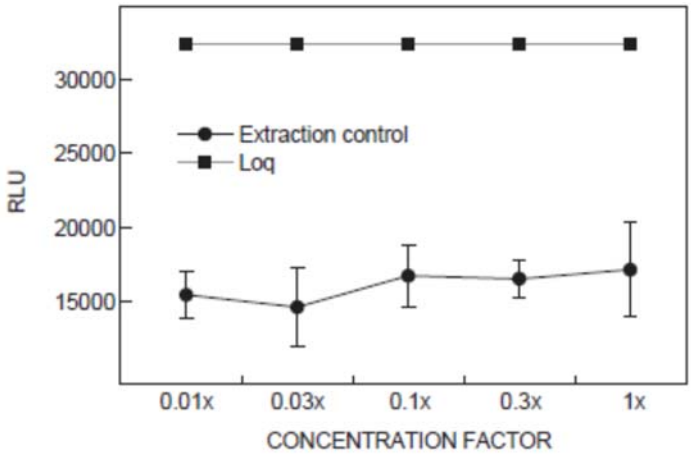

Figure 8: The response of the extraction control in the MDA-kb2 cells

Mancozeb analyses

Mancozeb was not detected in any of the water samples collected from the four sites.

Discussion

In vitro and *in vivo* studies reported on the endocrine disrupting properties of mancozeb (Runkle et al. 2017). After evaluating the endocrine disruptive properties of mancozeb using T47D-KBluc, MDA-kb2, and GH3-TRE-Luc bioassays, these properties could not be confirmed. In this study mancozeb did not show agonist activity or antagonist activity in any of the assays used. However, at the two highest test concentrations (100 mg l^{-1} and 200 mg l^{-1}), cytotoxicity was observed under the microscope and was confirmed with the resazurin assay. The cytotoxicity results are not surprising, because it was reported before by Lin and Garry (2000) in MCF-7 breast cancer cells and by Ghisari et al. (2015) in GH3 rat pituitary

tumour cells. Cytotoxicity can mask the oestrogenic, androgenic and thyroid activity under toxic concentrations (Griffero et al. 2018). Therefore, although no oestrogenic, androgenic or thyroid activity was seen, there is a chance that any of the above activity could have been missed, as a result of the cytotoxicity found. Griffero et al. (2018) noted that high levels of oestrogenic chemicals could be present in cytotoxicity sites, but not be detected.

Using the T47D-KBluc cell line, no oestrogenic or anti-oestrogenic properties were confirmed for mancozeb. This is similar to the study by Lin and Garry (2000) that reported no oestrogenic activity for mancozeb in the MCF-7 breast cancer cell line. Mancozeb can cause decreases in the number of oestrous cycles and the duration of pro-oestrus, oestrus, and met-oestrus with a parallel increase in the di-oestrus phase. In rats mancozeb caused atretic follicles and a decrease in the number of healthy follicles (Baligar and Kaliwal 2001). In parallel, it may cause a decrease in uterus weight and inhibits implantation. Recent evidence includes a net delay of the breeding and an effect on sexual maturity in the tadpoles of the green frog (*Rana* (Sana et al. 2015). Although this is not specifically for the female hormone it has to be assumed that breeding and sexual maturity is a characteristic held by both sexes. The discrepancy between the *in vitro* and *in vivo* studies may suggest that the oestrogenic effects of mancozeb might be non-receptor mediated.

Mancozeb is classified as an EDC and has anti-androgenic properties (Kjeldsen et al. 2013). A study by Archer and van Wyk (2015) confirmed the anti-androgenic properties of mancozeb in the recombinant yeast anti-androgen screen (anti-YAS) assay. Another report on the anti-androgenic properties of mancozeb came from Kjeldsen et al. (2013), using carcinoma MVLN and hamster CHO-K1 cells. These authors noted that at low concentrations mancozeb has an inhibitory effect on AR activity. It was expected that mancozeb may show anti-androgenic activity using the MDA-kb2 assay, but this could not be confirmed. However endocrine disruption can be through other mechanisms, such as interfering with metabolism of hormones, using a whole organism, such as vitellogenin assays and hormone measurements. The MDA-kb2 is a well-known and used bioassay to investigate (anti-) androgenic properties of chemicals. It has been used by Wilson et al. (2002), Xu et al. 2008), Blake et al. (2010), Du et al. (2010), Ermler et al. (2010), Kugathas et al. (2016) and König et al. (2017) to show androgenic properties of chemicals. Ksheerasagar and Kaliwal (2010) exposed Swiss male albinos to mancozeb for 30 d and detected toxicity in their testes, also a significant decrease in size.

