Investigating the presence of crop applied herbicide mixtures in aquatic systems and its possible risks

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LIST OF ACRONYMS / ABBREVIATIONS

2,4-D	2,4-dichloro-phenoxyacetic acid
A	
AAS	Atomic absorption spectroscopy
ADI	Acceptable daily intake
AhR	Aryl hydrocarbon receptor
AI	Active ingredient
AIDS	Acquired immune deficiency syndrome
ALP	Alkaline phosphatase
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AR	Androgen receptor
ARC	Agricultural Research Council
APG	Alkyl polyglucoside
APN	Aminopeptidase A
B	
BC	Blank control
Bt	*Bacillus thuringiensis*
C	
CA	Concentration addition
cAMP	Cyclic adenosine monophosphate
cdtFBS	Charcoal dextran treated foetal bovine serum
CEC	Cation exchange capacity
CHO cells	Chinese Hamster Ovary cells
CH_3COONa	Sodium acetate
CO_2	Carbon dioxide
Cry proteins	Crystalline proteins
CV	Coefficient of variation
D	
DAFF	Department of Agriculture, Forestry and Fisheries
DAHP	3-Deoxy-D-arabinoheptulosonate 7-phosphate
Dexa-EQ	Dexamethasone equivalents
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle's medium
DO	Dissolved oxygen
DRE	Dioxin response element
DTT	Dithiothreitol

E

EC	Effective concentration
ED	Endocrine disruption
EDC	Endocrine disrupting compounds
EDTA	Ethylene-diamine-tetra-acetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assays
EPSPS	Enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase
ER	Oestrogen receptor

F

FBS	Foetal bovine serum
FC	Fold change
Fludioxonil	4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile
FluEQ	Flutamide equivalents

G

GBH	Glyphosate based herbicides
GC/MS	Gas chromatography/mass spectrometry
Glyphosate	N-(phosphonomethyl)glycine]
GM	Genetically modified
G-protein	Guanine nucleotide-binding protein
GR	Glucocorticoid receptor
GST	Glutathione S-transferase

H

Ha	Hectares
HAH	Halogenated aromatic hydrocarbons
HEPA	High-efficiency particulate air
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HTS	High-troughput screening

I

IA	Independent action
IARC	International Agency for Research on Cancer
IC	Inhibition concentration
ICP-MS	Inductively coupled plasma-mass spectrometry
IRA	Immuno-immobilized androgen receptor assay

L

LAR	Luciferase assay reagent
LC/MS	Liquid chromatography coupled to mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
M	
MMTV	Murine mammalian tumour virus
MRL	Maximum residue limit
MSDS	Material safety data sheet
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	
NOEC	No Observed Effect Concentration
NRF	National Research Foundation
O	
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
P	
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + Tween
PC	Positive control
POEA	Polyethoxylated tallowamine
PR	Progesterone receptor
Q	
QA	Quality assurance
QC	Quality control
R	
R^2	R-square (correlation coefficient)
RDA	Redundancy analysis
RGA	Reporter gene assays
RLU	Relative light units
RR	Roundup Ready
S	
SC	Solvent control
SOD	Superoxide dismutase
T	
T	Testosterone
TCDD	2,3,7,8-tetrachlorodibenzo-*para*-dioxin

TCDD-EQ	TCDD equivalents
TDS	Total dissolved solids
TiPED	Tiered Protocol for Endocrine Disruption
TOC	Total organic carbon content
TR	Thyroid receptor
TTEQ	Testosterone equivalents
U	
USA	United States of America
USEPA	United States Environmental Protection Agency
W	
WHC	Water holding capacity
WHO	World Health Organisation
WWTP	Wastewater treatment plant
Y	
YAS	Yeast androgen screen

LIST OF FIGURES

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There are no tables in chapters 2, 5, 6, 7, 8.

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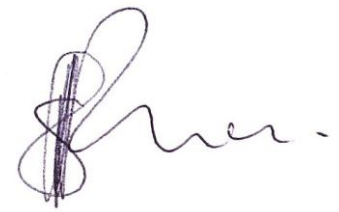
DECLARATION

I, Suranie Rachel Horn, hereby declare that this thesis entitled “Investigating the presence of crop applied herbicide mixtures in aquatic systems and its possible risks” submitted for the degree Doctor of Philosophy in Environmental Sciences at the North-West University (Potchefstroom Campus) has not previously been submitted by myself for any degree at any other tertiary institution. I am the sole author thereof, and it was my work in design and execution.



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Suranie Rachel Horn

ABSTRACT

Crop production is important to feed the growing global population. Over the last few decades, measures to improve crop production have been adopted around the world. This include the use of genetically modified maize to kill insect pests and resist glyphosate effects, (Bt and Roundup Ready) and also using various pesticides. The chemicals used in agricultural activities are mostly water soluble. These compounds therefore end up in the environment as complex mixtures. Their effects are unpredictable and they may act or interact differently when present in mixtures, than in their single capacity. The research question of this study was whether the herbicides, glyphosate and 2,4-D, and Cry proteins, from Bt maize, have endocrine disruptive effects when present in mixtures.

Previous work did not address mixtures containing this specific combination of compounds that was tested in the current study. In order to obtain a mixture of the specific target compounds, the idea was to conduct a field trial in which different cultivars were planted and sprayed with different combinations of the above-mentioned herbicides. This gave a better idea of the agricultural chemicals that were introduced into the field. After the field trial, soil was collected and extracted with rainwater to target the bioavailable fraction.

The endocrine potential of the mixtures containing the target compound was determined by using *in vitro* reporter-gene assays. The MDA-kb2 cells have both androgen (AR) and glucocorticoid (GR) receptors and were used to measure (anti)androgenic effects. H4IIE-*luc* cells determined the xenobiotic potential and an indirect endocrine disruptive potential of the compounds. The cells were exposed to: the single active ingredients (pure compounds) of glyphosate, 2,4-D and Cry1Ab; formulations Roundup®, and 2,4-D amine SL; environmentally relevant concentrations of the active ingredient, and formulations; as well as the rainwater extracts. The effect on cell proliferation of the same suite of compounds was assessed using the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay.

The results of the current study revealed that the environmental extracts that received a pre-and post-emergent Roundup® application facilitated androgen receptor binding. The testosterone equivalents (TTEQs) derived from AR activation and the dexamethasone equivalents (DexaEQs) derived from GR binding exceeded the drinking water trigger values specifically derived for bio-assays. Exceeding the trigger values act as a warning signal prompting further investigation. The environmental extracts containing 2,4-D suppressed AR activation, but only slightly, as these responses were not detected in the AR inhibition assay. The following

compounds caused inhibition of the AR from strongest to weakest inhibitor: Roundup® > Cry1Ab > 2,4-D > D:RR. Most of the compounds tested were responsible for increased cell proliferation presenting evidence that they could stimulate cancer cell growth.

Use of contaminated water sources can lead to chronic exposure to these mixtures and cause endocrine disrupting effects in humans or aquatic life. The findings of this study highlight the need for additional monitoring of water resources due to the effects the target compounds might pose to non-target organisms.

Keywords: 2,4-D; Cry proteins; endocrine disruption; glyphosate; reporter-gene assays

1. GENERAL INTRODUCTION

Agricultural activity is a potential source of a number of chemicals that end up in the environment. These include the pharmaceuticals used for treatment of livestock, the artificial fertilisers added to crops, and pesticides. Current-use pesticides, in contrast to the historically used persistent organophosphates, are made to be biodegradable, but the continuous use of high volumes cause contamination of the environment (Carvalho, 2017). Concerns have been raised that certain pesticides and especially their residues, may enter and pollute the rivers and dams where it is harmful to aquatic organisms. Humans and wildlife consuming the untreated water will also experience detrimental effects (Dabrowski, Shadung & Wepener, 2014).

Single chemical risk assessment underestimates the actual risks of pesticide mixtures (Köhler & Triebskorn, 2013), as agricultural remedies include biological and/or chemical agents used individually or in combinations to control or prevent various forms of pests (Dabrowski, Shadung & Wepener, 2014).

Biotechnology was applied to agriculture to reduce crop losses, reduce pesticide use, improve resistance to pests as well as abiotic stresses such as drought and cold. One of the methods was to make crops resistant to insect pests by creating genetically modified (GM) maize. The maize plant had been transformed by insertion of a gene into its genome that encodes for crystal (Cry) proteins found in *Bacillus thuringiensis* (Bt) and is now referred to as Bt-maize. The reason why this was done is that the bacterium forms protein inclusion bodies containing Cry proteins during sporulation and when certain insect species ingest the Cry proteins, they die (Betz, Hammond & Fuchs, 2000). The transgene expresses Cry proteins in the maize plants, which protect it from herbivore insect pests. The most common GM crops grown in the United States are maize and soybeans. South Africa has been planting Bt maize since 1998 and is among the top ten biggest GM maize producers in the world with 80% of crops planted in South Africa being GM (Kruger, Rensburg & Berg, 2009). Cry proteins therefore end up in the environment.

Another practice to reduce crop losses, is to spray herbicides. The use of herbicides has increased drastically over the past years, and further increases are scheduled to occur in the next few years (Benbrook, 2016). Roundup®, with the active ingredient (glyphosate [N-(phosphonomethyl)glycine]), is a broad spectrum, non-selective, post-emergent herbicide used for weed and vegetation control. It is the most used herbicide in the world (Dai *et al*., 2016). It only has one particular mechanism of action that disrupts plant metabolism and is therefore

deemed relatively safe to animals and humans. However, because of its mechanism of action it also kills the crops, and could therefore only be applied before or after the growing season.

This drawback was overcome by making crops tolerant to glyphosate (the active ingredient in Roundup®). Herbicide-resistant maize and soybeans with genetically engineered tolerance to glyphosate (Roundup®) were first introduced in the mid-1990s and are referred to as Roundup Ready® crops. Planting Roundup Ready® crops simplified weed management and enable farmers to spray larger quantities of glyphosate-containing herbicides, increase application rates, as well as spray them on the plants during the growing season while leaving the crops unharmed (Benbrook, 2012). Global use of glyphosate has increased by a factor of more than 10 over the last 20 years, but this reliance on glyphosate has led to 42 weed species developing resistance against glyphosate (Heap, 2018; Shaner, Lindenmeyer & Ostlie, 2012). To address this increasing tolerance of weeds towards glyphosate, farmers have to use herbicides with different mechanisms of action (Chahal & Jhala, 2015). One of the herbicides used in South Africa, against which 38 weed species are resistant to, is 2,4-dichloro-phenoxyacetic acid (2,4-D) (Landrigan & Benbrook, 2015; Benbrook, 2016). 2,4-D is a post-emergent auxin herbicide that has been around for more than 50 years and is used for the selective control of broadleaf weeds for lawns, golf-courses and grain crops.

As a consequence of the high usage of herbicide mixtures for weed control, a large number of compounds are released into the environment annually (Peixoto, 2008). This also includes other pesticides, for example, insecticides and fungicides. Pesticides are usually an ingredient in a formulation that contains chemical additives to improve absorption of the pesticide and translocation of the active ingredient. Manufacturers also recommend that most herbicides should be applied in conjunction with other herbicides (diuron, atrazine, bromoxynil) and also adjuvants such as surfactants (e.g. polyether-polymethylsiloxane co-polymer and ammonium sulphate) to increase the efficiency of the herbicide. These adjuvants have proven to make product formulations more toxic than active ingredients alone (Myers *et al*., 2016).

For pesticides to be registered, sold and distributed, toxicity studies in sentinel organisms should prove that a pesticide will not cause adverse effects on the environment and publish a material safety data sheet (MSDS) about the potential hazards. Toxicity tests primarily focus on the (single) active ingredient which means that mixture toxicity is often overlooked. Single toxicity testing leads to a gap in our knowledge about the effects of mixtures which reflect the real-life situation of pesticides application. When chemicals occur together in the environment, they can either interact in such a way that changes their toxicity characteristics or no interaction occurs. Interactions may lead to formation of new compounds, but newly formed compounds

may also cross-react with compounds that are already present in the environment where the chemicals/herbicides are applied. When no chemicals in the mixture affect the toxicity of the other chemical(s), toxicity can be predicted by how the chemicals act on their own (Könemann & Pieters, 1996), which means that there is no interaction between the compounds. This is referred to as “additivity”. When interaction does occur by either an increase or decrease in toxicity beyond the sum of the individual effects, it is referred to as “synergism” or “antagonism”, respectively (Donley, 2016).

Despite the usefulness of herbicides in controlling weeds, they may pose a risk to non-target organisms in the environment. Some studies have researched the toxic effects of herbicides (Williams *et al*. 2000; Bukowska 2006; González *et al*. 2006; Gasnier *et al*. 2009). However, there is limited research on the combined effects of glyphosate and 2,4-D, which are particularly relevant in the South African context. Moreover, residues of these two herbicides are present in environmental matrices in conjunction with: (i) residues of systemic seed treatments, especially neonicotinoid insecticides; (ii) residues of systemic insecticides and fungicides applied during the crop season, and (iii) Bt endotoxins or Cry proteins in the case of GM maize. All of these agricultural chemicals occur as mixtures in the environment, also in the aquatic environment, due to run-off and spray-drift. The interactions between all of the above-mentioned compounds are hardly studied at all, and mixtures can be expected to react differently than single substances, and may thus also cause undesirable toxic effects.

Due to pollution of the aquatic environment, humans and wildlife depending on the water resource are exposed to harmful chemicals. Continuous exposure to low-level cocktails of chemical substances may have health implications for man and environment, although not immediate (acute) toxicity, but rather in the form of chronic toxicity (Burkhardt-Holm, 2010). An example is the evidence of chronic exposure and effects (malfunction) in the hormonal systems of humans and wildlife malfunctioning (reviewed in Connolly *et al*. 2011; WHO, 2002). Chemicals capable of influencing the endocrine system is collectively known as endocrine disrupting compounds (EDCs). These substances have been defined by the US Environmental Protection Agency as: “Exogenous agents that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction development and/or behaviour” (WHO, 2002). EDCs are biologically active at very low concentrations (<1 ng/L) and are therefore harmful when present in the environment. Bio-assays have been developed to screen for biological effects caused by low-level environmental mixtures (Kortenkamp, 2008). Reporter-gene assays are one type of approach that can be applied to measure the potential endocrine effects of low-level environmental mixtures. These assays are more sensitive than instrumental analysis, but most

importantly, they measure overall biological activity and thus display combined effects of all the relevant chemicals in the tested environmental mixture (Wangmo *et al*., 2018). Moreover, these assays can determine the total agonistic or antagonistic activity of complex mixtures in the ng/L range (Van Der Linden *et al*., 2008). They can also detect and semi-quantify oestrogenic, progesterone, androgenic and glucocorticoid activity of single compounds or mixtures of compounds (Kiyama & Wada-Kiyama, 2015). The results are expressed as a reference-equivalent concentration.

The current project is unique in that it not only investigates the potential mixture-toxicity of the dominant herbicides (glyphosate and to a lesser extent, 2,4-D) and Cry toxins (from Bt maize) in the laboratory but also links observed effects from the laboratory to environmentally relevant studies. The three above-mentioned target compounds were chosen based on their high level of production, daily use and lack of adequate reports on their possible endocrine disrupting effects and presence in water resources.

Hypothesis

Residues of herbicides such as glyphosate and 2,4-D, and the insecticidal Bt toxin, Cry1Ab, are present in concentrations that may pose health risks to non-target organisms in water and soil. The combinatorial effects of these compounds might be different from the single compound's effect.

Aims and objectives

1. **A first assessment of glyphosate, 2,4-D and Cry protein residues in surface water of South Africa (manuscript to be submitted to the journal: South African Journal of Science).**

Objectives:

- Sample water from rivers in close proximity to agricultural fields growing herbicide-tolerant Bt maize, throughout the planting season to determine the extent and levels of the herbicides and Cry residues over time.
- Concentrate the water samples to enable the measurement of the concentration of Cry1Ab with the use of ELISA.
- Determine the concentrations of glyphosate and 2,4-D in water samples using enzyme-linked immunosorbent assays (ELISAs).

2. Determine the concentrations of glyphosate, 2,4-D and Cry1Ab toxin in field-trial soil systems

Objectives:

- Spray different maize varieties (GM lines expressing Cry proteins and non-GM iso-lines) with different combinations of Roundup® and 2,4-D to obtain the correct combinations of the target compounds in this environmental setting.
- Determine the concentrations of the three target compounds in the soil at the end of the growing season.
- Determine the concentrations of the residues of the three target compounds in the bioavailable fraction by extracting the soil samples using rainwater, which indicates the total concentrations that would end up in neighbouring surface water systems as they move off the fields during rainfall.

3. Investigate and compare endocrine disruption potential of the single compounds of glyphosate, 2,4-D and Cry1Ab, with herbicide formulations and mixtures in environmentally relevant concentrations.

Objectives:

- Determine the ability of the three target compounds to activate the expression of the P4501A1 enzyme Phase I biotransformation process using the H4IIE reporter gene bio-assay.
- Investigate the (anti)-androgenic and glucocorticoid activity of the target compounds, using a hormone receptor binding bio-assay.

4. Compare experimental observations with prediction models, and evaluate interactions between single compounds in mixtures

- Investigation and discussion of mixture effects of the three target compounds based on the following models:
    - Concentration addition (CA)
    - Independent action (IA)

MANUSCRIPT

This chapter is a separate entity and will be submitted as a manuscript to the South African Journal of Science.

A first assessment of glyphosate, 2,4-D, and Cry proteins in surface water of South Africa.

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Abstract

Agriculture plays a vital role in the South African economy, as well as producing maize to feed the population. Genetically modified (GM) crops were transformed by insertion of a gene that encodes crystal (Cry) proteins found in *Bacillus thuringiensis* (Bt) and is now referred to as Bt-maize. Ingestion of Cry1Ab (which is a specific type of Cry protein) causes the death of these insects. These crops, along with herbicides such as glyphosate and 2,4-dichlorophenoxyacetic acid are widely adopted as part of the South African farming regime.. These compounds end up in water sources. These compound levels are monitored worldwide, but not in South Africa. This study aimed to screen water sources in an agricultural area for the presence of the target compounds: Cry1Ab, glyphosate and 2,4-D using enzyme-linked-immuno-sorbent assays (ELISAs). No levels of Cry 1Ab were detected. Most of the sites had glyphosate levels <LOD and one had quantifiable traces of glyphosate after the spraying event. 2,4-D was detected at all the sites. This pilot study is the first to report on levels of these target compounds in South Africa. The presence of 2,4-D and glyphosate indicates the need for regular monitoring of these compounds in South African water resources, as many people are still dependent on untreated water resources, which may be contaminated by agricultural chemicals.

Keywords: GM crops, glyphosate, 2,4-D, Cry1Ab, ELISA, mixtures

Introduction

South Africa is an agricultural driven country and maize is grown on 2.8 million hectares, with the Free State, Mpumalanga and North West provinces accounting for approximately 84% of total maize production in the country (DAFF, 2017). Maize serves as the staple food for the majority of the South Africans and the country, therefore, relies on successful agriculture to meet the basic needs of its population (Jury, 2002).

The agricultural sector globally had major advances over the past 40 years.The genes that encode for Cry proteins, which are produced by *Bacillus thuringiensis* (*Bt*) and cause the death of the maize insect pests, were incorporated into maize, creating genetically modified (GM) crops. Ingestion of these proteins by a specific insect species leads to its death. Cry proteins are considered to be environmentally benign with little to no effects on non-target organisms. Cry1Ab proteins are not commonly found in water sources, but when they do occur, they readily partition to the clay and organic materials in the aquatic system (Strain, Whiting & Lydy, 2014). Another genetic modification of maize makes the crop plants resistant to the herbicide glyphosate (the active ingredient in Roundup®). These herbicide-tolerant crops are referred to as Roundup-ready (RR) maize and can be sprayed with glyphosate-based herbicides (GBH) in larger quantities and during a longer period of the planting season without causing damage to the crops (Benbrook, 2012).

Glyphosate [N-(phosphonomethyl)glycine] is the most used herbicide in the world (Dai *et al*., 2016). It is a broad spectrum, non-selective, post-emergence herbicide used for weed and vegetation control. Glyphosate is known to rapidly degrade and strongly adsorb to the soil (Simonsen *et al*., 2008). Glyphosate's mechanism of action is to inhibit the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) of the shikimate pathway. The shikimate (shikimic acid) pathway is responsible for the biosynthesis of folates and aromatic amino acids (phenylalanine, tyrosine, and tryptophan) in plants, bacteria, fungi, algae, and some protozoan parasites (Vivancos *et al*., 2011). Glyphosate is known to be non-toxic and has a low ecotoxicological potential, however, it was classified as a probable human carcinogen by the International Agency for Research on Cancer (IARC) (International Agency for Research on Cancer (IARC), 2015), but not by the European Food Safety Authority (European Food Safety Authority, 2015).

Insufficient crop management has led to glyphosate resistant-weeds (Shaner, Lindenmeyer & Ostlie, 2012). To address the tolerance of weeds towards glyphosate, farmers use herbicides with different mechanisms of action (Chahal *et al*., 2015). One of the herbicides used in South Africa, against which fewer weeds have developed resistance, is 2,4-dichloro-phenoxyacetic

acid (2,4-D) (Landrigan & Benbrook, 2015; Benbrook, 2016). 2,4-D is a post-emergent auxin herbicide and has been used as the selective control of broadleaf weeds.