Although mancozeb is known to act as a thyroid inhibitor (Cocco 2002; Pickford 2010) no (anti-) thyroid activity were found using the GH3-TRE-Luc cell line. In contrast, Ghisari et al. (2015) reported thyroid activity in the T-screen using GH3 cells. The activity of mancozeb was only detected at M with a relative proliferative effect of 7%. Compared with the activity induced by T3, higher concentrations were cytotoxic, similar to our study. The difference in the mancozeb between the two studies is that we used the commercial grade mancozeb and they used the reagent/analytical grade. Mancozeb contains zinc (Zn) and manganese (Mn). A recent study of Li et al. (2016) exposed TR yeast cells to cadmium (Cd), Zn, mercury (Hg), CuSO4, and MnSO4 and revealed that none of these elements exhibited TR-agonistic activities. They also exposed GH3 cells to the same metals and both TR yeast T-screen assays indicated the anti-thyroid activity on Zn, Hg and Cd ions.

Several studies focused on chemicals that have an effect on androgen and oestrogen hormones/receptors and less have researched about thyroid hormone, therefore more research on the effects of chemicals on thyroid receptors are needed (Kuster et al. 2010; Shi et al. 2012). Other chemicals that showed thyroid activity include *p,p'*-DDE, trans-nonachlor and oxychlordane exposure, but the activity was very poor and they also changed the levels of T4 and TSH (Jain 2014; Li et al. 2016) therefore we need to do more research on chemicals with thyroid activity. Mancozeb reduced T4 levels in dams in the study of Axelstad et al. (2011) and they also indicated that it may become a potential cause of the disruption in the thyroid of humans.

One of the challenges in the study of EDCs is that most of these chemicals react at low concentrations (Archer and van Wyk 2015), which might be below the detection limit. This might be one of the reasons mancozeb could not be detected. A study by Archer and Wyk (2015) analysed several pesticides using the recombinant yeast androgen screen (YAS). Mancozeb was the most potent anti-androgen and showed activity from 1.95 µM, whereas other pesticides only reacted from 1 mM, however, in the current study Mancozeb did not show any significant activity.

Mancozeb is not the only concern in water, other EDCs (such as DDT) that are present in the water might have an additive effect. Therefore, even though the levels are below the level of quantification, when several chemicals with the same effect are added together, the resultant activity may have a detrimental effect (Archer et al. 2017).

In this study the level of mancozeb at all four sites were below the level of quantification (10 µg l^{-1}). In comparison, the maximum mancozeb concentration was 39 µg l^{-1} in a simulation study that investigated point source contamination of pesticides from a vineyard farm in Italy (Fait et al. 2007). Approximately 1 km form the source of point contamination, the mancozeb concentration exceeded 0.1 µg l^{-1}.

Mancozeb is classified and detected as an organophosphate (Rodríguez et al. 2006). Rodríguez et al. (2006) detected organophosphates with the concentrations of up to 10 ng ml^{-1}, and similarly using a SPE method. However, when sampling, they maintained the pH at 6.8 before extraction. They used Oasis cartridges that were conditioned with ethyl acetate methanol and water (2 ml each). In this study, the pH was adjusted to 2–3 in the SPE extraction method for endocrine disruptors using Oasis HLB glass cartridges conditioned with 5 ml double-distilled H2O followed by 5 ml of methanol (HPLC grade) and 5 ml of double-distilled H_2O, but mancozeb was not detected. A different extraction method specific for organophosphates might be more appropriate to use.

Mancozeb is a pesticide with a short half-life of three to four days, as stated by (Mohapatra and Deepa 2012; Devi et al. 2015; Simakani et al. 2018). After sampling, the samples were kept in a −20 °C freezer for approximately a period of seven days for further analyses. Therefore, the delay of the analyses could have had an impact on the detection of the mancozeb, because of a possible degradation of its metabolites.

Oestrogenic activity was only detected at three of the four sites at very low levels (Table 1). In contrast, oestrogenic activity was reported in the Stellenbosch region with the E2

equivalent concentrations ranging between 0.082 and 0.029 μg l^{-1}, their sampling was done in summer and the results indicated oestrogenic activity in two of the 10 sampling sites using the recombinant yeast oestrogen assay (YES) (van Wyk et al. 2014). The samples did not suppress the oestrogenic activity either. Although the three positive samples were below the trigger value of 0.7 ng l^{-1} for oestrogenic activity of drinking water (Genthe et al. 2010), the fact that oestrogenic activity was detected is still a concern and needs to be monitored. Since mancozeb was not detected during the chemical analyses, the activity detected is probably from other sources, such as other types of EDCs from the sewage effluent or pharmaceutical effluent from a nearby hospital. The activity from the Nandoni Dam might also be because of personal care products, household products and industrial products entering the dam via various effluent points from the area, including the Mvudi-, Dzindi- and Luvuvhu Rivers, which receive agricultural and municipal effluents.

The oestradiol equivalents (EEq) values of extraction control and Tate Vondo Dam were below the limit of quantification whereas the ND, AD and XW had values above the limit of quantification, but still very low (table 1). Gumbo et al. (2016) studied the similarities between upstream and downstream of sampling sites that are downstream of municipal sewage plants. Upstream of the ND there are five sewage water treatment plants (SWTPs), of which one of them is the Elim SWTP discharging its effluents into the Muhohodi River, possibly ending up into the ND lower down. The effluents/pollutants from the remaining four SWTPs (Vuwani oxidation ponds, Waterval SWTP, Thohoyandou and Vuwani SWTPs) also finally discharge in the ND (Gumbo et al. 2016). Effluent from SWTPs may contribute to the oestrogenic activity in water samples, because most of these SWTPs are not fully functional.

Aneck-Hahn et al. (2009) did a study in the Limpopo province where they found positive oestrogenic activity in Molekane and Sekuruwe villages indicated by values above the detection limit of the assay. The EEq value of four sampling sites per area ranged between 0.68 and 2.29 ng l^{-1} and 0.63 to 2.48 ng l^{-1}, respectively. The EEq of the four sampling sites (different dams) from this study were all below 1 ng l^{-1} and ranged from 0.210 to 0.236 ng l^{-1} (Table 1). The ND was expected to have a high EEq, compared with other dams, because the water was collected at the edge of the dam where people sometimes dump their used disposable nappies and general household garbage. Dzaga's unpublished masters study indicated that a number of schistosomiasis infections were discovered among villagers situated on the shoreline of the Nandoni Dam (Dzaga 2012; Gumbo et al. 2016).

Unlike the ND and the AD, the inflow of the TD, which is situated in the Mutshindudi River catchment and in the area of tea plantations, and XW is prohibited. This means that in terms of dumping they are safe thus they have very low levels of oestrogenic activity. They also serve as drinking area for animals and humans. The fact that water samples were collected when the water was settled might also be the reason activity was not detected. Water from the TD and the extraction control both had values of EEq less than the limit of quantification. The AD receives run offs from a supposedly DDT free area, where there is no indoor residual spraying (IRS) (Barnhoorn et al. 2009). However, it is surrounded by commercial and subsistence farming practices and situated in the area where there is a direct discharge from wastewater treatment (Odiyo et al. 2012). Aneck-Hahn et al. (2008) collected water samples during a rainy season in the Limpopo Province and detected

oestrogenic activity in five out of seven sites with EEqs ranging from 0.3 ± 0.02 ng l^{-1} to 2.1 ± 0.18 ng l^{-1}. Two of the samples from this other area and the water from TD had no detectable oestrogenic activity with EEqs less than limit of quantification. The reason they were able to detect higher oestrogenic activity of the mancozeb, compared with this study, might be that they sampled in the reserve's catchment area, which is near industries, agricultural activities and settlements, and that some of the water was coming from SWTPs. They detected no oestrogenic activity at two sites. One of the sites was a natural spring protected from potential sources of contamination. At the second site, the sample was taken after the water had traversed through a natural peat wetland (Aneck-Hahn et al. 2008). However, more research is needed from these sites to verify the results found.

Additional studies are required in the natural water sources of the Vhembe District regarding the detection of oestrogenic activity. The results of this study were also very different from another study done in Pretoria, where oestrogen activity ranging from 0.000816 ng l^{-1} to 2.44 ng l^{-1} were detected from three different sampling sites (Mahomed et al. 2008). The difference might be the fact that they sampled at an urban site where there are dumping sites, industrial companies etc.

When comparing the results from the XW that had the highest EEq (0.237 ng l^{-1}) to our other sites, it was evident that the site was more affected. This might be, because the XW is situated within the DDT sprayed area (Marchand et al. 2008; Barnhoorn et al. 2009; Brink et al. 2012; Bornman et al. 2009). The water samples did not show any signs of androgenic activity at all sites, but it cannot be concluded that these four dams do not have characteristics of androgen activity, because the samples were taken during the middle of May 2017 during a dry period. There were no run-off points to the dams since the environment was dry.