South Africa is the biggest user of pesticides in sub-Saharan Africa and has more than 500 registered active ingredients. The use of herbicides on GM maize—of which 80% is the Roundup-ready version—has increased drastically over the past years, and further increases are expected to occur in the next few years (Benbrook, 2016). Generally, pesticides are developed to target specific pests and to be immobile, however, runoff, leaching and spray drift occur that spread the compounds into unintended sections of the environment, also to water sources. These compounds generally occur at low concentrations and if they are detectable, it is assumed that they do not have detrimental effects on non-target organisms. However, exposure to low levels of pesticides poses chronic human health effects which may include endocrine disruption, immune impacts, neurotoxicity, genotoxicity, carcinogenesis and mutagenicity (Brown *et al*., 2009).

In a water-scarce country such as South Africa, water contaminated with chemicals are of great concern because many residents are still dependent on untreated surface and groundwater resources (Dabrowski *et al*. 2014). This pilot study aimed to screen for the presence of the herbicides, glyphosate and 2,4-D, as well as Cry proteins that would leach from GM crops in water sources on farms in South Africa. These compounds are not monitored in South Africa and their persistence in the environment and the toxicity of these compounds are still under scientific discussion worldwide.

Materials and methods

Study area

The sampling sites were located on two farms in close proximity to the Renoster- and Vaal Rivers. Farm A is located in the Free State province and farm B on the border between the North West and Free State provinces, South Africa (Fig. 1). On both farms, the spraying regime consisted of pre-emergent Roundup® and post-emergent Roundup® as well as 2,4-D. The fields on farm A were planted with *Bt* & RR maize and on farm B only RR.

Sampling

Water was sampled at different intervals during the planting season: i) pre and – ii) post herbicide application, as well as, iii) after the harvest. Water was sampled from water bodies such as farm dams and rivers running through the farms. Water was sampled in 250 mL high-density polyethylene (HDPE) bottles (Nalgene™), protected from UV radiation and kept at 4°C

during transportation. It was assumed that the farmers applied the herbicides according to the manufacturer's guidelines.

The following sites and matrices were sampled from farm A: A1-water from the Renoster River; A2-water from a dam on the farm; A3-soil from the cultivated fields. Samples from farm B consisted of: B1-water from the Vaal River; B2-inflow dam on the farm where water is recycled from runoff after rainfall and irrigation, and used again for irrigation; B3-water from a dam on the farm used for recreational activities; B4-soil from the cultivated fields on the farm. No soil was analysed for this study.

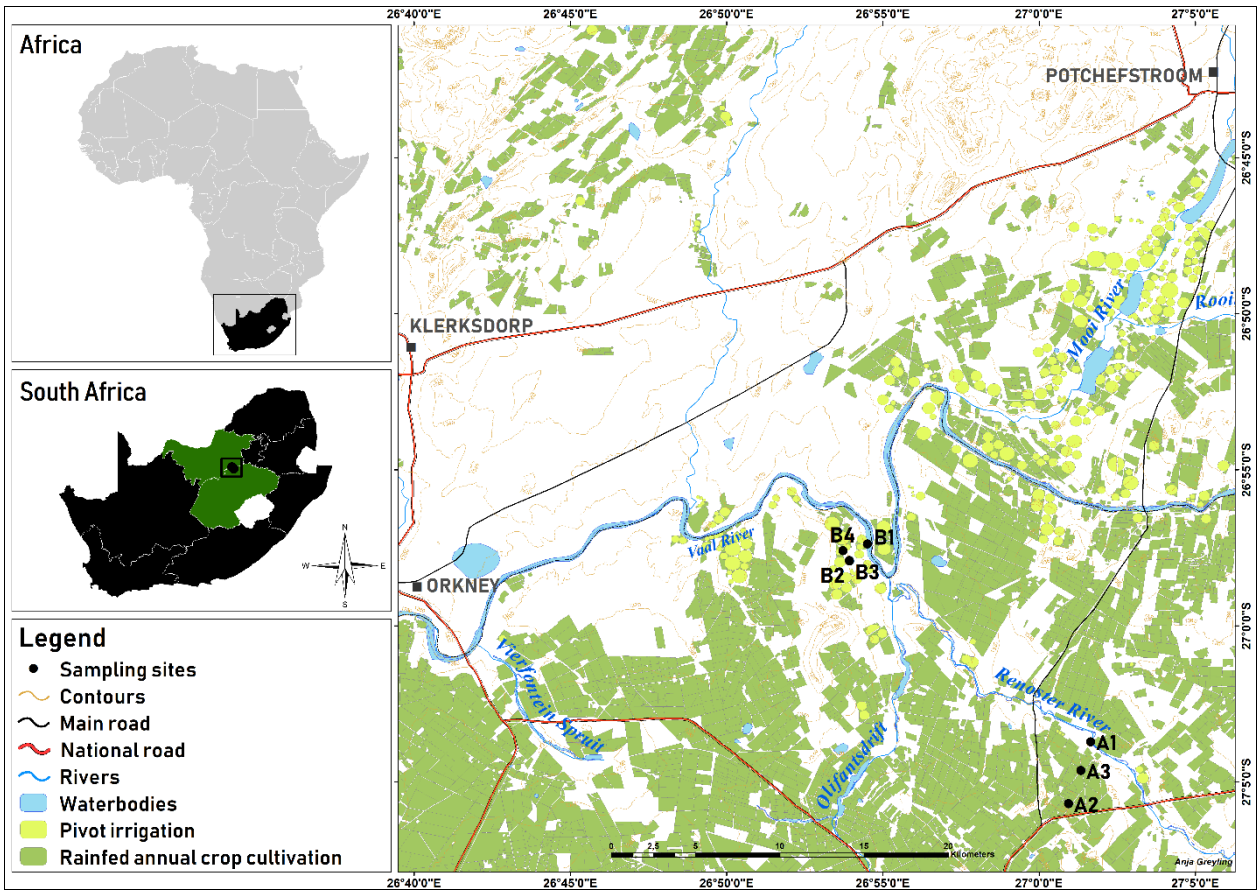

Figure 1:1 Map of the sampling sites situated on two farms: A and B.

Concentration of Cry1Ab proteins from water samples

Each water sample was concentrated using an Amicon® Ultra centrifugation tube (Millipore, Billerica, MA, USA) with a 30 000 molecular mass cut-off membrane. In short, a 15 mL aliquot of the sample was centrifuged at 870 g for 30 min. The eluent was discarded and the second 15 mL was added and again centrifuged at 870 g for 30 min. The Amicon® tubes were subjected to a third centrifugation cycle where after the membrane was rinsed with 1 mL phosphate buffered saline and Tween (PBST) assay buffer. The concentrate of the samples was refrigerated at 4°C and quantified within 24 h.

Enzyme-linked immunosorbent assays (ELISAs)

Over the past few years, ELISAs have demonstrated results comparable with LC/MS or GC/MS methods. These assays are therefore reliable and a good substitute to screen and quantify levels of contaminants in water sources (Szekacs, Mortl & Darvas, 2015).

Cry1Ab

The commercially available ELISA kit used for quantification of Cry1Ab in the water samples was from Envirologix (QualiPlate Kit for Cry1Ab/Cry1Ac Cat # AP003CRBS). The kit does not include a reference standard with a known concentration. The package insert advises that if the kit is to be used for quantification purposes, a reference standard should be obtained elsewhere. Lyophilised, activated Cry1Ab toxin prepared from Cry1Ab protoxin was acquired from Marianne Pusztai-Carey at the Department of Biochemistry, Case Western University, Cleveland, Ohio, United States of America (USA) (Pusztai-Carey *et al*., 1994). The lyophilised protein was re-suspended in 10 mM CAPS buffer at pH 10.5 at a concentration of 100 µg/mL and frozen at -80°C until use (Tank *et al*., 2010)***.*** The quantification of the Cry1Ab protein was determined by including two independent twelve-point standard curves ranging from 0–3.5 µg/L. The samples, blanks and calibrators (Cry1Ab) were loaded in triplicate on the 96-well-microtitre plate pre-coated with antibodies specific for Cry1Ab/Ac and containing Cry1Ab/Ac enzyme conjugate. The plates were left to incubate for 2 h and washed four times with 300 µL wash buffer. A substrate was then added, resulting in the formation of a blue colour produced by the hydrolysis of hydrogen peroxide by peroxidase. After 20 min, the stop solution containing 1 N HCl was added and the optical density (ODs) was measured at 450 nm and 650 nm (reference) using a multi-mode microplate reader (Berthold TriStar LB 941, Germany) (Strain, Whiting & Lydy, 2014).

Glyphosate

Glyphosate was quantified by use of the Abraxis ELISA kit (PN 500086) (Warminster, PA, USA). The method was performed according to the manufacturer's instructions. A six-point calibration curve that ranged from 0–4 µg/L was used to quantify the levels of glyphosate in the sample. In short, the samples, blanks and standards were derivatised and loaded into a 96-well plate coated with antibodies. A glyphosate antibody solution was added and incubated for 30 min. After incubation, the enzyme conjugate solution was added and the second incubation time was 60 min. Thereafter, the plate was washed three times with 250 µL wash buffer. A colour solution was added and after 30 min incubation, the stop solution was added. Absorbance was measured at 450 nm (Mörtl *et al*., 2013) (Szekacs, Mortl & Darvas, 2015).

2,4-D

To determine the levels of 2,4-D in the surface water an ELISA, specifically for 2,4-D (PN 54003A, Abraxis, Warminster, PA, USA) was employed. The 7-point calibration curved ranged from 0–80 µg/L. The water samples, standards and blanks were added to the test strips. The enzyme conjugate and antibody solution followed shortly after and the plate was incubated for 60 min. After the incubation period, the plates were washed three times using 250 µL of the wash buffer. After the washing step, a colour substrate was added and incubated for 30 min. The last step was to add a stop solution and read absorbance at 450 nm.

Quality control

All samples were quantified in triplicate using ELISAs specific for each target compound. The mean absorbance values were calculated and the coefficient of variation (CV) was determined for each sample, requiring a CV < 20%. The limit of detection (LOD) and limit of quantification (LOQ) were determined using a regression analysis of the calibration curves where LOD = $3S_b/b$ and LOQ = $10S_b/b$ with S_b = slope uncertainty and b = slope (Schoeman *et al*., 2015). The concentrations of glyphosate, 2,4-D and Cry1Ab were determined against the linear regression line of the calibration curve, with a correlation coefficient (R^2) as close as possible to 1.

Results and discussion

Quality control and quality assurance of ELISAs

Each water sample obtained from the 2 different farms, sampled over a maize growing season was subjected to ELISA plates in triplicate along with a blank and standards to obtain calibration curves. The CVs calculated for each sample, across the glyphosate, 2,4-D and Cry1Ab plates were deemed acceptable with good precision < 20%. The LODs and LOQs were determined for each target compound from the various ELISA plate tests (Table 1.1).

Table 1:1 The LOD and LOQ values for each of the target compounds

	2,4-D (µg/L)	Glyphosate (µg/L)	Cry1Ab (µg/L)
LOD (µg/L)	0.2	0.2	0.1
LOQ (µg/L)	0.7	0.4	0.5

LOD: Limit of detection; LOQ: limit of quantification

Levels of glyphosate, 2,4-D and Cry1Ab in water sources

Cry1Ab

The water samples tested for glyphosate and 2,4-D were analysed as is. There were no concentration or clean-up steps included. In contrast to this, the Cry1Ab samples were concentrated by the aid of an Amicon® Ultra centrifugation device designed to concentrate proteins with a 30 000 molecular mass cut-off membrane. Although the water samples were concentrated 30 times for the detection of Cry1Ab, there were still no levels of Cry1Ab above the LOD in any of the water sources analysed.

It is well-known that Cry1Ab proteins degrade quickly in water sources, and this was corroborated by the results of the current study (Table 1.2). In contrast to our results, Tank *et al*. (2010) detected Cry1Ab proteins in 23% of 215 water samples taken from streams near agricultural fields six months after harvest with a mean concentration of 14 ng/L and a maximum concentration of 32 ng/L. Whiting *et al*. (2014) detected no Cry1Ab in groundwater samples, but concentrations of 129 ng/L in run-off water between maize fields. The same research group also analysed soil and run-off sediment, but in contrast to the high levels in water, the maximum concentration of 9 ng/g, was detected during the pollination stage of the maize plants. Cry1Ab levels were detected in run-off water from a non-Bt field with levels from <ND–42 ng/L with the *Bt* field having higher levels with a maximum concentration of 130 ng/L (Strain & Lydy, 2015). The presence of Cry1Ab proteins in water, although at low levels, highlights the importance to investigate the potential long-term effects that these proteins might have on non-target organisms.

Glyphosate

The levels of glyphosate were below the LOD at most of the sites (Table 1.2). The water sampled from the dam (B3) on farm B had traces of glyphosate with levels between LOD and LOQ after the spraying event. Glyphosate levels of 0.42 µg/L were detected at the in-flow dam on farm B (B2) also after the spraying event. These levels decreased to <LOD at the end of the season (Table 1.2). Glyphosate is very water soluble and has been found in various water sources around the world, but it also degrades quickly which can be the reason for not detecting it. Some studies use the concentrations of aminomethylphosphonic acid (AMPA), the main metabolite of glyphosate, to explain the <LOD results and indicate that glyphosate degraded to AMPA. AMPA was excluded from this study because it is also formed by the degradation of phosphonic acids found in some household and industrial detergents and cleaning products (Battaglin *et al*., 2014). The levels of glyphosate are also highly influenced by precipitation and can change from year to year.

In contrast to the current study, studies from all over the world detected glyphosate in water sources. Sanchís *et al*. (2012), analysed 140 groundwater samples from Spain and found quantifiable levels for 41% of the samples. The mean concentration of glyphosate in the Spain study was 200 ng/L and the maximum concentration was 2.5 µg/L. Glyphosate concentrations of 663 ng/L were found in the Nottawasaga River watershed, in Canada (Van Stempvoort *et al*., 2016). According to Smith *et al*. (1996), 45 µg/L of glyphosate was detected in well water at the Massey Drive substation in the USA seven weeks after spraying. This station is built on a limestone bed that has high permeability emphasizing the fact that glyphosate is very mobile in water sources. In the USA, glyphosate has been detected in a stream and wastewater treatment plant (WWTP) effluent samples in a study by Kolpin *et al*. (2006). The maximum concentration they reported was 2.2 µg/L. Also in the USA, a very extensive study by Battaglin *et al*. (2014) reported glyphosate levels for different environmental matrices: 73 µg/L in streams; 2.03 µg/L in groundwater; 427 µg/L in ditches and drains; 3.08 µg/L in large rivers; 1 µg/L in soil water; 301 µg/L in wetlands, lakes, and ponds; 2.5 µg/L in precipitation; 476 µg/L in soil and sediment; and 0.3 µg/L in WWTP outfall. It is evident that glyphosate ends up in water sources.

2,4-D

According to Wilson *et al*. (1997) 2,4-D amine salts and 2,4-D esters are very mobile, but they are not persistent under most environmental conditions. 2,4-D does not adsorb to the soil but rather moves readily into water resources.

Most of the samples in the current study contained quantifiable levels of 2,4-D with a minimum of 0.72 µg/L and the maximum 1.08 µg/L. Before planting, the concentrations of 2,4-D were

below the LOD in both river samples and the dam at farm A. It was also detected at low quantifiable levels before planting at both dams from farm B. The highest concentrations were detected after the spraying event and decreasing towards the end of the season (Table 1.2).

Hernandez *et al*. (2011) detected 0.05 µg/L 2,4-D in Lake Chapala, Mexico which is an order of magnitude lower than the levels found in the current study. The concentrations of 2,4-D found in our study are in the same range than two studies from Europe: Rodil *et al*. (2012), detected levels of 0.062–0.2 µg/L 2,4-D in drinking and surface water in Spain and Tsaboula *et al*. (2016) reported of 1.16 µg/L in the Pinios River Basin, Greece. A few USA studies by Serrano & DeLorenzo (2008), Ensminger *et al*. (2013), and Wijnja *et al*. (2014), reported 2,4-D levels from Charleston in surface water, urban runoff, a freshwater pond and Kushiwah Creek from 0.1–11.5 µg/L. Rodil *et al*. (2012) published on 2,4-D detected in drinking and surface water in Spain at concentrations ranging between 62 and 207 ng/L. The estimated current environmental concentrations of 2,4-D in USA water sources range from 4–24 µg/L (Atamaniuk *et al*., 2013). These concentrations are much higher than the levels obtained in the current study. According to literature 91.7% of the applied 2,4-D eventually end up in water (Mountassif *et al*., 2008). This would explain the high levels detected in various countries.

The maximum residue limit (MRL) for pesticides in the Canadian drinking water guideline is 0.28 µg/mL, and 0.065 µg/mL for freshwater aquatic life. In the United States, the pesticide MRL for drinking water is 0.70 µg/mL and 0.1 ng/mL in the European Union (Rubio *et al*., 2003). The 2,4-D concentrations reported for the current study exceed the guidelines for Canadian and US drinking water.

According to Dabrowski *et al*. (2014), glyphosate-based herbicides are the most sold herbicide in South Africa with an estimated 23 million litres sold in 2012. The amounts of herbicides used in South Africa are far less than the top crop producers such as the United States, China, Brazil and Argentina (FAOSTAT, 2016).

Due to the lack of literature about Cry proteins, glyphosate and 2,4-D in South African water sources, it is assumed that these compounds have not previously been analysed for and is this the first report.

Table 1:2 Concentrations of the target compounds from various water sources after three different sampling events

		Cry1Ab (µg/L)	Glyphosate (µg/L)	2,4-D (µg/L)
Farm A				
River (A1)	Before planting	<LOD	<LOD	<LOQ
	After spraying	<LOD	<LOD	0.93 ± 0.08
	End of season	<LOD	<LOD	<LOQ
Dam (A2)	Before planting	<LOD	<LOD	<LOQ
	After spraying	<LOD	<LOD	0.72 ± 0.02
	End of season	<LOD	<LOD	0.72 ± 0.07
Farm B				
River (B1)	Before planting	<LOD	<LOD	<LOQ
	After spraying	<LOD	<LOD	1.02 ± 0.03
	End of season	<LOD	<LOD	0.96 ± 0.16
Inflow (B2)	Before planting	<LOD	<LOD	0.83 ± 0.10
	After spraying	<LOD	0.42 ± 0.04	1.08 ± 0.04
	End of season	<LOD	<LOD	0.99 ± 0.03
Dam (B3)	Before planting	<LOD	<LOD	0.74 ± 0.02
	After spraying	<LOD	<LOQ	0.90 ± 0.08
	End of season	<LOD	<LOD	0.92 ± 0.08

LOD: Limit of detection; LOQ: limit of quantification

Conclusion

Industrial agriculture increases global food production but involves the excessive use of herbicides. These compounds are developed to have such a specific mechanism of action so that they are not supposedly toxic to non-target organisms. Using less might decrease crop yield, leading to other global issues (Islam *et al*., 2017).

These target compounds are however mobile once released into the environment, and as use increases the levels in the environment will increase. Water scarcity will concentrate these compounds. Currently, research studies reveal the adverse health effects of Cry1Ab, glyphosate and 2,4-D exposure to non-target organisms, and for these reasons, water sources should be monitored to ensure safe drinking water for South African citizens. Humans, already battling with other health issues are especially at risk. To the author's knowledge, this is one of the first studies investigating the presence of Cry1Ab, glyphosate and 2,4-D concentrations in water sources in South Africa

Recommendations

From the results of this pilot study conducted over a single maize growing season it is recommended that follow-up studies be done which include more sampling locations across South Africa and perform monitoring over a longer period. It is also recommended to use ELISAs as a screening tool and confirm these results by using other analytical methods.

Acknowledgements

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References

For practical reasons the reference list for the short note was combined with the full reference list at the end of the thesis (see section 8: References).

2. LITERATURE REVIEW

2.1 Importance of maize production

The current total world production of maize is 1.07 billion metric tons (Statista, 2018) and the United States of America (USA), China, Brazil and Argentina are the top maize-producing countries (FAOSTAT, 2016). The consumption of these cereals varies widely by region with maize (also referred to as corn) as the preferred cereal in southern and eastern Africa, Central America, and Mexico.

The total maize production differs significantly between countries. The USA, China and Brazil produce 274, 208 and 71 million metric tons/year respectively. South Africa produces 12 million metric tons/year (FAOSTAT, 2016). For the last 30 years, maize in the USA has been used for human consumption, animal feed and ethanol production. As the world's biggest maize producer, the USA dominates the world maize trade. Countries such as South Africa, Ukraine, Brazil and Romania only export a significant amount of maize after big yields and when the international prices are attractive. Maize is one of the least expensive foods and food ingredients, and is also a staple food for Africa with a consumption rate of 52–328 g/person/day (Ranum, Peña-Rosas & Garcia-Casal, 2014). A wide variety of other crops are also produced in South Africa. These range from grains to sugar cane, deciduous, and citrus sub-tropical fruit (Dabrowski, Shadung & Wepener, 2014). In 2016 it was estimated that South Africa grows 2.66 million hectares of maize, soybean and cotton, an increase of 16% from 2015 (James, 2016).