The water samples from the four dams had values similar to that of the value of the extraction controls. All four sites had very low levels of androgenic activity, which were below detection limit. A study was done in summer in the Stellenbosch region, South Africa, in 10 dams and the results confirmed anti-androgenic activity in five of the ten sites using the YAS (van Wyk et al. 2014). The reason they found activity in five of their study sites might be that they sampled during summer, which is a rainy season, meaning more runoffs from surface to the dams.

None of the samples showed activity using the GH3-TRE-Luc assay. In comparison with other studies anti-thyroid activity associated with phthalate esters was detected in water sources from Yangtze River Delta using TH reporter gene assay (Shi et al. 2012).

Conclusion

Most studies examined the mode of action of mancozeb and detected anti-androgen and thyroid activity. However, no activity was detected in any of the bioassays in this study either oestrogenic, androgenic or thyroid. No mancozeb was detected at any of the sampling sites either. Several studies are emphasising that mancozeb has health effects and most *in vivo* studies are reporting its oestrogenic activity. Therefore, if any trace of mancozeb is found in water bodies it needs to be taken seriously, reported and mitigation plans put in place.

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ORCIDs
MC van Zijl: https://orcid.org/0000-0003-4950-5715
NH Aneck-Hahn: https://orcid.org/0000-0002-3649-8889
IEJ Barnhoorn: https://orcid.org/0000-0002-6635-304X

References
Aneck-Hahn NH, Bornman MS, de Jager C. 2008. Preliminary assessment of oestrogenic activity in water sources in Rietvlei Nature Reserve, Gauteng, South Africa. *African Journal of Aquatic Science* 33: 249–254. https://doi.org/10.2989/AJAS.2008.33.3.7.619.

Aneck-Hahn NH, Bornman MS, de Jager C. 2009. Oestrogenic activity in drinking water from a rural area in the Waterberg District, Limpopo Province, South Africa. *Water SA* 36: 245–252.

Archer E. 2018. Interaction of pharmaceutical and personal care products (PPCPs) and endocrine disrupting contaminants (EDCs) with microbial communities in South African wastewater treatment works and environmental waters. PhD thesis, Stellenbosch University, Stellenbosch.

Archer E and Van Wyk JH. 2015. The potential anti-androgenic effect of agricultural pesticides used in the Western Cape: *In vitro* investigation of mixture effects. *Water SA* 41: 129–138. https://doi.org/10.4314/wsa.v41i1.16.

Archer E, Wolfaardt GM, van Wyk JH. 2017. Pharmaceutical and personal care products (PPCPs) as endocrine disrupting contaminants (EDCs) in South African surface waters. *Water SA* 43: 684–705. https://doi.org/10.4314/wsa.v43i4.16.

Axelstad M, Boberg J, Nellemann C, Kiersgaard M, Jacobsen PR, Christiansen S, Hougaard KS, and Hass U. 2011. Exposure to the widely used fungicide mancozeb causes thyroid hormone disruption in rat dams, but no behavioral effects in the offspring. *Toxicological Sciences* 120: 439–446. https://doi.org/10.1093/toxsci/kfr006.

Baligar PN and Kaliwal BB. 2001. Induction of gonadal toxicity to female rats after chronic exposure to mancozeb. *Industrial Health* 39: 235–243. https://doi.org/10.2486/indhealth.39.235.

Barnhoorn IEJ, Bornman MS, Jansen van Rensburg C, Bouwman H. 2009. DDT residues in water, sediment, domestic and indigenous biota from a currently DDT-sprayed area. *Chemosphere* 77: 1236–1241. https://doi.org/10.1016/j.chemosphere.2009.08.045.

Blake LS, Martinovic D, Gray LE, Wilson VS, Regal RR, Villeneuve DL, Ankley GT. 2010. Characterization of the androgen-sensitive MDAkb2 cell line for assessing complex environmental mixtures. *Environmental. Toxicology and Chemistry* 29: 1367–1376.

Bornman MS, Aneck-Hahn NH, de Jager C, Wagenaar GM, Bouwman H, Barnhoorn IEJ, Patrick SM, Vandenberg LN, Kortenkamp A, Blumberg B, et al. 2017. Endocrine disruptors and health effects in Africa: A Call for Action. *Environmental Health Perspectives* 125: 085005–085010. https://doi.org/10.1289/EHP1774.

Bornman MS, Barnhoorn IEJ, Aneck-Hahn NH 2009. A pilot study on the occurrence of endocrine disruptive chemicals in a DDT-sprayed area. WRC Report No. KV220-09. Pretoria: Water Research Commission of South Africa.

Bornman MS, van Vuren JH, Bouwman H, de Jager C, Gentle B, Barnhoorn IEJ. 2007. The use of sentinel species to determine the use of endocrine disruptive activity in an urban nature reserve. WRC Report No. 1505/1/07. Pretoria: Water Research Commission of South Africa.

Brink K, Jansen van Vuren J, Bornman R. 2012. The lack of endocrine disrupting effects in catfish (*Clarias gariepinus*) from a DDT sprayed area. *Ecotoxicology and Environmental Safety* 79: 256–263. https://doi.org/10.1016/j.ecoenv.2012.01.006.

Burger AEC and Nel A. 2008. Scoping study to determine the potential impact of agricultural chemical substances (pesticides) with endocrine disruptor properties on the water resources of South Africa. WRC Report No. 1774/1/08. Pretoria: Water Research Commission of South Africa.

Cocco P. 2002. On the rumors about the silent spring: Review of the scientific evidence linking occupational and environmental pesticide exposure to endocrine disruption health effects. *Cadernos de Saúde P*ública 18: 379–402. https://doi.org/10.1590/S0102-311X2002000200003.

Dabrowski JM, Aneck-Hahn NH, Forbes PBC, Genthe B, Pieters R, Meyer JR. 2015. Investigation of the contamination of water resources by agricultural chemicals and the impact on environmental health. Volume 1: Risk Assessment of Agricultural Chemicals to Human and Animal Health. Pretoria: Water Research Commission of South Africa.

de Jager C, Aneck-Hahn NH, Barnhoorn IEJ, Bornman MS, Pieters P, Van Wyk JH, Van Zijl C. 2011. The compilation of toolbox of bioassays for detection of oestrogenic activity in water. WRC Report No.1816/1/10. Pretoria: Water Research Commission.

Devi PA, Paramasivam M, Prakasam V. 2015. Degradation pattern and risk assessment of carbendazim and mancozeb in mango fruits. *Environmental Monitoring and Assessment* 187: 4142. https://doi.org/10.1007/s10661-014-4142-6.

Diamanti-Kandarakis E, Bourguignon J, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. 2009. Endocrinedisrupting chemicals: An endocrine society scientific statement. *Endocrine Reviews* 30: 293–342. https://doi.org/10.1210/er.2009-0002.

Du G, Shen O, Sun H, Fei J, Lu C, Song L, Xia Y, Wang S, Wang X. 2010. Assessing hormone receptor activities of pyrethroid insecticides and their metabolites in reporter gene assays. *Toxicological Sciences* 116: 58–66. https://doi.org/10.1093/toxsci/kfq120.

Dzaga RA. 2012. An assessment of the impact of catchment activities on water quality: A Case study of Nandoni Dam, Vhembe District, Limpopo Province. MSc thesis. University of Venda, Thohoyandou, South Africa.

Ermler S, Scholze M, Kortenkamp A. 2010. The sensitivity of the MDA-kb2 cell *in vitro* assay in detecting anti-androgenic chemicals–identification of sources of variability and estimation of statistical power. *Toxicology In Vitro* 24: 1845–1853. https://doi.org/10.1016/j.tiv.2010.05.007.

Fait G, Nicelli M, Fragoulis G, Trevisan M, Capri E. 2007. Reduction of point contamination sources of pesticide from a vineyard farm. *Environmental Science & Technology* 41: 3302–3308. https://doi.org/10.1021/es062706k.

Fatoki OS, Bornman M, Ravandhalala L, Chimuka L, Genthe B, Adeniyi A. 2010. Phthalate ester plasticizers in freshwater systems of Venda, South Africa and potential health effects. *Water SA* 36: 117–126. https://doi.org/10.4314/wsa.v36i1.50916.

Genthe B, Le Roux WJ, Schachtschneider K, Oberholster PJ, Aneck-Hahn NH, Chamier J. 2013. Health risk implications from simultaneous exposure to multiple environmental contaminants. *Ecotoxicology and Environmental Safety* 93: 171–179. https://doi.org/10.1016/j.ecoenv.2013.03.032.

Genthe B, Steyn M, Aneck-Hahn NH, Van Zijl C, De Jager C. 2010. The feasibility of a health risk assessment framework to derive guidelines for oestrogen activity in treated drinking water. WRC Report No. 1749/1/09. Pretoria: Water Research Commission of South Africa.