2.2 The introduction of biotechnology to agriculture

2.2.1 Cry1Ab, expressed by Bt maize

Insecticidal crystalline (Cry) proteins were first discovered in 1901 and isolated from a microorganism, *Bacillus sotto,* from a diseased silkworm *(Bombyx mori)* larva (Ishiwata, 1901). When Ernst Berliner discovered a similar microorganism in a diseased Mediterranean flour moth larvae (*Anagasta kuchniella*) from grain, near the city of Thuringia, Germany, he formally named it *Bacillus thuringiensis* (Beegle & Yamamoto, 1992). *B. thuringiensis* is a gram-positive, soil-dwelling microorganism, but it is also commonly found in the gut of caterpillars of various moths and butterflies, in aquatic environments, animal faeces, flour mills and grain storage facilities (Clark, Phillips & Coats, 2005). It was discovered that a toxin in the form of bipyramidal crystals in sporulating *B. thuringiensis*, is partially responsible for its pathogenicity (Beegle & Yamamoto, 1992). The spores alone did not affect *Bombyx mori* larvae.

The discovery of the pesticidal potential of Cry proteins in the bacterium, together with the ability to create genetically modified organisms led to the rise of genetically modified (GM) crops. Maize was modified by inserting a gene into its genome that codes for crystal (Cry) proteins found in *B. thuringiensis* (Fig. 2.1A) (Clark, Phillips & Coats, 2005). There are different versions of Cry proteins that specifically target orders of insect pests for example Cry1Aa, Cry3Aa, and Cry4Ba toxins are toxic to different insect orders: Lepidopteran, Coleopteran and Dipteran respectively (Clark, Phillips & Coats, 2005). The engineered Bt maize produces Cry1A proteins that are toxic to lepidopteran orders of insects (Whalon & Wingerd, 2003).

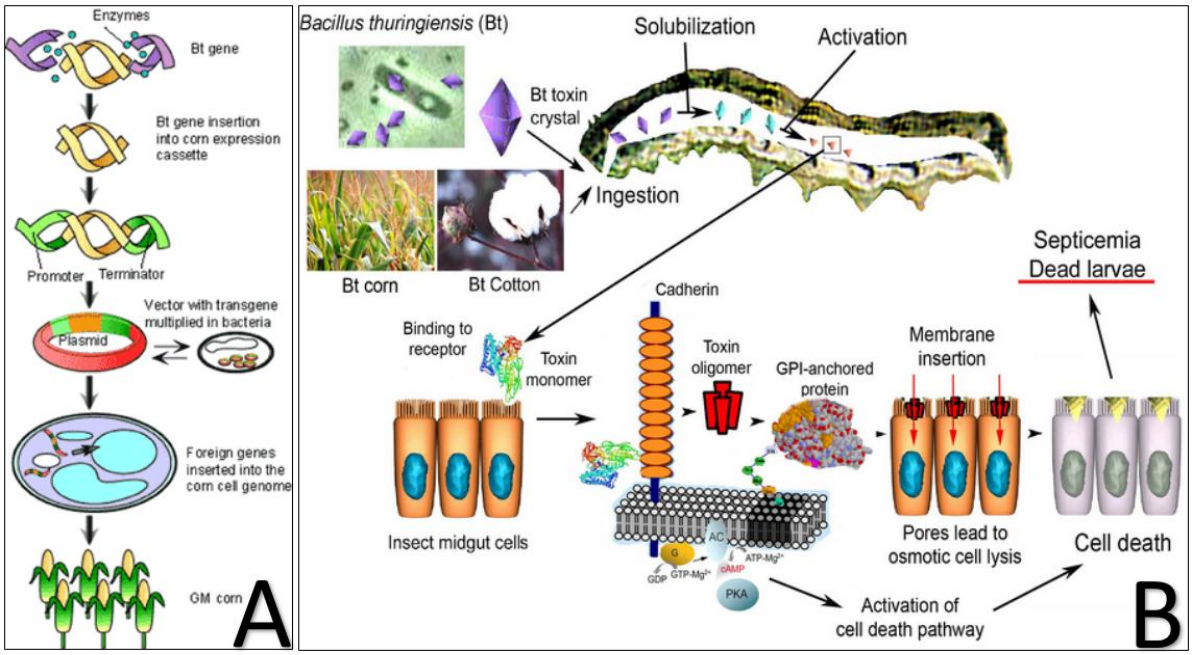

Figure 2:1 Simplified representation of A) gene insertion to create Bt maize (GM crop); B) ingestion of Cry toxins expressed by GM crops by a lepidopteran insect and the mechanism of action leading to death of the insect (Adapted from Jurat-Fuentes, 2018).

The specific mechanism of action of Cry protein toxins is still not clear. The scientific literature seems to agree that it is a multistep process, but controversy over the final step led to the rise of three different models. The initial steps are the same for these three models. The first step is the release of spores from *B. thuringiensis.* Thereafter, the Cry proteins (protoxin) need to be ingested by a susceptible host as the protoxin is not toxic in itself (Ujvary, 2001). The Cry proteins are solubilised inside the insect midgut. For lepidopteran and dipteran insects the gut is alkaline and for coleopteran insects it has a neutral/acidic environment (Ujvary, 2001). Protease enzymes cleave off portions of the N- and C-terminals, forming the activated toxin (Schnepf *et al.*, 1998) that is released into the stomach. The active toxin binds to one or more specific receptor(s) in midgut cells (Ujvary, 2001). It is after this step, that the three models deviate. In the first model, the proteins bind to primary receptors in the insect midgut, such as cadherin-like receptors (Pigott & Ellar, 2007; (Soberón, Gill & Bravo, 2009). The protein is cleaved and

undergoes oligomerisation into a tetrameric prepore, which binds to secondary receptors such as aminopeptidase A (APN) and alkaline phosphatase (ALP). The prepore inserts itself into the cell membrane creating multiple pores. This results in a loss of membrane potential and causes osmotic lysis leading to gut paralysis, feeding inhibition, starvation and sometimes septicaemia. The lysis of multiple cells results in the insect's death (Soberón, Gill & Bravo, 2009). In the second model, the Cry proteins bind to the same primary receptor, cadherin, but the binding activates a guanine nucleotide-binding protein (G-protein) (Pigott & Ellar 2007; Soberón *et al*. 2009). This triggers adenylyl cyclase that increases the cyclic AMP (cAMP) level and results in the activation of protein kinase A, leading to cytoskeleton and ion channel disruption, and ultimately death of the insect (Pigott & Ellar, 2007;Soberón, Gill & Bravo, 2009). The third model is a combination of the previous two models. This model proposes that toxicity of Cry1Ac to *Heliothis virescens* (tobacco budworm) is due to osmotic lysis and cellular signalling. Cry proteins bind to the cadherin-like protein HevCaLP, causing activation of an intracellular signalling pathway and protein oligomerisation at the same time. Both pathways are then responsible for the death of the insect (Fig 2.1B) (Pigott & Ellar, 2007) (Jurat-Fuentes & Adang, 2006).

The first transgenic crops conferring insect resistance for commercial use were approved in 1995 in the USA. The use of Bt maize has been adopted worldwide although to a lesser extent in Europe and even less so in Africa, but 81% of maize and 84% of cotton with insecticidal traits against a variety of insect pests have been planted in the USA in 2015 (United States Department of Agriculture, 2015).

2.2.1.1 Levels of Cry proteins in the environment

The adoption of Bt maize has led to increased release of Cry proteins into the environment. Several studies during the past decade have been conducted to determine the environmental fate of Cry proteins. Understanding the environmental fate is essential concerning the risks of Cry toxins to non-target organisms in environmental matrices (to be discussed in section 2.4). This section summarises the literature on the half-life and levels of Cry proteins detected in water sources and soil.

Cry proteins are expressed at various levels and stages in different GM crops. Plants express the highest concentration of Cry proteins at the seedling stage, but this concentration decreases throughout the season (Clark, Phillips & Coats, 2005). The largest amount of protein per acre was determined at anthesis (flowering period of the plant) when the plant biomass is greatest (USEPA, 2000). At the end of the season, the Cry proteins remaining in the maize debris, are

incorporated into the soil during post-harvest plant activities (Saxena, Pushalkar & Stotzky, 2010).

Various types of experiments have been performed to assess the half-life of Cry proteins in the soil. The terminologies “half-life” and “persistence” make comparisons difficult as “half-life” refers to the time it takes for 50% of the original amount to break down and “persistence” is described regarding detectable residues and bioactivity. In addition, other factors, not always mentioned, may play a role in the metabolism of Cry proteins in soil, such as environmental conditions, soil type, protein source and the specific Cry protein examined (Clark, Phillips & Coats, 2005). Sims & Holden (1996) reported Cry1Ab half-life of only 1.6 days in the soil. Palm & Seidler (1996) conducted microcosm experiments by incorporating pure cry toxins and maize leaves into different types of soil (fine sandy, coarse sandy and silt loam), as well as sterile and non-sterile versions of soil and found that Cry1Ab has a half-life of 2.2, 22, 30, 40, and 46 days under the different experimental conditions. The differences in half-lives published for Cry1Ab, are due to the fact that these proteins are sensitive to certain factors: they degrade when exposed to conditions such as a change in pH and high temperatures. The type of soil influences their adsorption, making them more persistent, but also decreasing their extractability. Other factors influencing the adsorption, transport, and activity of transgenic Cry proteins in agricultural soils were discussed by Madliger, Sander and Schwarzenbach (2010. Sander et al. (2010) concluded that: the electrostatic interactions control the adsorption of Cry1Ab to charged, polar surfaces; that the sum of electrostatic and Van der Waals interaction of Cry1Ab to negatively charged surfaces is weak at pH > 5 and constant ionic strength of 50 mM and causes reversible adsorption, and thirdly, that Cry1Ab has high conformational stability. Another discovery by this group, is that Cry1Ab retains its activity when absorbed to polar, charged surfaces in soils, which is important when assessing its potential adverse effects in agricultural systems (Madliger et al., 2011).

Zwahlen *et al*. (2003) detected 38% of the start-concentration of Cry1Ab after 40 days, and 20% after 60 days, but there were still Cry protein trace levels present the following spring. Baumgarte & Tebbe (2005) reported detectable levels of Cry1Ab in soil ranging from 0.1 to 10 ng/g. Levels of Cry1Ab protein (0.21 ng/g) was also detected in the soil seven months after harvest.

In the GM plant material, the Cry levels are higher: Cry levels were found in residues of leaves at 21 ng/g and in the roots at 183 ng/g (Baumgarte & Tebbe, 2005). Hopkins & Gregorich (2005) found t that Cry1Ab declines in fresh plant material from 6.8 µg/g to 0.82 µg/g, and reduces further to 0.026 µg/g in the soil. Gruber *et al*. (2012) determined the levels of Cry1Ab in

soil from four fields that had been cultivated for nine consecutive years with GM maize and only found levels above their (relatively high) detection limit (2.0 ng Cry1Ab protein/g) in soil after 6 weeks of harvest at one site with no detections in any of the other samples from the three remaining fields. In a study by Wang *et al*. (2006) at the Agricultural Experiment Station next to the Zhejiang University, China, the Cry1Ab protein in the shoots and roots of Bt transgenic rice was 3.23 and 0.68 mg/g (fresh mass), respectively. The half-lives of the Cry1Ab protein in the soils were 11.5–34.3 d. A laboratory investigation revealed the rapid dissociation of Cry1Ab and Cry1Ac from two different types of soil: sandy and sandy clay loam soil. After 35 days more than 80% of the Cry proteins from both soils were undetectable (Marchetti *et al*., 2007). Another study investigated the Cry1Ab protein accumulation from three different soils containing MON810 plants in pots under greenhouse conditions. After incorporating the plant biomass into the pots, the concentration of Cry1Ab declined steadily and did not persist or accumulate in the soil (Badea, Chelu & Lacatusu, 2010). Despite the short half-lives indicated by some of the studies, others report that a more substantial portion of the active protein is still present for a longer period, due to Cry1Ab's ability to adsorb to clay particles. This adsorption to clay reduces the bioavailability of the protein for microbial degradation, and these proteins, therefore, persist longer in the environment, retain their insecticidal activity and are still bioavailable to non-target organisms (Tapp & Stotzky, 1998).

Cry proteins end up in water sources through pollen deposition, are exuded from plant roots and ultimately in run-off to rivers and stream (Saxena & Stotzky, 2001). In a laboratory-based study, Douville *et al*. (2005) found that pure Cry1Ab degrades faster in water than soil with half-lives of four and nine days respectively. A survey conducted by Tank *et al*. (2010) detected Cry1Ab proteins in 23% of 215 water samples taken from streams near agricultural fields six months after harvest with a mean concentration of 14 ng/L and a maximum concentration of 32 ng/L. Whiting *et al*. (2014) investigated the levels of Cry1Ab in water samples. There were no Cry1Ab detected in the groundwater samples, but they found levels of 129 ng/L in run-off water. Several run-off samples contained Cry1Ab, indicating movement of Cry proteins from the field to aquatic ecosystems. This research group also analysed soil and run-off sediment, but in contrast to the high levels in water, the maximum concentration of 9 ng/g, was detected in the soil during pollination. Cry1Ab levels were detected in run-off from a non-Bt field at levels from below limit of detection (<LOD)–42 ng/L, i.e. lower than the levels in run-off water from the Bt field with a maximum concentration of 130 ng/L (Strain & Lydy, 2015).

The persistence of Cry1Ab protein for days in both soil and water, although at low-levels, highlights the importance to investigate the potential long-term effects that these proteins might have on non-target organisms.

2.2.2 Glyphosate (the active ingredient in Roundup®)

Glyphosate [N-(phosphonomethyl) glycine] was discovered by Dr Henri Martin, a Swiss chemist, in 1950. It was ultimately Dr John Franz, a Monsanto chemist, that identified the herbicidal activity of glyphosate in 1970. Glyphosate is active as a salt with various cations (Fig. 2.2) (e.g. the sodium or iso-propylamine salts).

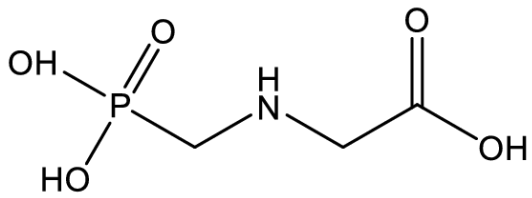

Figure 2:2 Chemical structure of glyphosate

The iso-propylamine salt of glyphosate was formulated, together with adjuvants, into the product Roundup®, and was first sold commercially by Monsanto in 1974 as a post-emergent, non-selective herbicide (Battaglin *et al*., 2009). Glyphosate's working mechanism is to inhibit the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) of the shikimate pathway (Fig. 2.3). The shikimate (shikimic acid) pathway is a multi-step metabolic process in plants, bacteria, fungi, algae, some protozoan parasites and is responsible for the biosynthesis of folates and aromatic amino acids (phenylalanine, tyrosine, and tryptophan) (Vivancos *et al*., 2011). Humans and animals lack the shikimate pathway. The exact mechanism of how glyphosate inhibits the shikimate pathways and kills the plant is not fully understood. The inhibition may cause insufficient amino acid production which is needed for protein synthesis. It has also been proposed that the increased carbon flow to the shikimate pathway due to deregulation of the pathway, results in carbon shortages at other essential pathways (Duke & Powles, 2008). Glyphosate appears to inhibit the EPSPS of all higher plants making it a non-selective herbicide to be used on an extensive range of plant species. Glyphosate is unique and no other alternative chemical targeting this enzyme has been commercialised (Duke & Powles, 2008).

The use of Roundup® steadily grew following its commercialisation, and it was sprayed in non-crop situations as well, to kill all vegetation (Benbrook, 2016). Glyphosate, the active ingredient in Roundup®, is a slow-acting herbicide which readily translocates to meristems distant from the treated foliage. This makes it an excellent weed-killer by only applying a small percentage on the weed's foliage. Glyphosate is also much more effective than other herbicides, for example, paraquat, by preventing re-growth from meristems. Although glyphosate has many advantages, its high level of phytotoxicity to crops, limited its use.

To combat this limitation, adjustments to the use of glyphosate were made. Application devices such as shielded sprayers and rub-on applicators were used to apply glyphosate as spot treatments to weeds and avoid contact with the crops. However, because of glyphosate's highly systematic properties, the risk of drift or direct application can result in damaging or killing the crop. This problem was solved by making the crops resistant to glyphosate via genetic modification (Benbrook, 2016).

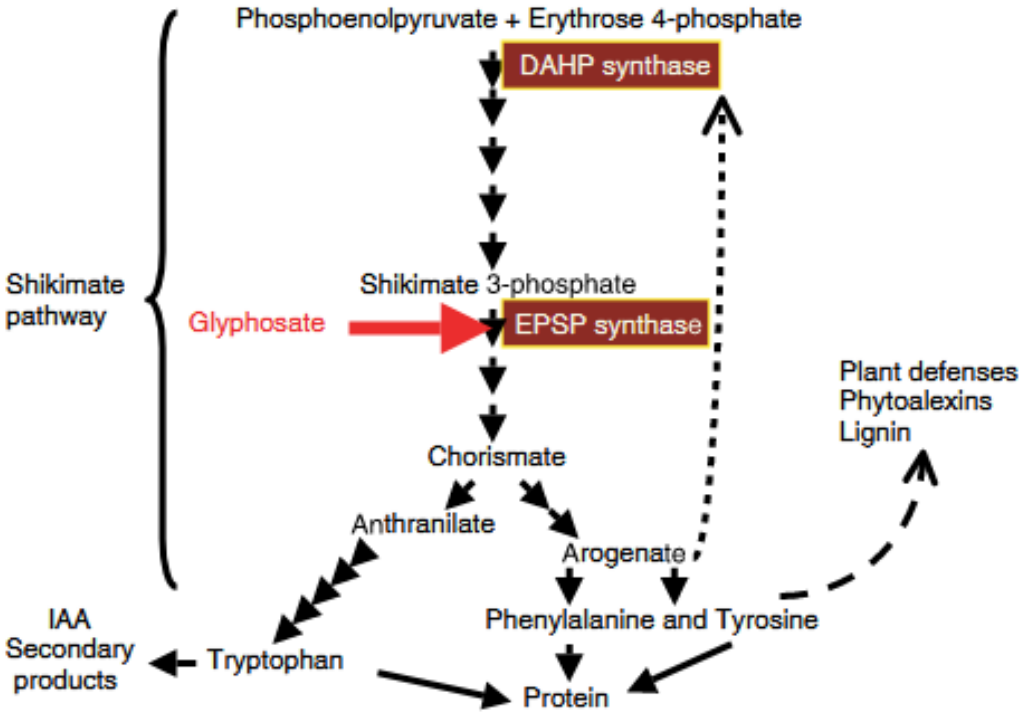

Figure 2:3 The shikimate pathway indicating the site that glyphosate inhibits. The dotted line indicates the product's pathway and regulatory feedback inhibition. (DAHP:3-deoxy-d-arabino-heptulosonate 7-phosphate; EPSP: enzyme 5-enolpyruvyl-shikimate-3-phosphate) (Duke & Powles, 2008).

2.2.2.1 Roundup® Ready Maize

In 1996, genetically engineered herbicide-tolerant soybean, maize, and cotton varieties, known as Roundup® Ready (RR), were approved in the USA (Benbrook, 2016). These crops were made resistant to glyphosate by introducing a homologue of the EPSPS, isolated from *Agrobacterium sp.,* which enables the transgenic enzyme to still function in the presence of (even high levels) of glyphosate and still allow the shikimate pathway to maintain the normal aromatic amino acid levels (Nandula *et al*., 2005; Dill, CaJacob & Padgette, 2008). Making crops resistant to glyphosate has made it a broadcast, post-emergent herbicide, thereby dramatically extending the period during which farmers can apply glyphosate-based herbicides (GBH) to eliminate weeds, and cause negligible crop damage (Duke & Powles, 2008). The use

and average concentration of the glyphosate applied has also increased drastically. Between 2005 and 2011 the total use of glyphosate increased with 12.9% per year (Benbrook, 2016). Of all the herbicide-resistant crops cultivated worldwide, 90% is glyphosate-resistant.

2.2.2.2 Levels of glyphosate in the environment

Glyphosate is a polar, non-volatile and amphoteric compound that binds strongly to soil, but is also highly water soluble (more than 10 g/L at 25°C). It also contains various active groups (phosphonate and carboxylate groups) which have a high affinity for metals (Eker *et al*., 2006). The parent compound of glyphosate undergoes little or no metabolism in most plants, and it is readily translocated into metabolic sinks including plant roots. It is eventually released into the rhizosphere, likely via a diffusion process along with sugars, amino acids, and other low molecular mass compounds (Kremer *et al*., 2005). Glyphosate is claimed to be environmentally benign because it tightly bounds to soil making it immobile with no soil activity and limited persistence (Reddy, 2001), however concerns whether this statement holds, will be discussed in the next section.

One of the significant glyphosate metabolites is alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) which is formed by microbial processes. AMPA is also highly water soluble and degrades more slowly than glyphosate, but AMPA is also formed by the degradation of phosphonic acids found in some household and industrial detergents and cleaning products (Battaglin *et al.*, 2014; Van Stempvoort *et al.*, 2016). AMPA ends up in water resources through the outfall of wastewater treatment plants (WWTPs) (Kolpin *et al*., 2006) and was therefore excluded as an indication of glyphosate contamination from this study.

In studies done between 1990 and 1992, it was found that the herbicide concentrations were the highest during the first run-off event after herbicide application. Lower levels of the run-off were detected for several weeks to months following the application. By late summer the herbicide concentrations are generally low, at less than 0.05 µg/L until the following planting season (Battaglin *et al*., 2003). In 1990, the half-life of glyphosate was reported to be 45–60 days in soil, with residues being below 6–18% of the initial glyphosate after 360 days (Feng & Thompson, 1990). It is estimated that up to 10% of the applied glyphosate can move to non-target plants (Eker *et al*., 2006). Different studies reported the half-life of glyphosate ranges from weeks to years (Nomura & Hilton, 1977; Feng & Thompson, 1990; Eker *et al*., 2006). And Roundup® Ready soybeans may still have high levels of glyphosate residues after two years in storage (Cuhra, Bohn & Cuhra, 2016).

Others report that glyphosate has a short environmental half-life due to microbial degradation (Duke & Powles, 2008). The half-life of glyphosate in soil and aquatic sources is 2–215 days and 2–91 days respectively (Grunewald *et al.*, 2001; Vera *et al.*, 2010; Battaglin *et al.*, 2014). The half-life of glyphosate in Norwegian soil at temperatures varying between −5°C and +5°C was estimated between 15–28 months (Laitinen *et al*., 2009). Laitinen *et al*. (2009) only found 14% of glyphosate in the topsoil surface 30–50 min after application. This was ascribed to movement along plant roots, and not leaching or colloidal transport since there was no water flow and no wind for spray drift to occur. After 38 days 25% glyphosate was left in the soil with the roots containing concentrations more than an order of magnitude higher than in the soil samples. Laitinen *et al*. (2006) concluded that the dissipation of glyphosate from the soil in their study was 11 months. This was a sandy loam site that had received glyphosate 1.5 years before their study. Glyphosate was detected along the French Atlantic coast at a peak concentration of 1.2 µg/L (Burgeot *et al*., 2007). A study by Skeff *et al*. (2015) reported concentrations of 0.028–1.7 µg/L in the water from German Baltic estuaries.

Glyphosate was also found in groundwater. In a study by Sanchís *et al*. (2012), 140 groundwater samples from Spain were analysed for glyphosate. The results reflected quantifiable levels for 41% of the samples with the maximum concentration of 2.5 µg/L and a mean concentration of 200 ng/L. Van Stempvoort *et al*. (2016) reported on glyphosate concentrations of 663 ng/L in rural groundwater in the Nottawasaga River watershed, Canada. According to Smith *et al*. (1996) 0.045 mg/L glyphosate was detected in well water seven weeks after spraying. This well is located at the Massey Drive substation, Newfoundland, Canada, where maintenance of the electric substation involves the management of vegetation by spraying Roundup®. This station is built on a limestone bed that is highly permeable and could allow contaminants sprayed on the weeds to move from the surface to groundwater. Börjesson & Torstensson (2000) conducted a study in Sweden where different concentrations of Roundup® were applied to railway weed and found levels of glyphosate in the soil ranging from <LOD to 2.7 mg/kg after 105 days. They also sampled groundwater along the railway and found levels ranging from 0.12 to 1.42 µg/L.

In the USA, glyphosate has been detected in a stream and WWTP effluent in a study by Kolpin *et al*. (2006) at a maximum concentration of 2.2 µg/L. A very extensive study conducted by Battaglin *et al*. (2014), also conducted in the USA, measured glyphosate levels in different environmental matrices and reported the following maximum levels per site type: streams: 73 µg/L; groundwater: 2.03 µg/L; ditches and drains: 427 µg/L; large rivers: 3.08 µg/L; soil water: 1 µg/L; lakes, ponds, and wetlands: 301 µg/L; precipitation: 2.5 µg/L; soil and sediment: 476 µg/L, and WWTP outfall: 0.3 µg/L.

2.2.2.2 Glyphosate-resistant weeds

Glyphosate is the most used herbicide in the world (Duke, 2018). The increased use of glyphosate and lack of effective weed management have caused weeds to become resistant to glyphosate. The existence of glyphosate-tolerant weeds has been reported in different countries, and their emergence is attributed to the overuse of glyphosate due to RR crops being resistant to glyphosate. Before the introduction of RR crops, there were no known glyphosate-tolerant weeds, and it was said that tolerance was unlikely to ever develop (Bradshaw *et al.*, 1997; Benbrook, 2012). The number of glyphosate-tolerant weeds across the world increased to 42 species since 2000 (Heap, 2018), which reduced the efficiency and sustainability of glyphosate (Salas *et al*., 2012). A few mechanisms that cause plants to develop resistance include reduced translocation of the active ingredient in the plant, reduced herbicide absorption by the crops, and alternations in the binding site of glyphosate (Nandula *et al.*, 2005; Beckie, 2011). It was also proposed that some weed species' tolerance development are due to higher amplification, expression and enzyme activity of the EPSPS (Salas *et al*., 2012).

To address this increasing tolerance of weeds to glyphosate, i.e. resistance evolution, integrated weed management is now implemented in which multiple types of herbicides are applied, especially herbicides with other mechanisms of action. An example of such a herbicide is 2,4-D, which kills broadleaf weeds.

2.2.3 2,4-dichlorophenoxyacetic acid

The herbicide, 2,4-D (Fig. 2.4) had been used for agricultural purposes for decades and is still extensively used in weed control programs around the world. Its use has increased over the last few years and it was therefore chosen to be included in the current study as one of the chemicals present in relevant environmental mixtures.

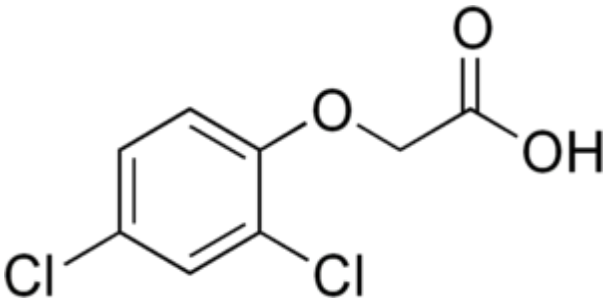

Figure 2:4 Chemical structure of 2,4-D.

2,4-D was discovered by a series of experiments by British and American researchers in 1940. The commercialisation of 2,4-D in 1945 revolutionised weed control. Other phenoxy-carboxylic acids herbicides such as MCPA,2,4,5-T, 2,4-DB, and dichlorprop were developed in the same

time frame, but 2,4-D has been the most widely used of the chlorophenoxy chemical family. Pure 2,4-D is a non-volatile, dry crystalline solid and only slightly soluble in water (44 g/L) (Gervais *et al*., 2008). For this reason, 2,4-D should be chemically modified into a formulation to form a suitable mixture with water. There are three types of formulations containing 2,4-D on the market, i.e. amine salts, esters and choline (Peterson *et al*., 2016). These different types of formulations vary in chemical properties, environmental behaviour, and toxicity (Islam *et al*., 2017).

2,4-D is a post-emergent herbicide used for selective control of broadleaf weeds. It is a synthetic, auxin herbicide with a structure similar to, and thus mimicking, the natural auxin indole-3-acetic acid in plants. Auxins are natural hormones which promote plant growth at low levels, but at high doses (such as the application of 2,4-D) it causes plant overgrowth which is uncontrollable and unsustainable, resulting in plant death (Song, 2014). From the long list of pesticides contaminating the aquatic ecosystems, the phenoxyacetic herbicides (which includes 2,4-D) is one of the largest groups of herbicides used worldwide. 2,4-D is found in more than 600 products on the market, and as a result of the intensive agricultural use of 2,4-D, it is estimated that high levels of 2,4-D are present in environmental water sources (Kubrak *et al*., 2013), which will be discussed in the next section.

2.2.3.1 Levels of 2,4-D in the environment

2,4-D is a polar molecule and the ester and amine versions of this herbicide are very mobile in water. Due to its low soil adsorption and the high water solubility of 2,4-D formulations, 2,4-D free acid is widely present in the environment. Although 2,4-D has other derivatives, the free acid is detected and monitored in water sources and its half-life of between 20 and 312 days is highly dependent on the environmental conditions. It is estimated that the highest levels of 2,4-D in water bodies occur during the planting period when farmers intensively apply herbicides for weed control. Adding adjuvants to 2,4-D are generally not recommended, but when 2,4-D is present in pesticide tank mixtures there will be adjuvants present that modifies some properties of the spraying solution to enhance its effectivity for penetration of the plant cells to reach the target site. These adjuvants also include surfactants that are responsible for lowering the surface tension between a liquid and a solid surface, to improve spreading droplet size and also penetration (Peterson *et al*., 2016).

2,4-D is relatively rapidly degraded by microbial action in soils in the ecosystem. It should be kept in mind that any fluctuation of the soil microbial system will influence the degradation and movement of 2,4-D (Islam *et al*., 2017).

Results from field studies found that 2,4-D only had a soil half-life of 1.7 to 13.1 days. The soil moisture content has a significant effect on the half-life, and the dissipation routes of 2,4-D appear to be oxidative microbial mediated mineralisation, photodegradation in water, and leaching of 2,4-D. Although 2,4-D amine salts and 2,4-D esters are very mobile, they are not persistent under most environmental conditions (Wilson, Geronimo & Armbruster, 1997).

In corroboration with Wilson *et al*. (1997), other authors reported that 2,4-D has a short half-life and is immobile in soil. A study in Canada showed no accumulative effect in soil over decades (Smith, 1988). Gupta *et al*. (2012) tested three plots using different irrigation methods. Plot 1 received 184 mm, plot 2 received 94 mm and plot 3 received 16 mm total irrigation after herbicide application. They concluded that 2,4-D degraded 90%, 70% and 60% within 40 days on plot 1, 2 and 3, respectively, indicating the influence of irrigation in the degradation or movement of 2,4-D.

Laboratory studies found that 2,4-D is potentially mobile, but rapid degradation in the soil and removal of this compound by soil uptake reduce leaching in the field. In the USA the estimated current environmental concentrations of 2,4-D in water sources range from 4–24 µg/L, and in agricultural fields it reaches a concentration of up to 4000 µg/L (Borges *et al.*, 2004; Atamaniuk *et al.*, 2013). 2,4-D has been detected in the effluents of a WWTP at concentrations ranging from 83–110 ng/L (Cardenas *et al*., 2016).

In a study by Rodil *et al*. (2012), 2,4-D was detected in drinking and surface water in Spain at concentrations ranging between 62 and 207 ng/L. A few studies, Serrano & DeLorenzo (2008), Ensminger *et al*. (2013), and Wijnja, Doherty & Safie, (2014) in the USA reported on 2,4-D levels in surface water, urban run-off, a freshwater pond and Kushiwah Creek, Charleston, ranging from 0.1–11.5 µg/L. Hernandez *et al*. (2011) detected 0.05 µg/L 2,4-D in Lake Chapala, Mexico, and Tsaboula *et al*. (2016) reported 1.16 µg/L in the Pinios River Basin, Greece.

In a multiple compound analysis study of river-, effluent- and drinking water from Spain, Kuster *et al*. (2008) found detectable levels of 2,4-D with a maximum concentration of 109 ng/L. Municipal wastewater in Australia contained 3 µg/L of 2,4-D (Cardenas *et al*., 2016). According to literature, 91.7% of all applied 2,4-D eventually end up in water (Mountassif *et al*., 2008).

2.2.4 Herbicide-resistant weeds in South African agriculture

The preceding section highlighted the presence of the afore mentioned herbicide residues in the environment worldwide, but there is no infomation available on their levels for South Africa in spite of their wide use in South Africa. The over-use of these compounds lead to development of glyphosate-tolerant weeds: rigid ryegrass (*Lolium rigidum*) (resistance reported in 2001); hairy fleabane (*Conyza bonariensis*) (reported in 2003) and buckhorn plantain (*Plantago lanceolata*) (reported in 2003) (Nandula et al., 2005) (Heap & Duke, 2018). There are no official reports on the weeds resistant to 2,4-D in South Africa, except for a unpublished note that wild radish (*Raphanus raphanistrum*) are resistant to synthetic auxins (such as 2,4-D) (Pieterse, 2010).

Successful weed management is an essential agronomic practice as weeds can reduce crop yield up to 90%. Weed infestations can cause yield losses due to weeds that interfere with the crop's growth and development. These mechanisms of action include competition for nutrients, water and light, and allelopathy, through phytotoxic chemicals (allelochemicals) which weeds release into the environment. In southern Africa, weed management consist of labour intensive use of the hand hoe or use of herbicides (Thierfelder et al., 2018). Development of weed-resistance often leads to new herbicide combinations or increased used of previously effective herbicides. This in not only more expensive but more detrimental to the environment (Pieterse, 2010).

2.3 GM crops and herbicides

Genetically modified (GM) crops were developed to improve agricultural practices by providing more cost effective and easier weed control for farmers, which in turn increases the crop yield (Brookes & Barfoot, 2018). The worldwide use of transgenic crops increased 100-fold from 1.7 million hectares (ha) in 1996 to 170 million ha in 2012 (James, 2012). Although GM maize was originally banned from European and African countries, importing it is now accepted. In 2012, the European Union imported 30 million tons of GM crops that were mostly used to feed livestock, as humans reject the use of GM crops and the food chain is GM-free (Twardowski & Małyska, 2015).

For a country like South Africa, using and living off GM foods, credible biosafety systems should be in place and decisions about GM crops should be based on scientific evidence of risks, costs and benefits of these technologies (Gouse *et al*., 2005). Farmers who plant Bt maize also use pesticides with different mechanisms of action such as Roundup® and 2,4-D to reduce the evolution of any pesticide resistant species (Petzold-Maxwell *et al*., 2013). South Africa is the

biggest user of pesticides in sub-Saharan Africa due to the vast amount of agriculture in the country. There are over 500 active ingredients registered for use. The high usage of herbicides causes pollution of neighbouring environments as most of these compounds are water soluble, mobile, and can accumulate somewhere else (Chang, Simcik & Capel, 2011).

Glyphosate is highly water soluble and may therefore leach through surface run-off into water bodies. Although 2,4-D is sparingly soluble in water, this compound is weakly absorbed by soil, and therefore it prefers to rather move to surface and groundwater where it is detected (section 2.2.3). Another concurrent environmental contaminant is Cry1Ab that enters water systems through many possible routes, but its concentration rapidly decreases in water. It is however released into soils where it has a high binding affinity for humic acids and clay, but not sand which is the most preferred soil for agricultural activities (Du Plessis, 2003).

The previous sections confirmed the presence of Cry1Ab, glyphosate and 2,4-D in the environment. This contamination may potentially harm exposed non-target vegetation and wildlife. In addition, the herbicides that are also carried by run-off into the local river systems may affect the health of aquatic organisms. Industrialised agriculture is aimed at improving global food production, but is largely based on excessive use of herbicides. Unfortunately, it may be difficult to decrease the use of these agrochemicals without reducing crop yields. Therefore, any unintended effects of these agrochemicals, or mixtures of these, should be investigated and evaluated. Food, feed and foodstuffs are contaminated by pesticides (Bai & Ogbourne, 2016), but this study was about the contaminated environmental matrices to which humans and animals are exposed to.

2.4 Side effects of these compounds

Herbicides were developed to target weeds, but broad-spectrum herbicides, detected in the environment may have adverse effects on non-target organisms, especially long-term effects resulting from chronic exposure to low concentrations of chemicals. In recent years, there has been increased concern regarding the safety of agricultural toxicants because there are many conflicting results reported in the scientific literature, about Cry proteins, glyphosate/Roundup® as well as 2,4-D.

2.4.1 Cry1Ab

There is a large volume of literature on the effects of Cry proteins on certain insect species and microorganisms. In this section, I only highlight and mention a few examples of the studies done in the past few years.

When registering Bt maize traits as plant protection products, companies are required to conduct several tiers of toxicity testing to non-target species and submit these results to regulatory agencies that evaluate the data make decisions based on the results obtained. This data is available to the public. Both the bacterium as well as the plant material expressing the Cry genes were tested on humans, rats, mice, cattle, swine, northern bobwhite quail, mallard duck, sheepshead minnow, bluegill sunfish, rainbow trout, catfish, daphnia, grass shrimp, and copepods. No adverse effects have been found in these acute toxicity tests (OECD, 2007). These results are conflicting, as effects have been observed (discussed in next sections) before and after 2007.

The effects on insect predators were investigated because of their essential role in agroecosystems. The various species were exposed to a broad spectrum of Cry1Ab concentrations where diets were fortified with Cry proteins. Moser, Harwood and Obrycki (2008) found that exposure to Cry1Ab from Bt maize causes a delay in development of juveniles of *Coleomegilla maculate* (ladybird beetles). Limited studies have been done on the effect of Bt proteins on parasitoid wasps that feed on herbivorous hosts that are dependent on transgenic plants. The United States Environment Protection Agency (USEPA) reported on studies that showed no adverse effects (USEPA, 2000) however, this was not the case for other studies which found that parasitoids were susceptible to Cry1Ab (Lövei, Andow & Arpaia, 2009). Although Cry1Ab has a specific action to control lepidopteran insects, controversial results showed mortality to juveniles in studies conducted on non-target organisms: the lacewing *Chrysoperla carnea*, a neuropteran (Hilbeck *et al.*, 1998) and for the ladybug *Adalia bipunctata*, a coleopteran (Hilbeck *et al*., 2012).

A number of studies indicate that Cry toxins have negative effects on non-target organisms, i.e. showing that Cry toxins can affect organisms beyond the expected target groups. For example, the aquatic invertebrate species *Daphnia magna* was shown to be negatively affected by feeding on Cry1Ab maize as well as purified Cry toxins (Bøhn *et al*., 2008; Bøhn, Traavik & Primicerio, 2010; Raybould & Vlachos, 2011; Bøhn, Rover & Semenchuk, 2016). Cry1Ab had negative effects on growth of the brown garden snail**,** *Cantareus asperses* (Kramarz *et al*., 2009), and earthworm species, *Eisenia fetida* (Clark & Coats, 2006; Shu *et al*., 2017) and *Lumbricus terrestris* (Zwahlen *et al*., 2003). Aquatic species affected by Cry1Ab from GM maize were *Lepidostoma liba* (one of the little brown sedges) in which the juvenile's development time was affected (Chambers *et al*., 2010) and Cry causing mortality of the rusty crayfish, *Orconectes rusticus* (Linn & Moore, 2014). Van Wyk *et al.*, (2017) proved exposure of soil microorganisms to Cry1Ab (from GM maize) caused a difference in their species diversity.

Insect communities, specifically the species of Scarabaeidae communities (Campos & Hernández, 2014) and *Pycnopsyche sp., Caecidotea communis* (Swan *et al*., 2009) were affected by Cry toxins that led to differences in community composition and abundance. A number of authors: Losey, Rayor & Carter (1999a), Hansen Jesse & Obrycki (2000), Hellmich *et al*. (2001), Felke, Lorenz & Langenbruch (2002) and, Shirai & Takahashi (2005) found Cry1Ab to be toxic to butterfly and moth larvae causing lower body mass, reduced survival, reduced consumption rates and increased development time, whereas others reported no acute negative effects to butterfly larvae (Wraight *et al*., 2000).

2.4.2 Glyphosate

Glyphosate and glyphosate-based herbicides have been investigated extensively for having adverse effects on human and ecosystem health (Williams, Kroes & Munro, 2000; Govindarajulu, 2008). The safety of glyphosate and GBH is increasingly questioned and recent studies indicate that glyphosate is not as safe as previously assumed (Paganelli *et al.*, 2010; Guilherme *et al.*, 2012; Koller *et al.*, 2012). During the application of herbicides, not only the target species is exposed, and the residual active ingredients and surfactants in soil and water may pose health risks (Sihtmäe *et al*., 2013).

Because of its primary specific mode-of-action, glyphosate is considered to be almost non-toxic to vertebrates and non-target organisms (Sihtmäe *et al*., 2013) and GBHs such as Roundup®, are advertised as such and regarded as environmentally friendly. The material safety data sheet of Roundup® states that glyphosate has no mutagenic, carcinogenic, reproduction or developmental effects *in vitro* and *in vivo.* However, Roundup® was reported to be moderately toxic to the bluegill sunfish (*Lepomis macrochrirus*), the common carp (*Cyprinus carpio*) and *Daphnia magna* (Cuhra, Traavik & Bøhn, 2013). Moreover, it is highly toxic to green algae (*Selenastrum capricornutum*) with acute toxicity after 72 h exposure with an EC50 of 0.46 mg/L. In contrast, the formulation is practically non-toxic to honey bees (*Apis mellifera*) or earthworms (*Eisenia foetida*). Glyphosate causes slight dietary toxicity to bobwhite quail (*Colinus virginianus)* and mallard duck (*Anas platyrhynchos*) as was previously mentioned.

The results from many studies indicate that the formulation is more toxic than the active ingredient itself (Mesnage, Bernay & Séralini, 2012). One of the surfactants in Roundup® is polyethoxylated tallowamine (POEA), and Pettitt & Buhr (1998) and Glover *et al*. (1999) reported on the ability of POEA to increase the permeability of cell membranes. However, when registering herbicides, the toxicity is only tested as the active ingredient and mostly for acute effects. Thus, effects on non-target organisms caused by chronic exposures to low concentrations, as found in the environment, may have been overlooked.