Ghisari M, Long M, Tabbo A, Bonefeld-Jørgensen EC. 2015. Effects of currently used pesticides and their mixtures on the function of thyroid hormone and aryl hydrocarbon receptor in cell culture. *Toxicology and Applied Pharmacology* 284: 292–303. https://doi.org/10.1016/j.taap.2015.02.004.

Griffero L, Gomes G, Berazategui M, Fosalba C, Teixeira de Mello F, Rezende C, Bila D, García-Alonso J. 2018. Estrogenicity and cytotoxicity of sediments and water from the drink water source-basin of Montevideo city, Uruguay. *Ecotoxicology and Environmental Contamination* 13: 15–22. https://doi.org/10.5132/eec.2018.01.02.

Gumbo JR, Dzaga RA, Nethengwe NS. 2016. Impact on water quality of Nandoni water reservoir downstream of municipal sewage plants in Vhembe District, South Africa. *Sustainability* 2016: 597. https://doi.org/10.3390/su8070597.

Jain RB. 2014. Association between thyroid function and selected organochlorine pesticides: data from NHANES 2001–2002. *The Science of the Total Environment* 466–467: 706–715. https://doi.org/10.1016/j.scitotenv.2013.07.087.

Kjeldsen LS, Ghisari M, Bonefeld-Jorgensen EC. 2013. Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity. *Toxicology and Applied Pharmacology* 272: 453–464. https://doi.org/10.1016/j.taap.2013.06.028.

König M, Escher BI, Neale PA, Krauss M, Hilscherová K, Novák J, Teodorović I, Schulze T, Seidensticker S, Kamal Hashmi MA, et al. 2017. Impact of untreated wastewater on a major European river evaluated with a combination of *in vitro* bioassays and chemical analysis. *Environmental Pollution* 220: 1220–1230. https://doi.org/10.1016/j.envpol.2016.11.011.

Ksheerasagar RL and Kaliwal BB. 2010. Effect of mancozeb on thyroid, testis, accessory reproductive organs and biochemical constituents in albino mice. https://www.cabdirect.org/globalhealth/abstract/20103347305. [Accessed 26 June 2019].

Kugathas S, Audouze K, Ermler S, Orton F, Rosivatz E, Scholze M, Kortenkamp A. 2016. Effects of common pesticides on prostaglandin D2 (PGD2) inhibition in SC5 mouse Sertoli cells, evidence of binding at the COX-2 active site, and implications for endocrine disruption. *Environmental Health Perspectives* 124: 452–459. https://doi.org/10.1289/ehp.1409544.

Kuster M, Díaz-Cruz S, Rosell M, López de Alda M, Barceló D. 2010. Fate of selected pesticides, estrogens, progestogens and volatile organic compounds during artificial aquifer recharge using surface waters. *Chemosphere* 79: 880–886. https://doi.org/10.1016/j.chemosphere.2010.02.026.

Leusch FD, Aneck-Hahn NH, Cavanagh JAE, Du Pasquier D, Hamers T, Hebert A, Neale PA, Scheurer M, Simmons SO, et al. 2018. Comparison of in vitro and in vivo bioassays to measure thyroid hormone disrupting activity in water extracts. *Chemosphere* 191: 868–875. https://doi.org/10.1016/j.chemosphere.2017.10.109.

Leusch FD, Neale PA, Hebert A, Scheurer M, Schriks MC. 2017. Analysis of the sensitivity of in vitro bioassays for androgenic, progestagenic, glucocorticoid, thyroid and estrogenic activity: suitability for drinking and environmental waters. *Environment International* 99: 120–130. https://doi.org/10.1016/j.envint.2016.12.014.

Li J, Liu Y, Kong D, Ren S, Li N. 2016. T-screen and yeast assay for the detection of the thyroid-disrupting activities of cadmium, mercury, and zinc. *Environmental Science and Pollution Research International* 23: 9843–9851. https://doi.org/10.1007/s11356-016-6095-5.

Lin N and Garry VF. 2000. *In vitro* studies of cellular and molecular developmental toxicity of adjuvants, herbicides, and fungicides commonly used in Red River Valley, Minnesota. *Journal of Toxicology and Environmental Health Part A* 60: 423–439. https://doi.org/10.1080/00984100050033494.

London L, Dalvie, MA, Cairncross E, Solomons A. 2000. The quality of surface and groundwater in the rural western Cape with regard to pesticides. WRC Report No. 795/1/00. Pretoria: Water Research Commission of South Africa.