Glyphosate is classified as non-carcinogenic for humans by the European Food Safety Authority (European Food Safety Authority, 2015). However, glyphosate has been classified as probably carcinogenic to humans (Group 2A) by the International Agency for Research on Cancer (IARC) (IARC, 2015) and has been associated with an increased risk of non-Hodgkin's lymphoma (Chang, Simcik & Capel, 2011). Glyphosate and GBHs disrupt endocrine-signalling systems *in vitro*, including multiple steroid hormones, which play vital roles in the biology of vertebrates (Walsh & Stocco, 2000; Gasnier *et al*., 2009). Thongprakaisang *et al*. (2013) found oestrogenic additive effects between genistein, a phytoestrogen in soybeans, and glyphosate when they tested the combination in *in vitro* models (T47D-KB*luc* cells with an oestrogen receptor). The same results were reported by Mesnage *et al*. (2017).

Various authors reported that GBH and glyphosate have a cytotoxic effect on human cells, and cause endocrine disruption, specifically through inhibition of oestrogen synthesis (Richard *et al*., 2005; Benachour *et al*., 2007; Benachour & Séraline, 2009). Glyphosate formulations may be responsible for defects or adverse reproductive effects in vertebrates or contribute to a variety of human diseases (Daruich, Zirulnik & Sofía Gimenez, 2001; Dallegrave *et al*., 2003; Paganelli *et al*., 2010; Samsel & Seneff, 2013). Other abnormal effects due to glyphosate have also been reported in the livers of pregnant rats and their foetuses (Beuret, Zirulnik & Giménez, 2005), muscle and liver cells in dairy cows (Kruger *et al*., 2013) and the reproductive system in ducks (Oliveira *et al*., 2007).

There are many more studies done on the effects of glyphosate on non-target organisms, but for this review on the effects, I have only focused on the effects with regards to reproduction.

2.4.3 2,4-dichlorophenoxyacetic acid

According to the data from the material safety data sheet for 2,4-D, it is practically non-toxic to fish and amphibians, only slightly toxic to aquatic invertebrates, and practically non-toxic to honeybees and earthworms. The 2,4-D formulations are considered to vary between "somewhat toxic" to "practically non-toxic" to the mallard duck and bobwhite quail in 8-day dietary studies (USEPA, 2005b). Evaluation of 2,4-D's effect on avian reproduction (quail) the no-effects concentration was higher than 1 000 mg/L, practically non-toxic for a wide range of measurements such as egg laying and shell thickness (USEPA, 2005b). Similarly, most formulations of 2,4-D have been shown to be practically non-toxic to aquatic invertebrates (WHO, 1997; Peterson *et al.*, 2016).

The USEPA considers 2,4-D not likely to be carcinogenic to humans. However, a Canadian study found a significantly increased risk of men developing cancer (non-Hodgkins' disease)

when exposed to 2,4-D (McDuffie *et al*., 2001). Some studies reported that 2,4-D could reduce growth rates, induce reproductive problems, and produce changes in appearance or behaviour, or could cause death of non-target species, including plants, animals and microorganisms (Gervais *et al*., 2008).

Multiple studies have indicated the genotoxic potential of 2,4-D. Filkowski *et al*. (2003) proved that 2,4-D caused a concentration-dependent increase in homologous recombination and A to G point mutations in thale cress (*Arabidopsis thaliana*). Only 0.1 mg/L 2,4-D was sufficient to increase the frequency of A to G mutations by 49.5%. 2,4-D is also known for its interference on a molecular level of organisms (Madrigal-Bujaidar, Hernández-Ceruelos & Chamorro, 2001; Zeljezic & Garaj-Vrhovac, 2004). 2,4-D causes adverse effects on the superoxide dismutase (SOD) and glutathione S-transferase (GST) activity of fish (Nile tilapia (*Oreochromis niloticus)* and the common carp (*Cyprinus carpio)* although this varies depending on the species and dosage (Ozcan Oruc, Sevgiler & Uner, 2004). In another study, 2,4-D caused free radicals associated with oxidative stress in yeast (Teixeira *et al*., 2004).

Here follows a brief overview of the toxicity of 2,4-D on human health: The USEPA concluded that 2,4-D has low acute toxicity via the oral, dermal and inhalation routes of exposure (Toxicity Category III or IV). 2,4-D is not metabolised in the human body and is rapidly and completely excreted in urine (Van Ravenzwaay *et al.*, 2003; Timchalk, 2004). Epidemiology data are inadequate to report on a relationship between 2,4-D exposure and cancer with no evidence of carcinogenicity (Von Stackelberg, 2013; Goodman, Loftus & Zu, 2015). A recent study examined the systemic toxicity, developmental neurotoxicity, developmental immuno-toxicity, reproductive toxicity, endocrine modulation, and thyroid effects in humans (Marty *et al*., 2013). Reproductive toxicity, developmental toxicity and neurotoxicity were detected after exposure to 2,4-D and its amine salts and esters at dose levels that were at or higher than the threshold of saturation of renal clearance (Peterson *et al*., 2016). The debate of whether the use of herbicides is safe continues as a there may be unknown long-term effects on human health and the environment (Green, 2012).

The preceding section contains a lot of data relating to single compound effects. There are hardly any data available on the combinatorial effects that these compounds might pose. This includes combined substances as they occur in stacked crops as well as the herbicides sprayed (Cuhra, Bohn & Cuhra, 2016). The combinatorial effects may pose health risks that have not been tested before and cannot be predicted (Mesnage & Antoniou, 2018). This is a big gap and therefor this study aims to investigate these mixture effects that have not been tested elsewhere.

2.5 Exposure to contaminants

It is estimated that there are currently 83 000 and 60 000 chemicals listed in the USA and European Union respectively. Humans are exposed to many chemicals as part of daily life through water and food ingestion, air inhalation and dermal absorption (Olujimi *et al*., 2010). A vast number of these chemicals also end up in the environment through various sources such as wastewater treatment plants, run-off, irrigation and spray-drift. These substances range from natural compounds (e.g., hormones, phytoestrogens and mycotoxins) to synthetic compounds (e.g., pesticides, pharmaceuticals and industrial or process chemicals) (Connolly, Ropstad & Verhaegen, 2011). Therefore, mixtures of contaminants are widely distributed in the environment and may pose health effects to non-target organisms. Some chemicals can persist in the environment causing prolonged exposure at low levels, they may accumulate, biomagnify in the food chain and cause detrimental effects. Only a small portion of the chemicals is cherry-picked based on single exposure toxicity and used for risk assessment and monitoring programmes (Svingen & Vinggaard, 2016). Risk assessment is predominantly testing acute toxicity, but in this investigation, the focus is on chronic toxicity and specifically on endocrine disruption.

2.5.1 The endocrine system

The endocrine system consists of various glands (adrenal, thyroid, pituitary, parathyroid, islets of Langerhans of the pancreas, and gonads—testes and ovaries) that secrete hormones. Hormones typically act by binding to specific hormone receptors. Protein and amine hormones bind to membrane receptors to initiate effects via another messenger system. Gene expression is regulated by the binding of steroid and thyroid hormones by nuclear receptors. Hormones travel through the bloodstream to tissues and organs to regulate major body functions such as development and functioning of the reproductive organs, sexual characteristics and libido, growth and developmental processes, metabolism, homeostatic processes, body responses to stress and immune responses (WHO, 2002). A healthy endocrine system is essential for normal development and reproduction. The disruption of these processes may lead to adverse human health effects.

Over the last few years, the production and use of synthetic chemicals have increased drastically. Studies have indicated that these chemicals may have endocrine disrupting properties and that are the cause of endocrine-related effects in humans and wildlife.

2.5.2 Endocrine disrupting compounds (EDCs)

Endocrine disruptors (EDs) or endocrine disrupting compounds (EDCs) are defined as compounds which disrupt the endocrine system. The World Health Organization (WHO) formally defined EDCs as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations” (WHO, 2002). This definition is widely accepted and useful in the context of environmental and human risk assessment.

ECDs form part of various chemical classes, which makes them a challenging group to study. Generally, many endocrine disruptors have one or more aromatic rings and some are chlorinated (Byrne *et al*., 2009).

EDCs can be divided into different groups:

- Natural hormones and their metabolites (e.g. 17β-estradiol (E2), estriol (E3), estrone (E1)
- Synthetic hormones (e.g. diethylstilbestrol; sex steroids in contraceptive pills)
- Food additives (e.g. preservatives, tartrazine used as yellow food colouring)
- Pharmaceuticals and personal care products (e.g. oxybenzone in sunscreen, triclosan in antimicrobial in soap, cosmetics etc.)
- Phyto- and mycoestrogens (e.g. zearalenone, isoflavones, lignans)
- Industrial and household chemicals and combustion by-products (e.g. polychlorinated biphenyls, polybrominated biphenyls, dioxins, polycyclic aromatic hydrocarbons (PAH))
- Pesticides and metabolites (e.g. DDT, methoxychlor, lindane, endosulfan).
- Plasticisers, flame retardants, paints (e.g. such as phthalates, bisphenol A,)
- Metals (e.g. mercury, cadmium, lead) (Burkhardt-Holm, 2010).

EDCs can travel through air, water, sediment and live tissue and some such as pesticides have been detected thousands of miles away from their source and in areas where they were never used (Diamanti-Kandarakis *et al.*, 2009; Futran Fuhrman, Tal & Arnon, 2015). EDCs are ubiquitous, making human exposure to environmental EDCs unavoidable.

2.5.3 Health effects linked to EDC exposure

Exposure to EDCs has been linked to adverse health effects related to development, reproduction and fertility, as well as the nervous and immune systems in humans and wildlife (Connolly, Ropstad & Verhaegen, 2011). Endocrine disruption has different effects in adults than in embryos. The effects during foetal development are much more devastating and are irreversible, whereas in adults, homeostasis can be maintained and the effects may be restored (Sikka & Wang, 2008). Some of the detrimental effects include the development of cancers

such as breast, ovarian, endometrial, vaginal and thyroid, prostate, and testicular (Diamanti-Kandarakis *et al*., 2009; WHO and UNEP, 2012; Yang *et al*., 2015); liver dysfunction, and cardiovascular diseases; reproductive disorders and malformations such as cryptorchidism and poor semen quality in males, endometriosis and polycystic ovarian syndrome in females and infertility; learning and behavioural problems; immune-related disorders relating to higher incidences of asthma, allergies and greater susceptibility to infections (WHO and UNEP, 2012), and higher prevalence of obesity and type 2 diabetes (Schug *et al.*, 2011; Chamorro-García and Blumberg, 2014; Chevalier and Fénichel, 2015).

Endocrine-related effects are also evident in wildlife populations. These effects include developmental abnormalities, reproductive abnormalities and the decline in numbers of some wildlife populations. The increase in reproductive diseases and decline in reproductive function have been detected in a relatively short time frame (since the mid-20th century) and can therefore not only be explained by genetic changes (Connolly, Ropstad & Verhaegen, 2011).

2.6 Methods to detect endocrine disruption (ED) effects

New EDCs are introduced into the market continuously, increasing their occurrence in the natural environment. EDCs complicate environmental risk assessment by having low-dose effects and non-monotonic dose-response curves (Vandenberg *et al*., 2012). Another concern is that, while some of these EDCs have been assessed as relatively safe to consume at low levels individually, they may act/combine with other low-level EDCs to create a low-level cocktail or mixture effects (Kortenkamp *et al*., 2007). These low-levels cause chronic exposure where toxicity is only detected after years of exposure. Effects due to low-levels are overlooked in the current guidelines for toxicity testing, where the doses used for these tests exceed human exposure and environmental related levels by orders of magnitude (Vandenberg *et al*., 2013).

Generally instrumental analysis is used to determine the levels of certain compounds present in the environment, not to predict the effects of these chemicals. This is where *in vitro* assays offer a sensitive and rapid way in screening low-levels mixtures for specific effects. *In vitro* bioassays have the benefit of closely-mimicking natural systems without the use of whole animal testing and can detect compounds based on their effects rather than presence (Connolly, Ropstad & Verhaegen, 2011). There are a few types of *in vitro* bio-assays used to measure ED effects. These assays include enzyme activity assays (using microsomes or recombinant enzymes) (Ekuase *et al*., 2011), co-factor or co-repressor binding assays (Johnson *et al*., 2011), cell proliferation assays (Soto *et al*., 1995) and hormone biosynthesis screens (e.g., steroidogenesis in adenocarcinoma cells: H295R cell line) (Hecker *et al*., 2006). *In vitro* assays investigating the interaction with hormone receptors include receptor binding assays using yeast-, bacterial- or

mammalian cell reporter gene assays (RGAs) (Chakraborty *et al*., 2011; Liang, Zhou & Liu, 2011). These assays measure different end-points indicating ED activity such as e.g. agonism/antagonism of receptors, the proliferation of cells, gene expression and hormone production (Leusch *et al*., 2010). It is therefore recommended to use a suite of bioassays.

EDCs have, in general, more than one mechanism of action which can be receptor-dependent or receptor-independent (Fisher, 2004). EDCs can interact with steroid hormone receptors as agonists or antagonists. Among them are the oestrogen receptor (ER), androgen receptor (AR), thyroid receptor (TR), progesterone receptor (PR), peroxisome proliferator-activated receptor, glucocorticoid receptor (GR) and the aryl hydrocarbon receptor (AhR). An activation or blocking of the receptor causes up- or down-regulation of the receptor, resulting in quantitative or qualitative differences in the downstream events (Leusch *et al*., 2010).

The Endocrine Disruptor Screening and Testing Advisory council, based at the USEPA, implemented methods such as *in silico* and *in vitro* assays with the idea to complement *in vivo* assays by expanding the results and target population beyond humans to include animal wildlife. They have a tiered approach to use molecular-based high-throughput screening assays to prioritise and screen for endocrine disruption which includes the following *in vitro* assays as tier battery 1 (Hartig *et al*., 2003):

- Oestrogen receptor: (ER) binding and (hERα) transcriptional activation - Human
- Androgen receptor (AR) binding
- Steroidogenesis – Human cell line (H295R)
- Aromatase – Human recombinant microsomes

The Tiered Protocol for Endocrine Disruption (TiPED) was created by researchers from the USA specifically for EDCs. In this software, Tier 1 is a computation-based assessment that utilises statistical, computer and mathematical models to predict possible EDC properties based on the structure of the molecules. In the second tier, high-throughput cell-based and cell-free *in vitro* screens, mostly based on receptor binding, are included. Tier 3 uses other sophisticated *in vitro* whole cell assays to examine the practical outputs following receptor binding and activation. Tier 4 includes the assessment of fish and amphibians and Tier 5 refers to the mammalian whole animal assessment. The fifth tier is needed to study EDC effects on complex mammalian physiological processes, *in utero* exposure and behavioural effects (Schug *et al*., 2013). Chemicals that are identified through TiPED to have potential EDC effects will be removed from the product list and re-evaluated. The USEPA also developed ToxCast™ which is a database containing high-throughput screening (HTS) data with information on over 9 000 chemicals and

from more than 1 000 high-throughput assay endpoint components. This information aids users to evaluate the safety and effects caused by various chemicals and is not limited to endocrine disrupting chemicals (Richard *et al*., 2016).

In vitro assays also form part of an Organisation for Economic Co-operation and Development (OECD) conceptual framework to determine mammalian and non-mammalian toxicity. These level 2 assays provide information regarding endocrine mechanisms, and pathways using mammalian and non-mammalian pathways. These established and validated methods include oestrogen receptor transactivation, androgen or thyroid transactivation, steroidogenesis *in vitro,* MCF-7 cell proliferation assays (ER (anti)-agonist).

All of the organisations mentioned above use *in vitro* assays to screen chemicals for toxic effects in order to protect the humans and wildlife of their countries. These *in vitro* assays were used in the current study to evaluate the potential effects of environmental matrices to human and wildlife.

2.6.1 *In vitro* bioassays

Reporter gene bioassays are sensitive, very specific and are used to determine the total agonistic or antagonistic activity of complex mixtures in the ng/L range (Van Der Linden *et al*., 2008). They can be used to detect and semi-quantify receptor-mediated activity of single compounds or mixtures of compounds (Kiyama & Wada-Kiyama, 2015). One example is the MDA-kb2 reporter gene bioassay (Wilson *et al*., 2002) with the ability to measure (anti)androgenic and glucocorticoid receptor-mediated responses. Antagonistic activity is measured by incubating the sample extracts with the agonist control. Another cell line employed to measure endocrine disruption indirectly is the H4IIE-*luc* cells that quantify AhR mediated toxicity and also measures the CYP1A1 activity as an endpoint (Sanderson *et al*., 1996). These bioassays consist of cells that have a steroid receptor coupled to a reporter gene. Under normal circumstances receptor binding takes place in the cell, and this complex moves into the DNA where transcription takes place. Subsequent translation will occur, producing proteins, for example hormones (Fig. 2.5). This description is incomplete and simplified, and there are many more components involved in the process.

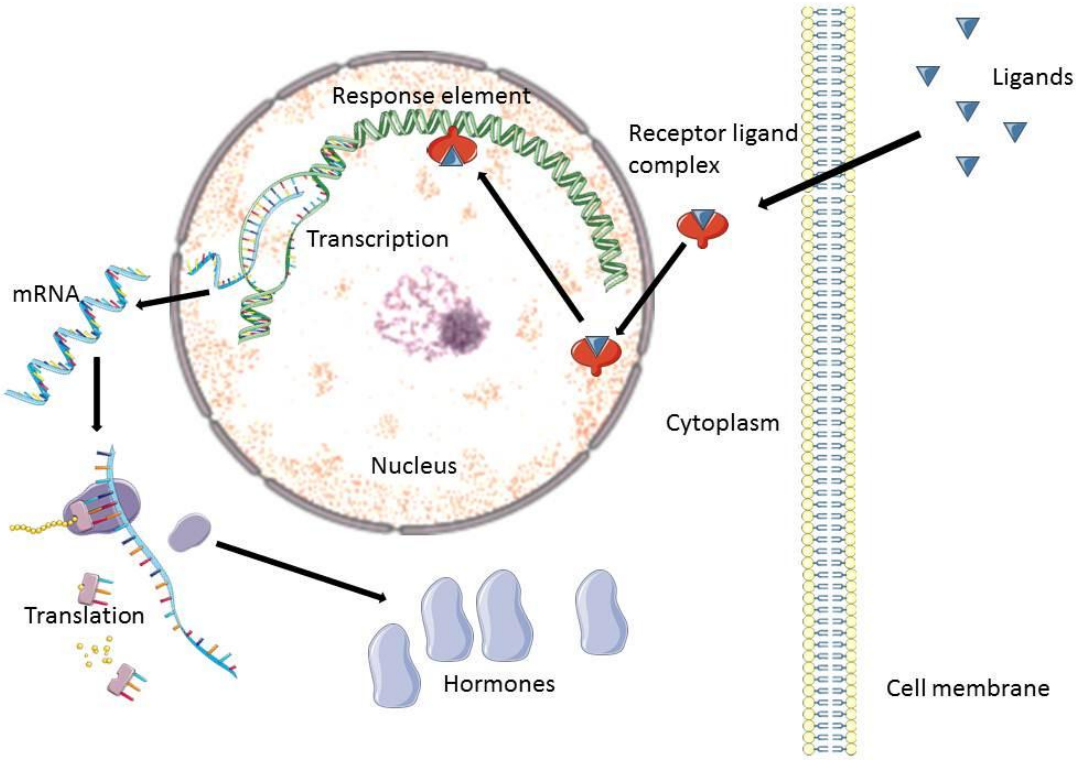

Figure 2:5 Receptor-ligand activity in which target gene expression causes protein synthesis (e.g. hormones) (Adapted from Hilscherova *et al.* 2000).

2.6.1.1. Aryl-hydrocarbon receptor

The H4IIE-*luc* cells have an aryl hydrocarbon (AhR) receptor. These cells are rat hepatoma cells that have been genetically modified with a firefly *luc*iferase reporter to be able to indirectly measure cytochrome P450 induction, which is an endpoint in the AhR mediated response (Aarts *et al*., 1995; Denison *et al*., 2004). A luciferase gene was transcribed downstream of the cytochrome genes and the dioxin response element (DRE) in the H4IIE-*luc* cells. When the substrate luciferin is present, light is produced and is directly proportional to the amount of AhR agonists present in the sample (Hilscherova *et al*., 2000). A positive control, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is known to activate the AhR, is included in the assay and the sample responses are expressed in terms of the control as TCDD-equivalents. The cellular activity and pathways displayed in Fig. 2.5 is a simplified version in order to indicate the difference of gene-expression and products between normal cells (Fig. 2.5) and genetically modified cells (Fig. 2.6).

The in-depth pathway for AhR activation begins when the ligand-bound AhR moves to the nucleus, to form a heterodimer with the AhR nuclear translocator (Arnt) resulting in AhR:Arnt. This complex interacts with the dioxin responsive element (DRE) on the DNA strand (Villeneuve *et al*., 1999; Denison *et al*., 2004; Whyte, Schmitt & Tillitt, 2004; Baston & Denison, 2011). This

interaction causes expression of AhR-responsive genes and the production of mRNA, which is translated in the cytoplasm to form the detoxification enzymes CYP1A1, CYP1B1 and CYP1A2 (Stronkhorst, Leonards & Murk, 2002; Yoshinari *et al*., 2006). Several compounds have been identified that activate the AhR receptor and include drugs such as omeprazole, flutamide, and atorvastatin, industrial pollutants such as dioxins, polycyclic aromatic hydrocarbons (PCBs), and polyaromatic hydrocarbons (PAHs), and natural products like cruciferous vegetables, carotenoids, and green tea polyphenols, have been shown to activate the AhR pathway (Hilscherova *et al*. 2000). This pathway is also connected to the detoxification process in liver cells.

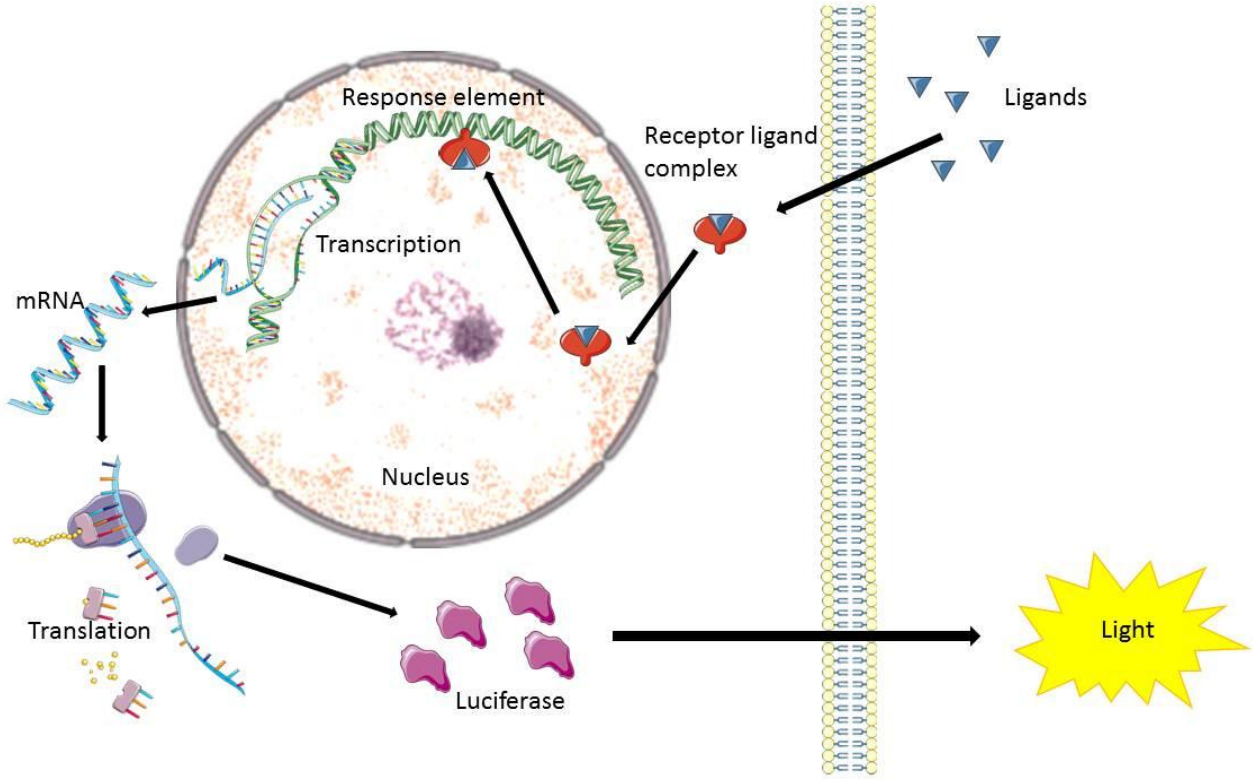

Figure 2:6 A simplified representation of the genetically modified cells that produce luciferase and light is quantified by luminescence (Adapted from Hilscherova *et al*. 2000).

2.6.1.2. (Anti)androgenic and glucocorticoid receptors

The MDA-kb2 cell line was derived from the MDA-MD-453 human breast cancer cell line that expresses high quantities of the androgen (AR) and glucocorticoid (GR) receptors. Normally, during the androgen/glucocorticoid-dependent gene transcription, the androgen/glucocorticoid hormone binds to the AR/GR where after the AR/GR-hormone complex moves to the nucleus and binds to the androgen/glucocorticoid responsive elements on the DNA. The following step is target gene expression and protein synthesis (Janošek *et al*., 2006). However, scientists succeeded in genetically transforming the MDA-kb2 cells with the firefly luciferase reporter

plasmid which is driven by the MMTV promoter (androgen-responsive and very robust) (Wilson *et al*., 2002). The firefly *luc*iferase system used was isolated from the *Photinus pyralis* firefly species. It is bioluminescent and responsible for light emission as a result of an enzyme catalysed chemical reaction (Leitão & Esteves da Silva, 2010).

The AR and GR can activate the MMTV promoter in the *in vitro* MDA-kb2 reporter gene bio-assay when specific ligands are present in a sample. These compounds will enter the cell, bind to the AR/GR which will activate the MMTV promoter. This subsequently causes the expression of the luciferase enzyme (positive response). A substrate, luciferin, is added and the luciferase catalyses the ATP-dependent oxidation of the substrate (Nguyen, Morange & Bensaude, 1988; Leitão & Esteves da Silva, 2010).

Moreover, similar to the H4IIE-*luc* bioassay, the light emitted is recorded as relative light units with a multimode spectrophotometer. The response of the samples is compared to a positive control (which is receptor specific). When light is quantified during the AR assay it may be difficult to distinguish whether the AR and/or GR was activated. This can, however, be resolved by adding an AR antagonist, e.g. hydroxyflutamide, to block the AR, and then the light emitted can be ascribed to GR activity (Wilson *et al*., 2002).

Another function of this assay, other than activation of the AR, is its ability to determine antagonistic effects. The cell line is exposed to an androgen agonist such as testosterone which activates a small portion of the receptor, before adding the sample. The antagonistic compounds will compete with testosterone for binding, and can therefore block binding to the AR. This reaction decreases the amount of light emitted and can be measured as the negative response (Wilson *et al*., 2002).

Biological assays can only semi-quantify responses and not identify the specific compounds, but has the advantage to detect effects of mixtures which can not be determined by chemical analysis (Blake *et al.*, 2010). Other advantages include short exposure periods and small sample volumes. In addition to using these *in vitro* assays to investigate endocrine disruptive effects caused by specifically Cry1Ab, glyphosate and 2,4-D mixtures, some statistical models can predict the effects of mixtures and their interactions.

2.7 Models to predict the toxicity of pesticide mixtures

It is evident that pesticides end up in water sources as mixtures. Thus, it is more effective and realistic to test combinations of herbicides than single substances. In particular, the relevance of testing mixtures is higher when the chemicals have different mechanisms of action. As the

spraying of multiple herbicides continues to increase, e.g. with GM plants with stacked traits, herbicides 'cocktails' become ubiquitous in the aquatic environment, leading to exposure to non-target organisms. This complicates the interpretation of possible effects of the mixtures because only single compounds are generally subjected to toxicity tests. The potential interaction of the co-occurring chemicals may lead to unexpected toxicities that are not identified by the toxicity test of the single compounds. Based on the literature, mixtures and their toxic effects have gained much attention over the last few years. However, the toxicity of the individual compounds within mixtures is assumed to be additive (no interaction effects), until it has been proven otherwise by experimental testing (Boedeker *et al*., 1993). There is increasing evidence of methods to detect interactions such as synergism, independent and antagonist effects. In an attempt to improve the predictive assessment of mixtures in a scientific way, the PREDICT project of the European Communities was launched and investigated the use of several types of mixtures in different types of toxicity tests (Faust *et al*., 2001).

Currently there are two mixture models that exist. The one is termed concentration addition (CA) which is when toxicants have a similar mode of action, and they act on the same target. The second model is referred to as independent action (IA) (Cedergreen *et al*., 2007) and the toxicants have different mechanisms of action.

Compounds detected in aquatic environments occur mostly at lower concentrations than would cause statistically significant different responses in laboratory toxicity tests. Environmental levels are usually below the so-called No Observed Effect Concentrations (NOEC). There are a few assumptions to consider when applying the prediction models: "Under the assumption of concentration addition (Eq. (2.1), section 2.7.1), any concentration of any mixture component is expected to contribute to the overall toxicity of a mixture; there would be no threshold concentration other than zero. Under the alternative hypothesis of independent action (Eq. (2.2), section 2.7.2), the situation is different. Only those concentrations of individual toxicants that cause individual effects greater than zero (E(*ci*)>0) are expected to contribute to the overall toxicity" (Faust *et al*., 2001). No Observed Effect Concentrations can be applied by the alternative use of regression-based statistical estimates of low-effect concentrations, so-called ECx point estimations (Faust *et al*., 2001).

2.7.1 Concentration addition (CA)

The concentration addition method determines the risk of a mixture by summing up all risks of components in the mixture. The CA model is generally defined by the formula from Faust *et al*. (2000):

$$ECx_{mix}=1/\left(\sum_{k=0}^{n}\frac{Pi}{ECx_i}\right)$$ (Equation 2.1)

where:

ECx_{mix}: total concentration of the mixture that causes x% effect

Pi: the proportion of component i in the mixture

n: number of components in the mixture

ECxi: concentration of component i that would cause x effect

2.7.2 Independent action (IA)

In contrast to the CA model a multi-component mixture of substances, defined by the formula from (Faust *et al*., 2001):

$$(E(c_{mix})=1-\prod_{i=1}^{n}(1-E(ci)))$$ (Equation 2.2)

where:

E(ci): effects of the individual constituents

E(cmix): the total effect of the mixture.

E: effects expressed as fractions (x%) of a maximum possible effect.

2.8 Summary of literature review

There is an increase in the use of toxic agricultural chemicals and the adoption of GM crops worldwide. These compounds are released into soil and aquatic environments in which the biodiversity and non-target organisms may be harmed. Toxicity testing and risk assessment only assess the single chemicals or formulations for acute toxicity testing at high concentrations. This preceding information creates knowledge gaps that need further investigation. These gaps refer to the low-level effects posed by these compounds as well as the mixtures, which is present in the environment, and reflect realistic exposure. Compounds found at low-levels and mixtures have proven to have endocrine disrupting effects after chronic exposure, in which the effects are not detected immediately. In this study I attempted to address some of these gaps: I have measured concentrations of mixture chemicals in agricultural regions in South Africa and also extended work with bioassays that specifically test some of the most relevant combinations of chemicals (Cry1Ab, glyphosate and 2,4-D) in the current agricultural practices. The bioassays are a powerful tool to investigate the mixture effects that these combinations might have on non-target organisms specifically with regards to endocrine disruption.

3. MATERIALS AND METHODS

The first phase of this study was laboratory-based but there is an environmental application section in phase two. In the laboratory part of the study, the ability of the herbicides and Cry protein to bind to the selected cellular receptors were investigated: cell lines were exposed to pure active ingredients and formulations. The environmental component consisted of a field trial, in which different combinations of formulations were applied on different maize cultivars. The soil samples were extracted for the target compounds where after the cells were exposed to these extracts. Flowchart to clarify work-flow (Fig 3.1):

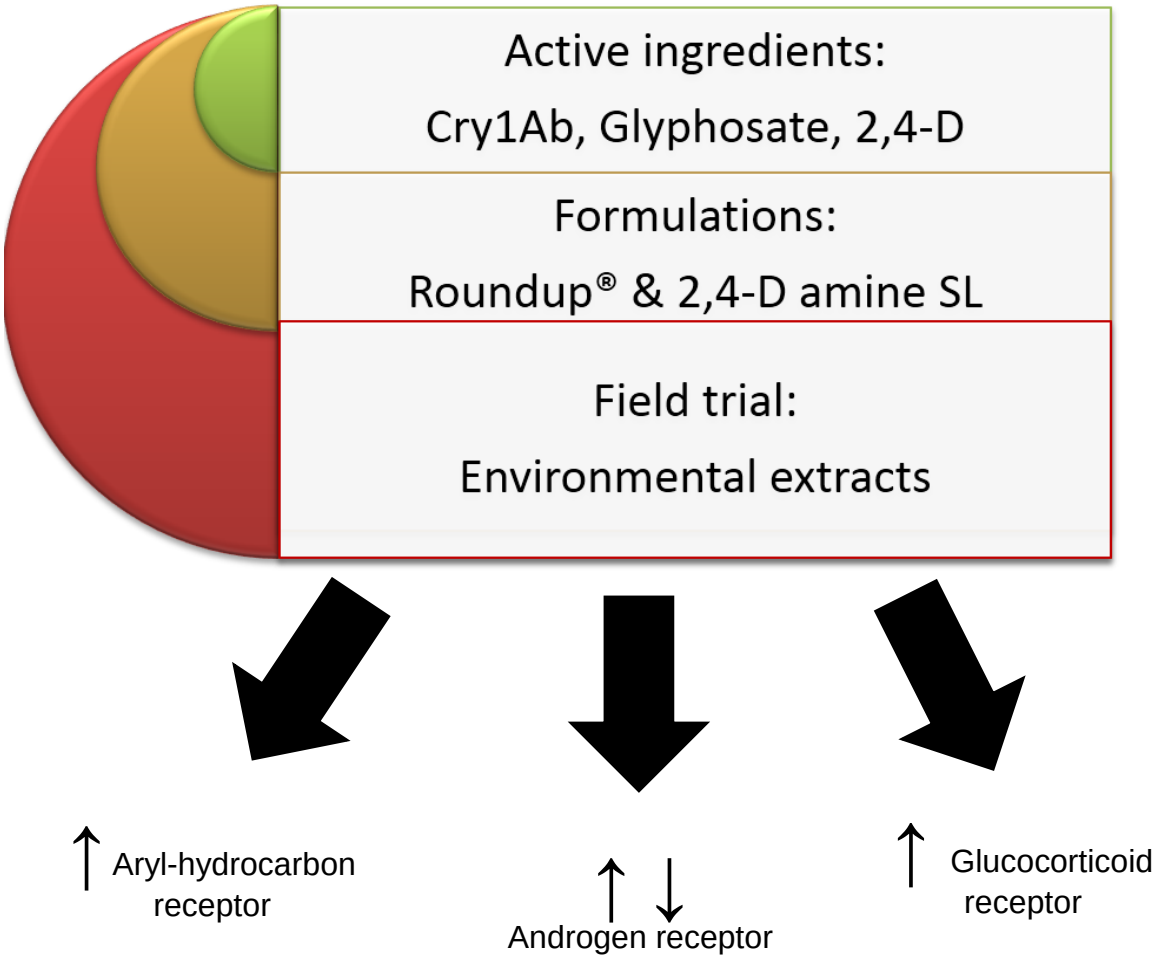

Figure 3:1 Representation of the workflow

3.1 Chemicals

3.1.1 Active ingredients

The target compounds used in this study are glyphosate, 2,4-D (2,4-dichlorophenoxyacetic acid) and Cry1Ab. Analytical reference standards (≥ 99% purity) of glyphosate (CAS# 1071-83-6) and 2,4-D (CAS# 94-75-7) were obtained from Sigma, Germany. Double distilled, nanopure, water (18.2 MΩ) was obtained from an in-house ELGA water purification system. Analytical grade ethanol was acquired from Sigma, Germany. Stock solutions of 1 mg/mL of glyphosate and 2,4-D were prepared in water and ethanol respectively. Stock solutions were prepared in amber glass vials with solid screw lids and kept at -20°C until further use. The Cry1Ab protein standard was purchased from Marianne Carey from Case Western University, Cleveland, Ohio, United States of America (USA). The protein was expressed as a single gene product in *Escherichia coli*. Inclusion bodies, containing Cry1Ab protoxin, were dissolved and trypsinised and the activated Cry1Ab toxin was isolated using high-performance liquid chromatography (HPLC) (Pusztai-Carey *et al*., 1994). The lyophilised protein was resuspended in 10 mM CAPS buffer at pH 10.5 at a concentration of 1.8 mg/mL and frozen at -80°C until use.

3.1.2 Formulations

The formulations tested in this study were Roundup®PowerMax (540 g/L) (Monsanto) (referred to as Roundup® from here onwards, as there are many different versions of Roundup® or glyphosate-based herbicides on the market available) and 2,4-D amine SL (480 g/L) (Villa Crop).

3.2 Field trial

South Africa is an agriculture driven country and various pesticides regimes are applied to crops countrywide. Unfortunately, information on these uses, application rates and times of application, as well as concentrations sprayed, are not always available when sampling on farms. Another drawback of sampling on farms is the availability of the exact combination of target compounds to coincide with the aims of this study. It was for these two reasons that I conducted a field trial on a plot to mimic environmental conditions of farming. Glyphosate, 2,4-D and Cry1Ab were extracted from environmental matrices of the field trial. In the field trial the amount and the times when herbicides were applied as well as the exact concentrations could be controlled. Specific cultivars could be selected and the option to plant and spray various combinations/mixtures became available in the field trial which would not necessarily be the case on farms.

3.2.1 Site description

The field trial was conducted in the crop growing season (January–May) of 2017 at the Agricultural Research Council – Grain Crops Institute (ARC), Potchefstroom, North West Province, South Africa. The field is one of many that are regularly used for experimental agricultural research. Within these plots (Fig. 3.2) maize (with and without genetic modification) as well as unmodified sunflower and sorghum are planted and cultivated under current agriculture practice procedures. A 5 280 m^2 field was ploughed to be used for the field trial.

Figure 3:2 Photograph of the field with markers set in place. The trial markers were set out on the day before planting commenced.

3.2.2 Cultivation practices and herbicide applications

The experimental layout consisted of a randomised block design (Fig. 3.2 & Fig. 3.3). Common agricultural practices were performed with the cultivation and management dates given in Table 3.1. Fertilizer (PetrowAgri) 3:2:1 (25) + 0.28% Zn was added to the soil 24 h before maize was planted. The four different maize (*Zea mays*) varieties used, were purchased from Pannar Seeds (Pty) Ltd (Greytown, South Africa) and included: 1) iso-line (non-Bt) (BG 3292), 2) Bt (BG 3492B), 3) Bt and RR (BG 3792BR) and 4) RR (BG 3592R) (Fig. 3.3).

Herbicide treatments were applied as farmers would in their maize fields. It consisted of pre-emergence Roundup® (Monsanto, Crop Science) and early post-emergence (one-trifoliolate leaf stage (14 d after planting) < 30 cm) Roundup® and 2,4-D application (Table 3.1). The commercial formulations Roundup® Powermax and 2,4-D amine SL were used. Roundup® was applied at 2 L/ha and 2,4-D at 0.5 L/ha. Herbiboost containing ammonium sulphate was added to the Roundup® Powermax mixture as a wetter (to make Roundup® rain fast) and were applied at 4 L/ha. An untreated control was also included. Four different spraying regimes (B, C, D, E) were applied (Fig. 3.3). A: control (no herbicides sprayed); B: Only Roundup® pre-and post-emergent (2x); C: Roundup® pre-and post-emergent (2x) and 2,4-D; D: 2,4-D and Roundup® pre-emergent (1x); E: Only 2,4-D post-emergent.

Herbicides were applied with Knapsack e-band nozzles that were calibrated to deliver 234 L/ha. The spraying boom had tapered edges spraying pattern and overlapped 30%. The nozzles used for pre-emergence spray were 80 04 and achieved droplet wash in the soil. The post-emergence nozzles were 80 02 and delivered a fine spray to wet the leaf surface (Everitt & Keeling, 2007). The boom sprayer was kept at a constant height of 40 cm above the ground. Spraying took place in the early morning with clear skies and no wind.

There were 20 different treatments in total. The plots consisted of a 10 x 5 m area, with three rows planted and an inter-row and intra-row spacing of 1 m (Fig. 3.3). During planting and spraying the treatments were clearly labelled with trial markers (Fig.3.2) (Erasmus, Marais & Van den Berg, 2016).

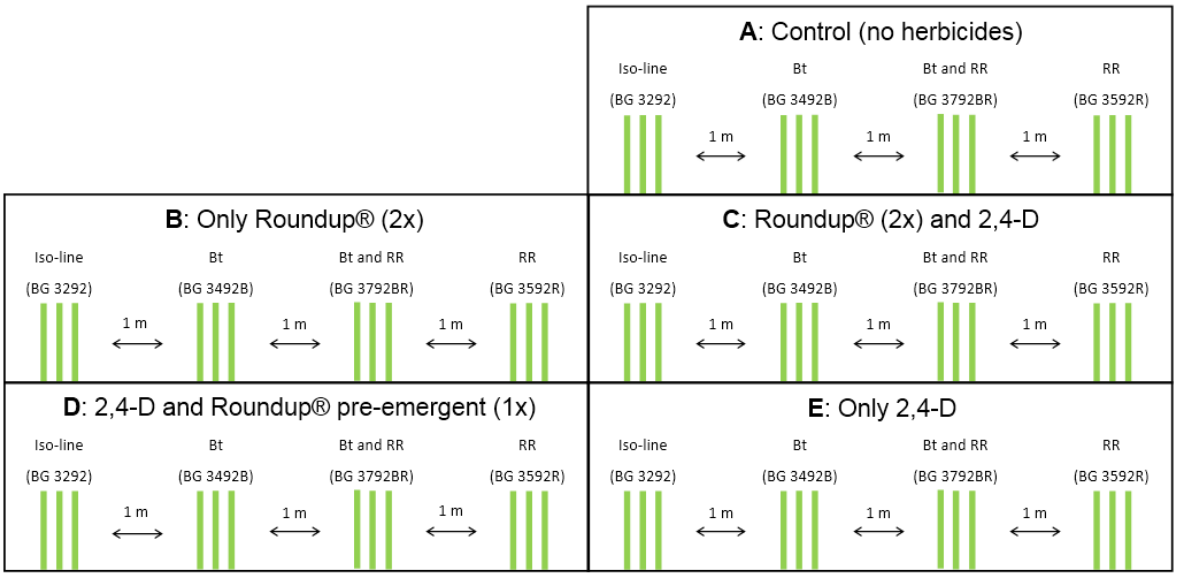

Figure 3:3 Representation of the field plot layout showing the combination of different cultivars and the spraying regime that was applied.