Mahomed SI, Voyi KW, Aneck-Hahn NH, de Jager C. 2008. Oestrogenicity and chemical target analysis of water from small-sized industries in Pretoria, South Africa. *Water SA* 35: 357–363.

Manickum T and John W. 2015. The current preference for the immuno-analytical ELISA method for quantitation of steroid hormones (endocrine disruptor compounds) in wastewater in South Africa. *Analytical and Bioanalytical Chemistry* 407: 4949–4970. https://doi.org/10.1007/s00216-015-8546-0.

Marchand MJ, Pieterse GM, Barnhoorn IEJ. 2008. Preliminary results on sperm motility and testicular histology of two feral fish species, *Oreochromis mossambicus* and *Clarias gariepinus*, from a currently DDT-sprayed area, South Africa. *Journal of Applied Ichthyology* 24: 423–429. https://doi.org/10.1111/j.1439-0426.2008.01141.x.

Mohapatra S and Deepa M. 2012. Residue dynamics of fenamidone and mancozeb on gherkin under two agro climatic zones in the state of Karnataka, India. *Bulletin of Environmental Contamination and Toxicology* 88: 507–510. https://doi.org/10.1007/s00128-012-0558-2.

Monneret C. 2017. What is an endocrine disruptor? *Comptes Rendus Biologies* 340: 403–405.

Ngcobo S. 2017. The suitability of oestrogen and androgen bioassays for the measurement of endocrine activity in different water matrices. PhD thesis, University of Pretoria.

O'Connor JC, Frame SR, Davis LG, Cook JC. 1999. Detection of the environmental antiandrogen *p,p*-DDE in CD and long-evans rats using a tier I screening battery and a Hershberger assay. *Toxicological Sciences* 51: 44–53. https://doi.org/10.1093/toxsci/51.1.44.

Odiyo JO, Phangisa JI, Makungo R. 2012. Rainfall-runoff modelling for estimating Latonyanda River flow contributions to Luvuvhu River downstream of Albasini Dam. *Physics and Chemistry of the Earth 5*0–52: 5–13. https://doi.org/10.1016/j.pce.2012.09.007.

Pickford DB. 2010. Screening chemicals for thyroid-disrupting activity: A critical comparison of mammalian and amphibian models. *Critical Reviews in Toxicology* 40: 845–892. https://doi.org/10.3109/10408444.2010.494250.

Power KA and Thompson LU. 2003. Ligand-induced regulation of ERα and ERβ is indicative of human breast cancer cell proliferation. *Breast Cancer Research and Treatment 8*1: 209–221. https://doi.org/10.1023/A:1026114501364.

Rebel P, Poblete C, van Zyl G, Wessels JPB, Coetzer C, McLeod A. 2019. Determining mancozeb deposition benchmark values on apple leaves for the management of *Venturia inaequalis*. *Plant Diseases* 104: 168–178. https://doi: 10.1094/PDIS-04-19-0873-RE.

Reinecke AJ, Maboeta MS, Vermeulen LA, Reinecke SA. 2002. Assessment of lead nitrate and mancozeb toxicity in earthworms using the avoidance response. *Bulletin of Environmental Contamination and Toxicology* 68: 779–786. https://doi.org/10.1007/s00128-002-0023-8.

Robson L, Barnhoorn IEJ, Wagenaar GM. 2017. The potential effects of efavirenz on *Oreochromis mossambicus* after acute exposure. *Environmental Toxicology and Pharmacology* 56: 225–232. https://doi.org/10.1016/j.etap.2017.09.017.

Rodríguez I, Calvo F, Quintana JB, Rubi E, Rodil R, Cela R. 2006. Suitability of solid-phase microextraction for the determination of organophosphate flame retardants and plasticizers in water samples. *Journal of Chromatography A* 1108: 158–165. https://doi.org/10.1016/j.chroma.2006.01.008.

Rossi G, Buccione R, Baldassarre M, Macchiarelli G, Palmerini MG, Cecconi S. 2006. Mancozeb exposure *in vivo* impairs mouse oocyte fertilizability. *Reproductive Toxicology (Elmsford, N.Y.)* 21: 216–219. https://doi.org/10.1016/j.reprotox.2005.08.004.

Rossouw CJ, Fourie PH, Van Zyl JG, Hoffman JE, McLeod A. 2018. Rainfastness of mancozeb on apple seedlings determined through deposition quantification of mancozeb residue and a fluorescent pigment. *Crop Protection (Guildford, Surrey)* 106: 93–102. https://doi.org/10.1016/j.cropro.2017.12.008.