3.3.3 Soil sampling

The soil sampling took place as described in Table 3.1. Composite soil samples were collected from the tillage layer of each variety for every herbicide application (4 varieties and five different herbicide applied plots) in triplicate in 50 mL centrifuge tubes (Falcon). Sampling equipment was rinsed with water, acetone and hexane between each soil sample collected. The samples were transported back to the laboratory at 4°C and stored at -20°C until extraction commenced. The samples were transferred to the -80°C freezer and subsequently freeze-dried for 48 h (FreeZone®, 77530 series, Labconco Corporation) (Laitinen *et al*., 2006)**.**

Table 3:1 Logbook of cultivation, spraying and sampling

Date	Activity
24 January 2017	Place trial markers in the field
	Fertilizer added to the soil
	Sampled soil
25 January 2017	Planting commenced
1 February 2017	Spray pre-emergence Roundup® on treatments (B, C, D)
2 February 2017	Sampled soil
16 February 2017	Spray post-emergence one-trifoliolate leaf stage (14 d after planting) < 30 cm
	Treatment C sprayed with Roundup® and 2,4-D. mixture
	Treatments D and E only 2,4-D
17 February 2017	Sampled soil
21 May 2017	Sampled soil
22 May 2017	End of field trial

3.3.4 Soil properties

The soil samples were subjected to various analyses to investigate possible interactions and absorption of that target compounds to soil. Different types of soils and characteristics of soil indicate the ability of the soil to retain pollutants, that would influence extraction using rainwater and herbicide movement in the field.

3.3.4.1 Soil particle size

In order to describe the nature of the soil from the overall plot used in the field trial, a subsample of 50 g of freeze-dried soil from each treatment was sieved using an Endecott dry-sieving system to collect fractions: mud (<50 µm), fine sand (53–212 µm), medium sand (212–500 µm), coarse sand (500–2 000 µm), very coarse sand (2 000–4 000 µm) and gravel (> 4 000 uM) (ISO 14688, 2016). The sieves were placed on top of one another from the largest mesh size to the

smallest, with a final collection pan at the bottom. The samples were shaken for 10 minutes whereafter the soil contents of each sieve was weighed and recorded, then divided by the initial mass (~50 g) to determine the percentage composition of each particle size of the total soil sample (Trask, 1959).

3.3.4.2 Water holding capacity and moisture content

The water holding capacity (WHC) of the soil samples was determined according to ISO 11268-2 (2012) (Eq. (3.1); section 3.3.4.2). Five grams of freeze-dried soil was placed in a plastic tube, open at both ends. The bottom of the tube was covered with a piece of filter paper and with this end facing down, gradually pushed down into a water bath until the surrounding water level was higher than the soil. The tubes were left overnight to absorb the water. Because the soil capillaries cannot retain all the water absorbed, the soil-containing tubes were left to drain for two hours the following day on a bed of wet, finely ground quartz sand in a closed vessel (to prevent drying). The wet soil samples were weighed and dried at 105°C in an infrared moisture analyser (Sartorius, MA35). The water holding capacity (WHC) of the soil samples were calculated as follow:

$$\text{WHC (in \% of dry mass)} = [S/D] \times 100 \qquad \text{[Equation 3.1]}$$

where:
S = mass of water-saturated soil sample
D = mass of the dry soil

3.3.4.3. Total organic content

The total organic carbon content (TOC) of the soil samples was determined because the carbon indicates the rate of degradation of herbicides by microbial mineralisation (Eq. (3.2); section 3.3.4.3). One gram of dried soil was weighed and transferred into a clean and pre-weighed ceramic crucible. The crucibles containing the soil were incinerated at 600°C for six h. After cooling, the crucibles were weighed again and the carbon percentage was determined using the following formula:

$$\text{TOC (\%)} = [(Mb-Ma)/Mb] \times 100 \qquad \text{[Equation 3.2]}$$

where:
Mb = mass before incineration
Ma = mass after incineration

The organic carbon content was categorised into the following classes: very low (<0.05%), low (0.05–1%), moderately low (1–2%), medium (2–4%) and high (>4%) (Gerber *et al*., 2015).

3.3.4.4 Acid digestion of soil for metal analysis

The soil samples were subjected to metal analysis to investigate whether the metals would retain the compounds of interest, and thereby influencing their bio-availability. A sub-sample of the dry soil were weighed (~200 mg), placed in a Teflon bomb to which 9 mL of 30% HCl and 3 mL of 65% HNO_3 were added (USEPA, 1994). Procedural blanks and certified reference materials (NCS DC 73310 for stream sediment) were included in triplicate. The samples were digested at 1 000 W and 200°C in a Milestone Ethos Easy microwave digestion system for 45 min and then decanted into 50-mL glass volumetric flasks. The bombs were washed twice with ultrapure water and subsequently filtered through cellulose filter paper (0.45 µm) (Sartorius). Metal analysis was performed by inductively coupled plasma mass spectrometry (ICP-MS) using standard methods. Calibration curves were set-up using Ultraspec Certified Element Standards (De Bruyn Spectroscopic Solutions, SA (Kemp *et al*., 2017). The metals analysed included silver (Ag), aluminum (Al), arsenic (As), gold (Au), boron (B), barium (Ba) beryllium (Be), bismuth (Bi), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), potassium (K), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), palladium (Pd), platinum (Pt), rubidium (Rb), antimony (Sb), selenium (Se), strontium (Sr), titanium (Ti), thorium (Th), thallium (Tl), uranium (U), vanadium (V), and zinc (Zn).

3.3.4.5 Cation exchange capacity

The cation exchange capacity (CEC) refers to the available fraction of the exchangeable cations neutralising the charge in the soil. The CEC of the soil was determined because of its influences the adsorption and extractability of glyphosate from soil (Pessagno, Torres Sánchez & Dos Santos Afonso, 2008). The Bower method was performed and contained the following steps. The dried and sieved soil samples (5 g) were placed into a centrifuge tube. A 1 N sodium acetate solution (NaOAc) was added to the tube and the pH was adjusted to 8.2. The mix was shaken for 5 minutes and centrifuged at 1 000 g until the supernatant was clear. The supernatant was discarded and the soil extraction was repeated for a total of four times. The soil was washed four times with 95% ethanol to remove the excess NaOAc. The adsorbed Na^+ was replaced with $NH4^+$ by extracting the soil sample with three aliquots of 1 N ammonium acetate at pH 7, in a similar process as the NaOAc treatment. However, the supernatants were saved and combined and its Na content was determined by atomic absorption spectroscopy (AAS) (Ocampo & Pratt, 1997).

3.3 Extraction of target compounds from field trial soils

Although there are many other methods to extract the target compounds–glyphosate, 2,4-D and Cry1Ab–from soil, these are all for analytical purposes, i.e. what is the exact concentration of these compounds. These methods usually involve methods “forcing” target compounds into a solution that would otherwise not have been bio-available. However, to mimic the transport of the target compounds from the vegetation and field to rivers, as close as possible, the soil samples were extracted with rainwater. The assumption was made that biota would be exposed to the bioavailable fraction, i.e. the water-soluble fraction.

3.3.1 Extraction of soil from field trial

The soil samples collected after the growing season at the end of the trial were extracted for the target compounds. The extractions of glyphosate, 2,4-D and Cry1Ab have been optimised, validated and performed by many laboratories across the world. In an attempt to mimic environmental conditions as close as possible, the soil was extracted with rainwater. Quality assurance and quality control (QA/QC) procedures included a blank, as well as soil samples spiked with low and high concentrations of Cry1Ab (0.1 ug/L and 3 ug/L), glyphosate (0.1 ug/L and 3.5 ug/L) and 2,4-D (2 ug/L and 50 ug/L). The QA/QC samples were treated in the same manner as the samples. The percentage recovery for the spiked samples was also determined (although the main aim was not to obtain maximum recovery, but rather realistic bio-available concentrations).

A volume of 20 mL rainwater was added to a sub-sample of 10 g soil in a 50 mL centrifuge tube (Falcon). The tube was shaken for 1 h on a mechanical shaker. The tubes were centrifuged at 3 000 g for 20 min. This process was performed twice and the aqueous supernatants were pooled and used for enzyme-linked immunosorbent assays (ELISA) analysis and tissue culture exposures.

3.3.1.1 Rainwater parameters

Rainwater was collected in large, clean plastic containers on the roof of a three storey building on campus. The pH, dissolved oxygen (DO), total dissolved solids (TDS) and temperature of the rainwater were determined using a water testing probe (Lovibond®, Tintometer® Group). Macronutrients were analysed with the HACH DR2800 and included free chlorine (Cl_2), nitrate (NO_3^-), nitrite (NO_2^-), phosphorus (PO_4^{3-}), sulphate (SO_4^{2-}), sulphide (S^{2-}) and ammonia (NH_3) (HACH Company, CO, USA). The analysis consisted of measuring these parameters according to the manufacturer’s instructions. The metal concentrations in the rainwater and rainwater sample extracts were also determined using ICP-MS analysis (section 3.3.4.4).

3.4 Quantification of glyphosate, 2,4-D and Cry1Ab using ELISAs

Due to the ease of use, immuno-assays are becoming more sensitive and are already used as a screening tool. For quantification purposes, the correct reference compound is needed to ensure accurate results. In this study, enzyme-linked immunosorbent assays (ELISAs) were used to determine the levels of glyphosate, 2,4-D and Cry1Ab in the rainwater extracts. ELISAs are more time and cost effective than conventional instrumental analysis such as liquid and gas chromatography coupled to mass spectrometry (LC/MS and GC/MS). The ELISAs used were all commercial kits and information about some of the reagents are proprietary.

3.4.1 Cry1Ab

The concentration of Cry1Ab in the extracts was quantified using the commercially available ELISA kit from Envirologix (QualiPlate Kit for Cry1Ab/Cry1Ac Cat # AP003CRBS). The kit is sold as a qualitative kit but can be used quantitatively if the appropriate reference compound (Cry1Ab protein standard) could be acquired. The quantification of the Cry1Ab protein was based on a 12-point standard curve ranging from 0–3.5 µg/L. The Cry1Ab protein standard used was the activated Cry1Ab toxin as explained before (section 3.1.1) (Pusztai-Carey *et al*., 1994). The extracts, blanks and standards were loaded in triplicate on a 96-well-microtitre plate pre-coated with antibodies specific for Cry1Ab/Ac and containing 50 µL Cry1Ab/Ac enzyme conjugate. The plates were left to incubate for 2 h at 21°C, whereafter they were washed with 300 µL wash buffer four times. The wash buffer included in the kit was phosphate buffered saline plus Tween (PBST), that consisted of 0.14 M sodium chloride, 8.1 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM potassium chloride and 0.05% Tween-20, at pH 7.4. A substrate was added to the wells, resulting in the formation of a blue colour when peroxidase hydrolysed hydrogen peroxide. After 20 min, the stop solution containing 1 N HCl was added and the optical densities (ODs) were read at 450 nm (and 650 nm as reference) wavelength by means of a multimode microplate reader (Berthold TriStar LB 941, Germany) (Strain, Whiting & Lydy, 2014).

3.4.2 Glyphosate

Glyphosate was quantified using the Abraxis ELISA plates (PN 500086) (Warminster, PA, USA). The method was performed according to the manufacturer's instructions. In short, the extracts, blanks and standards were derivatised and loaded into a 96-well plate coated with antibodies. A glyphosate antibody solution was added and the plates were incubated for 30 min at 21°C. After incubation, the enzyme conjugate solution was added and incubated for 60 min. After that, the plates were washed three times with 250 uL washing buffer. A colour solution

was added and after 30 min of incubation, the stop solution of 0.5 M sulphuric acid was added. Absorbance was measured at 450 nm. A six-point calibration curve that ranged from 0–4 µg/L were used to quantify the levels of glyphosate in the soil extracts (Mörtl *et al*., 2013; Szekacs, Mortl & Darvas, 2015).

3.4.3 2,4-D

To determine the levels of 2,4-D of the rainwater soil extracts, an ELISA, specifically for 2,4-D (PN 54003A, Abraxis, Warminster, PA, USA) was employed. The extracts, standards and blanks were added to the test strips in 96-well plate format. The enzyme conjugate, and antibody solution followed shortly after and the plate was incubated for 60 min at 21°C. After the incubation period, the plates were washed three times with 250 µL washing buffer solution. After the washing step, a substrate was added and incubated for 30 min. The last step was to add a stop solution and read absorbance at 450 nm. The 7-point calibration curved ranged from 0–80 µg/L.

3.4.4 ELISA quality control and quality assurance

All the extracts were quantified in triplicate using ELISAs specific for each target compound. The mean absorbance values were calculated and the coefficient of variation (CV) was determined for each sample, requiring a CV < 20%. The concentration of glyphosate, 2,4-D and Cry1Ab were determined against the linear regression line of the calibration curve, with an R-square as close as possible to 1. The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the regression analysis of the calibration curves for each ELISA, where LOD = $3S_b/b$ and LOQ = $10S_b/b$ with S_b = slope uncertainty and b = slope (Schoeman *et al*., 2015)

3.5 Exposure list

From this stage of the chapter onwards, the exposures to the reporter-gene bio-assays will be discussed. For simplicity, all the active ingredients, formulations and mixes of these two, as well as environmental extracts to which the cells were exposed to in the bio-assays, are listed in table 3.2. The concentrations of the active ingredient (AI) and formulation mixes were based on the environmental concentrations obtained from the environmental rainwater extracts, to be comparable to one another.

Table 3:2 The list of active ingredients (pure compounds) and formulation mixes used in the bio-assay exposures. *Concentrations that remained constant during the entire dose-response exposures. The varying ingredient was diluted twice. Six dilutions were tested.

Compounds	Concentrations (µg/L)		
	Glyphosate	**2,4-D**	**Cry1Ab**
	Active ingredients		
Cry1Ab	0	0	7
2,4-D	0	220	0
Glyphosate	13	0	0
AI Mix 1	13	220*	4*
AI Mix 2	13*	220*	4
AI Mix 3	13*	220	4*
AI Mix 4	13	220	4
AI Mix 5	1.2	220	3
	Formulations		
	Glyposhate (from Roundup PowerMax)	2,4-D (from 2,4-D amine 480 SL)	Cry1Ab (pure compound)
Roundup® PowerMax	13	0	0
2,4-D amine 480 SL	0	220	0
Formulation mix 1	13	220*	4*
Formulation mix 2	13*	220*	4
Formulation mix 3	13*	220	4*
Formulation mix 4	13	220	4
Formulation mix 5	1.2	220	3
	Environmental extracts		
20 treatments	Unknown mix	Unknown mix	Unknown mix

AI: active ingredient

3.6 Bio-assays

Two different cell lines (H4IIE-*luc* and MDA-kb2) were used in reporter-gene bioassays to measure endocrine disruptive effects of herbicide formulations, the active ingredients, as well as the environmental extracts containing mixtures. These cells have different nuclear receptors (Table 3.3) and therefore measure different endpoints.

3.6.1 Maintenance of cells

The H4IIE-*luc* and MDA-kb2 cell lines were a gift from John Giesy (University of Saskatchewan, Canada). An aliquot of 1 mL thawed cells were initiated from liquid nitrogen storage and added to 11 mL of medium. The various cell lines were grown and maintained in 60.1 cm^2 tissue culture dishes (TPP, Separations) and their specific culturing nutrient media (Table 3.3) containing foetal bovine serum (FBS). The FBS provides essential minerals, lipids, hormones, transport proteins and aids in the attachment-, growth- and spreading factors needed for the *in vitro* culturing of animal cells. Both cell lines were kept in an incubator fitted with a high-efficiency particulate air (HEPA) filter to prevent bacterial contamination. The humidified atmosphere within the incubators were kept at 37°C and supplemented with 5% carbon dioxide (CO_2), except for the incubator housing the MDA-kb2 cell line which received no CO_2. The cell cultures were handled in a sterile laminar flow hood which was carefully cleaned with 70% ethanol before and after use. The laboratory and laminar flow were also sterilised with UV-radiation once a week.

The cells' media were replaced with fresh media every 2–3 days and the cells were passaged when the plates were 80% confluent. When passaging the cells, the cell debris was removed by washing the plates three times with Dulbecco's phosphate buffered saline (PBS; Sigma Aldrich), which contained no added salts.

Table 3:3 The specific nutrient media and growth conditions for each cell line used

Cell line	Nuclear receptor	Nutrient media
H4IIE-*luc*	aryl hydrocarbon (AhR)	Dulbecco's Modified Eagle's Media (DMEM), with 10% FBS
MDA-kb2	androgen (AR) and glucocorticoid (GR)	Leibovitz L-15 media, with phenol red and 10% FBS

FBS: foetal bovine serum

Afterwards, the cells were treated with 1.5 mL trypsin to loosen them for sub-culturing. The cells were employed in bioassays to determine the effects of active ingredients, formulations and environmental extracts (Table 3.3). Each cell line's assay has differences that will be discussed in the following sections.

3.6.2 Reporter-gene assays

The assays were performed in 96-well microtitre, white-walled, clear bottom plates (Bio-Greiner One, Lasec). The assays were done in dosing medium which means that the FBS in the nutrient medium, used to grow the cells, were stripped of hormones using dextran treated charcoal

(cdtFBS) (HyClone, Separations) to prevent false positive results. The cells were only seeded in the inner 60 wells at their respective cell concentrations. The outer 36 wells were filled with PBS to create the same micro-environment across all cell-containing wells (Figure 3.4). Each cell line had a positive control for activation or inhibition of its specific receptor (mentioned in sections 3.6.3.1, 3.6.3.3 and 3.6.3.4). Each assay plate was dosed in triplicate with a solvent control (SC) (water) to control for potential solvent related effects and blank control (BC) only containing cells and dosing medium proving the cell's well-being during the assay. The cells were simultaneously exposed to controls, formulations, active ingredients and environmental extracts of the target compounds. The concentrations of these compounds are summarised in Table 3.2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	S2 1x	S2 1x	S2 1x	S2 1x	S2 1x	S2 1x	SC	PC 1	PC 1	PC 1	PBS
C	PBS	S2 2x	S2 2x	S2 2x	S2 2x	S2 2x	S2 2x	SC	PC 2	PC 2	PC 2	PBS
D	PBS	S2 4x	S2 4x	S2 4x	S2 4x	S2 4x	S2 4x	SC	PC 3	PC 3	PC 3	PBS
E	PBS	S2 8x	S2 8x	S2 8x	S2 8x	S2 8x	S2 8x	BC	PC 4	PC 4	PC 4	PBS
F	PBS	S2 16x	S2 16x	S2 16x	S2 16x	S2 16x	S2 16x	BC	PC 5	PC 5	PC 5	PBS
G	PBS	S2 32x	S2 32x	S2 32x	S2 32x	S2 32x	S2 32x	BC	PC 6	PC 6	PC 6	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Figure 3:4 Layout of the 96-well plate. The outer 36 wells are grey and filled with PBS, where the inner 60 wells contain the receptor specific positive control (PC), solvent (SC) blank (BC) control and test compounds (S2 and the 2x dilution range of the samples).

3.6.3 Preparation of the exposure-medium from extracts

The environmental extracts to which the cells were exposed to, were prepared as follows: 10 mL of the aqueous extracts were aliquoted into centrifuged tubes for the different bio-assays. These extracts were used to prepare dosing medium for each cell line, by weighing the correct amount of powdered media and salts, add it to the extract, as well as cdt-FBS. These tubes were shaken until the medium was dissolved. The exposure-medium was sterilised by using a sterile disposable syringe and filtering the extract with a syringe filter (0.22 µm). The extracts were 2x serially diluted and given to the cells by replacing the media, on the dosing day of each specific assay.

3.6.3.1 H4IIE-luc assay

The H4IIE-*luc* cells are rat hepatoma cells that have been genetically modified and transfected with a firefly luciferase reporter gene (Sanderson *et al*., 1996). The H4IIE-*luc* assay has the ability to screen for aryl hydrocarbon receptor (AhR) activity and indirectly measure cytochrome P450 induction, which is an endpoint in the AhR mediated response (Denison *et al*., 2004). In

the 5-day assay, the cells were stripped of hormones and given 10% cdtFBS (HyClone, Separations) in DMEM. On day 1 of the assay, the cells were seeded at 80 000 cells/mL in 250 µL and incubated to attach for 24 h. After attachment, the cells were dosed, in triplicate with the positive control: 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD). The cells were exposed to the 4x dilution series resulting in concentrations of 0.05, 0.19, 0.75, 3, 12, 48 ng/mL. The exposure time was 72 h (Chan *et al*., 2013).

3.6.3.2 MDA kb2 (anti-) androgenic activity

The parent cell line, MDA-MB-453 human breast cancer cells, were stably transfected with murine mammalian tumour virus (MMTV)-luciferase neo-reporter-construct into the MDA-kb2 cell line (Wilson *et al.*, 2002; Klopcic & Dolenc, 2017). The 5-day MDA-kb2 reporter gene bio-assay can detect both the (anti-)activation of the androgen receptor (AR) and the activation of the glucocorticoid receptor (GR) (Aït-Aïssa *et al.*, 2010; Blake *et al.*, 2010).