Runkle J, Flocks J, Economos J, Dunlop AL. 2017. A systematic review of mancozeb as a reproductive and developmental hazard. *Environment International* 99: 29–42. https://doi.org/10.1016/j.envint.2016.11.006.

Sana B, Reda DM, Houria B, Amel A, Marwa B, Fateh ZM, Rima A. 2015. Toxicological impact of a mimetic estrogen specie: mancozeb on a tadpoles of the green frog (*Rana saharica*). *Journal of Biodiversity and Environmental Sciences* 6: 307–314.

Schutte GC, Beeton KV, Kotze JM. 1997. Rind stippling on Valencia oranges by copper fungicides used for control of citrus black spot in South Africa. *Plant Disease* 81: 851–854. https://doi.org/10.1094/PDIS.1997.81.8.851.

Schutte GC, Mansfield RI, Smith H, Beeton KV. 2003. Application of azoxystrobin for control of benomyl-resistant *Guignardia citricarpa* on 'Valencia' oranges in South Africa. *Plant Disease* 87: 784–788. https://doi.org/10.1094/PDIS.2003.87.7.784.

Shi W, Zhang FX, Hu GJ, Hao YQ, Zhang XW, Liu HL, Wei S, Wang XR, Giesy JP, Yu HX. 2012. Thyroid hormone disrupting activities associated with phthalate esters in water sources from Yangtze River Delta. *Environment International* 42: 117–123. https://doi.org/10.1016/j.envint.2011.05.013.

Simakani P, Abolhasani MH, Hoseini SM. 2018. Determination of mancozeb toxicity and biochemical effects in common carp (*Cyprinus carpio*). *International Journal of Aquatic Biology* 6: 157–161.

Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guilette LJ, Jegou B, Jensen TK, Jouannet P, Keiding N, et al. 1996. Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives* 104: 741–803.

Van Wyk JH, Archer E, Babalola OO, Truter JC, Jansen van Rensburg E, Dabrowski J. 2014. Pesticides as endocrine disruptors in South Africa: *Laboratory and field studies* (Report No. 1932/1/14). Pretoria: Water Research Commission.

Van Zijl MC, Aneck-Hahn NH, Swart P, Hayward S, Genthe B, de Jager C. 2017. Estrogenic activity, chemical levels and health risk assessment of municipal distribution point water from Pretoria and Cape Town, South Africa. *Chemosphere* 186: 305–313. https://doi.org/10.1016/j.chemosphere.2017.07.130.

Vermeulen LA, Reinecke AJ, Reinecke SA. 2001. Evaluation of the fungicide manganese-zinc ethylene bis (dithiocarbamate) (Mancozeb) for sublethal and acute toxicity to *Eisenia fetida* (Oligochaeta). *Ecotoxicology and Environmental Safety* 48: 183–189. https://doi.org/10.1006/eesa.2000.2008.

Watanabe T, Junzheng W, Morikawa K, Fuchigami M, Kuranami M, Adachi I, Yamaguchi K, Abe K. 1980. In vitro sensitivity test of breast cancer cells to hormonal agents in a radionucleotide-incorporation assay. *Japanese Journal of Cancer Research 8*1: 536–543. https://doi.org/10.1111/j.1349-7006.1990.tb02603.x.

Wehmas LC, Cavallin JE, Durhan EJ, Kahl MD, Martinovic D, Mayasich J, Tuominen T, Villeneuve DL, Ankley GT. 2011. Screening complex effluents for estrogenic activity with the T47D-KBluc cell bioassay: Assay optimization and comparison with in vivo responses in fish. *Environmental Toxicology and Chemistry* 30: 439–445. https://doi.org/10.1002/etc.388.

Wilson VS, Bobseine K, Gray LE Jr. 2004. Development and characterisation of a cell line that stably expresses and oestrogen-responsive luciferase reporter for the detection of oestrogen receptor agonists and antagonists. *Toxicological Sciences 8*1: 69–77. https://doi.org/10.1093/toxsci/kfh180.

Wilson VS, Bobseine K, Lambright CR, Gray LE Jr. 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: 69–81. https://doi.org/10.1093/toxsci/66.1.69.

Xu LC, Liu L, Ren XM, Zhang MR, Cong N, Xu AQ, Shao JH. 2008. Evaluation of androgen receptor transcriptional activities of some pesticides in vitro. *Toxicology 2*43: 59–65. https://doi.org/10.1016/j.tox.2007.09.028.