3.6.3.3 Activation of AR and GR

At the start of the assay the cells were seeded at 120 000 cells/mL in 250 µL dosing medium (Leibovitz L-15 media supplemented with 10% cdtFBS). The plates were incubated for 48 h to allow attachment of the cells. Thereafter the cells were dosed with the test compounds (Table 3.2) and a positive control. The positive control for AR activation is testosterone that was serially diluted in MeOH to yield a dose-response curve. These concentrations were: 0.001; 0.004; 0.018; 0.092; 0.46; 2.3 ng/mL. The assay exposure time was 48 h and the plates were treated as described in section 3.6.4.

This cell line has two receptors: AR and GR that can be activated. If the samples elicited a response during the AR activation assay, a second assay was performed in which the AR is blocked with (0.2 µg/mL) flutamide (AR inhibitor), to clarify whether binding took place to the AR or GR. The positive control for the GR, dexamethasone, is also included in the plate at with six serial dilutions of 0.375 µg/mL diluted 5x.

3.6.3.4 Inhibition of the AR

For the AR inhibition (anti-androgenic activity) of the test compounds, the activation method for the AR was slightly modified. The cells were seeded with a known background concentration of testosterone (2.3 ng/mL) (AR agonist) which resulted in cells with activated ARs. The fold change between the responses of the SC wells and samples was calculated and used to determine inhibition. A serial dilution of the AR antagonist, flutamide, was also dosed at 0.032, 0.16, 0.8, 4, 20 and 100 µg/mL.

3.6.4 End of the reporter-gene assays

After exposure, all the plates were visually examined under a phase contrast microscope to check confluency and for signs of cytotoxicity such as detachment and vacuolization and also bacterial infection (if present) (Wilson *et al*., 2002). These changes were noted if needed for data interpretation. Another sign of toxicity was luminescence readings significantly below (10%) of the vehicle control level (0%) in the agonist mode (Bou-Maroun *et al*., 2017). The culture media were removed and the plates were washed with PBS supplemented with Mg^{2+} and Ca^{2+}. The membrane proteins of the cells were then lysed by adding 25 µL lysis buffer (Promega). The plates were stored at -80°C for rapid freezing to ensure that the cells have ruptured the cell membranes and the release of cell content.

A Berthold multimode microplate reader (model LB941) was used to measure luminescence. After the plates were thawed the plate reader injected 100 µL luciferase assay reagent (LAR) (20 mM tricine (Sigma-Aldrich), 1.07 mM $Mg(CO_3)_2Mg(OH)_2{\cdot}5H_2O$ (Sigma-Aldrich), 2.67 mM $MgSO_4{\cdot}7H_2O$ (Sigma-Aldrich), 0.1 mM EDTA (ethylene-diamine-tetra-acetic acid)-disodium salt (Sigma-Aldrich), 33.3 mM dithiothreitol (DTT) (Sigma-Aldrich), 270 µM coenzyme A (Sigma-Aldrich), 530 µM ATP (Sigma-Aldrich) and 470 µM beetle luciferin (Melford)) (Villeneuve *et al*., 1999). The luciferin cleaved by the luciferase produced light in each well—which was recorded as relative light units (RLUs)—used in further statistical analyses to express the sample's responses quantitatively. The amount of light elicited is directly proportional to the number of agonists present in the test compound (Denison *et al*., 2004).

3.6.5 Viability assay (MTT)

Simultaneously to the reporter-gene assays, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the viability of the cells after exposures. These results were used to validate the reporter gene bio-assay data by preventing false negative responses since low responses (*i.e*. no luminescence) do not necessarily indicate the presence of antagonists or absence of agonists, but possible cytotoxicity. Mossman (1983) developed the MTT viability assay and it has since been used as a method to quantify the viability of cells that have been exposed to toxic compounds. The principle of the assay is based on an enzyme reaction during which the yellow tetrazolium salt (MTT) is transformed into a purple crystalline, insoluble formazan product by succinate dehydrogenase (an enzyme present in metabolically active mitochondria that cleaves the tetrazolium ring). When the formazan salts are dissolved they can therefore be quantified spectrophotometrically (Mossman, 1983).

All the MTT plates were handled and dosed in the same way as its respective reporter-gene assay. For the procedure, the cells were seeded in clear 96-well plates, in the same manner as

in the reporter-gene assays, although the wells in column 12 did not receive PBS as indicated in Figure 3.3, but cells. These cells represented the negative control and were killed with 200 µL methanol at the end of the assay for 5 minutes. At the end of the assay, the plates were visually inspected and washed three times with PBS. Each well received 100 µL of MTT solution (0.5 mg/mL MTT in stock media) (Sigma, Germany) and were incubated for 30 minutes at 37°C. The MTT was removed and 200 µL of dimethyl sulfoxide (Merck, RSA) was added and the plates were incubated at room temperature for 30 minutes to dissolve the formazan crystals**.** Absorbance was measured at 560 nm in a multimode microplate reader (Berthold TriStar LB 941, Germany). The optical density of the solubilised formazan is directly proportional to the number of living cells per well (Ulukaya *et al*., 2008). The mean of the optical density (OD) values obtained from the negative control (MeOH killed cells) were subtracted from the mean OD of all the cells that were exposed. This value was expressed in terms of the OD values of the solvent control (100% viable cells) and percentage viability was calculated. A viability percentage lower than 70% was subjected to non-parametric tests (section 3.7) to determine if these samples were statistically significant cytotoxic which may affect reporter-gene response interpretations.

3.7 Data processing and statistical analysis

The data for the cellular receptor activation and inhibition assays to determine TCDD, testosterone-, flutamide- and dexamethasone- equivalents (TCDD-EQ, TTEQ, FluEQ and DexaEQ) were exported to Microsoft Excel. The mean, standard deviation and CV values were calculated for all exposures. The RLUs from the rainwater control was subtracted from the samples' RLU values. As part of the quality control for each assay, the fold induction between the maximum concentration of positive control and vehicle control was determined and should be >5 (Wilson *et al.*, 2002; Aït-Aïssa *et al.*, 2010). This was done by dividing the mean RLU of the highest concentration of the positive control with the mean RLU of the vehicle control. The LOQ was set at an effective concentration where 10% activity occurred (EC_{10}) and RLU values below this were not considered in further analysis. In contrast, the LOQ for the AR inhibition assay was set at an inhibition concentration (IC_{90}) (where 90% activity occurred) and RLU values above this were not considered for further analysis. The data (above EC_{10} and below IC_{90}) was imported into GraphPad Prism v. 7, where the concentrations of the various positive controls (depending on receptor assay) were logarithmic transformed and a sigmoidal dose-response (variable slope) was generated. From these dose-response curves the correlation coefficient (R^2) and EC_{50} values for the positive controls were determined and the sample's responses interpolated in terms of the positive control, where after the TCDD-EQ, TTEQ, FluEQ and DexaEQ's for each compound with a quantifiable response was obtained. The conversion to the bio-assay-equivalents was done with a back calculation based on the initial quantity of

sample extracted which were concentrated into a final dosing volume (e.g. 10 g soil and 40 mL used in the bio-assay).

The inhibition data were reported as fold change after dividing the RLU of the samples with the RLU of the solvent control. A fold change < 1 indicates possible inhibition and a fold change > 1 show activation of the AR. Statistical significant differences for these responses were investigated using the SPSS statistical package, version 24 (IBM). The responses obtained from the cells after exposure was subjected to the Kolmogorov-Smirnov test for normality and homogeneity of variance was assessed by use of Levine's test. The sample size, unequal variance and data that were not normally distributed dictated that a non-parametric test (Mann-Whitney U) had to be performed (significance = $p < 0.05$). Statistical significant differences between each exposure compounds' viability were also determined using Mann-Whitney U with significance at $p < 0.05$.

Spearman's correlations were performed between bioassays equivalents (TTEQs) and the concentrations of Cry1Ab, glyphosate and 2,4-D measured in the rainwater extracts from the field trial. The same correlations were also applied to the inhibition data by comparing the fold changes obtained by each treatment of the field trial to the concentrations of the target compounds.

The multivariate statistics were done with Canoco version 5 (Leps & Smilauer, 2003). A principle component analysis (PCA) was applied as it uses a linear response model to explain the variation between species data and environmental variables e.g. TTEQ (species) and metals and levels of herbicides and Cry1Ab in the environmental extract (environmental variable). This allows new combinations of variables that explain the greatest variation in the data set. Factor 1/axis 1 explains the greatest amount of information from the data and Factor 2/axis 2 explains the second largest part of the data (Van den Brink, Van den Brink & Ter Braak, 2003). In the PCA biplots the angles between the vector arrows show the correlation between each environmental variable and/or species data arrow: an angle close to 0° is a positive correlation between the variables; an angle closer to 90° indicates no correlation, and an angle approaching 180° indicates a negative correlation between the variables. The vertical line between a sample symbol and a particular species arrow can be used to estimate the value of that sample regarding the variable it is vertical too.

4. RESULTS

In this study the endocrine disruptive effects of single compounds and mixtures prepared by using pure active ingredients and formulations were investigated. Another exposure compound included the water-soluble fraction (environmental extract) from the field trial that had been conducted. In the field trial 4 different maize varieties were planted and each type of maize cultivar (iso-line, Bt, Bt and RR, and only RR) were sprayed with different combinations of herbicide mixtures (section 3.2.2). The soil samples and rainwater extracts were subjected to various analysis to characterise them and the results obtained are discussed in this chapter.

4.1 Soil characteristics

The characteristics of soil from the field plot were determined to evaluate the influence of their inherent chemical properties on the extractability of the target compounds from soil using rainwater. These parameters included grain size, total organic carbon content (TOC), water holding capacity (WHC) and the cation exchange capacity (CEC).

4.1.1 Grain size

The grain sizes of the soil were measured using different sieve sizes and determining the percentage of each fraction. The results showed that the soil had the same grain size across the different plots in the field (Table 4.1). The size class contribution is in declining order as follows: coarse sand>medium sand>fine sand>gravel>very course sand>mud (clay). This sandy type of soil profile is the preferred type for crop cultivation. In South Africa typical agricultural fields are known to have a high sand fraction (Du Plessis, 2003). Because the plot used for the field trial is a small piece of land, the grain sizes across the plot are very similar.

4.1.2 CEC, WHC and TOC

Determining the CEC of the soil can explain adsorption and extractability of glyphosate from soil (Pessagno, Torres Sánchez & Dos Santos Afonso, 2008). The %WHC relates to the Cry1Ab content in the soil. The %TOC was measured to evaluate to what extent compounds would adsorb to the carbon fraction. The carbon content also indicates the rate of degradation of the herbicides by microbial activity (La Cecilia & Maggi, 2018). Microbial activity is related to %TOC as it is the main activity responsible for degradation of glyphosate and 2,4-D. The results for the soil properties that include the grain sizes, WHC, TOC and CEC were mostly the same across all the treatments in the field trial plot (Table 4.2). Therefore, these parameters of the soil were

not considered to have contributed to the effects found between the sampling sites and were not further discussed in this thesis.

Table 4:1 Results for grain sizes of the soil obtained from the field trial (%)

Mesh size (µm)	>4 000	2 000–4 000	500–2 000	212–500	53–212	<53
Soil type	Gravel	Very coarse sand	Coarse sand	Medium sand	Fine sand	Mud (clay)
A: Iso-line	18.93	11.11	30.05	20.18	16.99	2.74
A: Bt	21.92	8.06	27.39	19.54	19.00	4.06
A: Bt + RR	18.38	15.17	34.42	20.68	22.14	5.16
A: RR	15.62	16.00	33.35	16.71	16.44	3.55
B: Iso-line	11.53	9.61	30.01	22.54	22.72	4.30
B: Bt	10.21	11.63	34.84	23.32	23.98	4.93
B: Bt + RR	17.65	11.89	31.23	21.54	18.08	3.40
B: RR	29.61	6.22	26.11	21.03	23.66	4.60
C: Iso-line	19.49	10.01	26.81	18.83	19.93	4.60
C: Bt	9.54	11.48	25.27	22.20	23.22	3.68
C: Bt + RR	8.72	9.96	30.20	20.08	17.24	2.68
C: RR	17.00	15.58	38.93	18.32	16.06	4.56
D: Iso-line	9.18	11.37	34.33	22.89	17.64	3.51
D: Bt	21.99	11.91	29.45	17.77	19.91	4.50
D: Bt + RR	29.82	14.94	32.66	15.77	15.05	3.98
D: RR	8.31	11.63	35.86	25.20	26.39	5.31
E: Iso-line	18.87	15.20	33.63	16.16	15.90	3.83
E: Bt	15.36	12.90	33.07	17.19	15.54	3.17
E: Bt + RR	20.50	14.54	30.25	18.56	15.80	0.00
E: RR	13.02	11.38	37.65	22.47	21.20	4.61

Table 4:2 The water holding capacity (WHC), total organic carbon content (TOC) and the cation exchange capacity (CEC) of soil samples for each spraying regime from the field trial.

Soil type	CEC (cmol(+)/kg)	WHC (%)	TOC (%)
A: Bt + RR	34.00	33.75	1.90
B: Bt + RR	27.91	33.54	2.69
C: Bt + RR	24.38	32.69	2.87
D: Bt + RR	32.49	29.04	2.45
E: Bt + RR	30.21	35.77	2.41

4.1.3 Metals

The soil samples from each spraying treatment and the rainwater extracts were subjected to metal analysis. The was done to evaluate if the metal levels in the soil influence the extractability of the Cry1Ab, glyphosate and 2,4-D. Due to the chemical nature of glyphosate, it has the capability to form insoluble complexes with cationic micronutrients for example Fe, Zn and Mn, which influences its bio-availability (Eker *et al*., 2006). Herbicide tank mixtures and formulations may also contain metals (Defarge, Spiroux de Vendômois & Séralini, 2018) and therefore the metal concentrations of each of the soil spraying treatments (A–E) were measured to determine differences between treatments. The metals in the environmental aqueous extracts were also measured because the cells were exposed to these extracts and metals have been found to bind to nuclear receptors (Stoica, Katzenellenbogen & Martin, 2000).

The soil samples were digested using acid and analysed by ICP-MS. The concentrations of the metals in the rainwater and rainwater extracts (to which the cells were exposed to) were also measured by ICP-MS.

4.1.3.1 Quality control/Quality assurance for metal analysis of soil

The CRM contains only a portion of the metals analysed in the samples. The percentage recovery was calculated by comparing the results obtained, to the values published in the CRM (NCS DC 73310) certificate. The percentage recovery of the CRM metals ranged from 34.93–119.4% (Table 4.3). Beryllium, Ba, Ti and Sr were only recovered at 34, 36, 43 and 43.8% respectively. The overall recovery was 71% which is acceptable and considered as a good recovery percentage (Liu, Zhou & Wilding, 2004). The instrumental detection limit is reflected in table 4.4.

4.1.3.2 Metal concentrations in the soil

For most of the metals, the levels were in the same order across all the treatments. There were however some deviations (by 3 to 4 times higher or lower): Au was higher in treatment E; Ba and Cd higher in treatments A; Sb were lower in treatments C and D; Ag, Cr, Se and Ti were higher in treatment C (sprayed with pre- and post-emergent Roundup® and 2,4-D) (Table 4.4). Aluminium, Bi, Ca, K, Mg, Mn, Na, Ni, P, Pb, V and Zn were much lower in treatment C when compared to the other treatments. The results reflect that the metal concentrations were different for some metals in the different spraying treatments, which confirms that the metals are not only from the soil.

Table 4:3 Metals (µg/g) extracted from the sediment CRM (NCS DC 73310), the certified metal concentrations (µg/g) and the percentage recovery of the experimental procedure

Element	Experimental value			Certified values			Recovery (%)
As	80.07	±	1.00	115.00	±	9	69.63
Ba	74.85	±	5.83	206.00	±	23	36.33
Be	2.86	±	0.09	8.20	±	1.1	34.93
Bi	8.23	±	0.26	10.90	±	1.3	75.49
Cd	3.56	±	0.05	4.00	±	0.4	88.94
Co	7.90	±	0.01	8.80	±	1.1	89.81
Cu	1197.60	±	5.52	1230.00	±	51	97.37
Mn	1076.88	±	9.83	1400.00	±	73	76.92
Mo	8.55	±	0.07	8.40	±	0.9	101.75
Ni	9.41	±	0.10	12.80	±	1.9	73.54
P	280.60	±	1.98	235.00	±	34	119.40
Pb	229.48	±	1.84	285.00	±	16	80.52
Sb	16.62	±	0.04	24.00	±	4	69.26
Sr	10.52	±	0.40	24.00	±	4	43.84
Th	17.49	±	0.20	21.40	±	1.7	81.74
Ti	649.29	±	12.29	1510.00	±	70	43.00
U	4.87	±	0.00	7.80	±	1	62.49
V	28.72	±	0.63	46.00	±	5	62.43
Zn	347.03	±	3.42	498.00	±	27	69.69

4.1.3.3 Metal concentrations of each treatment's rainwater extract

The metal concentrations of each rainwater extract, that formed part of the environmental mixtures to which the cells were exposed, were determined by ICP-MS analysis (Table 4.5). These metal results represent the extractable portion in the water-soluble and bioavailable fraction.

Table 4:4 Metal concentrations (µg/g) in soils from each of the plots that received different herbicide applications (A–E).

	Detection limit (µg/L)	A: Control	B: Pre- and post emergent Roundup®	C: Pre- and post emergent Roundup® and 2,4-D	D: Post emergent Roundup® and 2,4-D	E: Only 2,4-D
Ag	0.011	0.18	0.15	0.32	0.17	0.13
Al	1.660	32765.24	36063.22	21361.64	38956.90	28484.28
As	0.029	5.48	4.21	4.79	5.62	4.00
Au	0.001	0.08	0.73	1.21	0.04	2.97
B	0.937	6.43	6.17	6.94	7.06	5.59
Ba	0.019	190.88	121.31	140.95	154.90	155.21
Be	0.007	1.36	1.23	1.36	1.42	1.16
Bi	0.002	0.58	0.50	0.16	0.81	0.43
Ca	0.359	6922.71	6764.85	2110.30	6126.35	5537.31
Cd	0.040	0.48	0.10	0.10	0.09	0.07
Co	0.031	24.05	15.75	14.64	22.57	19.87
Cr	2.978	366.78	314.66	582.24	424.34	341.15
Cu	0.025	44.67	36.45	34.16	46.25	37.33
Fe	1.671	45079.21	33860.15	30209.86	45592.56	34091.98
Hg	0.008	0.18	0.16	0.14	0.23	0.22
K	3.116	3516.56	3716.48	2298.44	4194.42	3033.79
Mg	0.192	3574.17	3572.80	2087.12	3685.11	3374.00
Mn	0.048	1105.38	730.36	694.24	964.50	1005.40
Mo	0.004	0.94	0.69	0.83	0.94	0.78
Na	0.555	769.56	800.77	592.73	940.01	648.05
Ni	0.036	65.46	52.61	42.70	65.57	53.92
P	0.662	533.37	449.71	349.44	482.37	444.63
Pb	0.025	17.69	12.03	10.84	14.10	13.19
Pd	0.007	0.54	0.76	0.55	0.53	0.63
Pt	0.019	0.00	0.24	0.00	0.00	0.07
Rb	0.004	49.02	52.16	43.39	58.06	47.61
Sb	0.004	2.34	1.02	0.38	0.54	1.37
Se	0.383	1.50	1.38	4.43	1.53	1.49
Sr	0.004	12.77	13.00	14.06	14.75	10.75
Th	0.010	6.48	5.82	4.25	6.17	5.88
Ti	0.096	428.47	549.57	1047.83	660.14	389.02
Tl	0.007	0.23	0.24	0.22	0.28	0.24
U	0.003	1.13	0.86	0.77	1.05	0.90
V	0.014	95.87	78.28	67.13	104.09	72.85
Zn	0.680	61.88	43.70	28.06	41.26	35.15

Table 4:5 Metal concentrations (µg/L) in each environmental rainwater extract.

	Detection limit (ng/L)	A: Iso-line	A: Bt	A: Bt + RR	A: RR	B: Iso-line	B: Bt	B: Bt + RR	B: RR	C: Iso-line	C: Bt	C: Bt + RR	C: RR	D: Iso-line	D: Bt	D: Bt + RR	D: RR	E: Iso-line	E: Bt	E: Bt + RR	E: RR
Ag	9.8	0.1	0.0	0.0	n.d.	0.1	n.d.	n.d.	0.0	0.1	n.d.	0.0	0.1	n.d.	n.d.	0.0	n.d.	n.d.	0.0	0.1	0.0
Al	1771.0	3032	1722	2324	1998	901	1088	2653	1242	937	729	1069	1696	1865	988	2011	1510	572	428	1567	1000
As	12.2	4.1	1.4	1.5	1.2	0.5	0.6	1.2	1.0	1.0	0.8	0.9	1.2	1.2	0.6	1.4	1.3	0.2	0.7	1.2	0.7
Au	10.4	0.4	0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B	2462.0	23.4	18.3	20.4	19.3	6.1	16.7	15.2	16.6	28.3	16.0	8.4	9.8	17.8	10.6	15.1	16.2	9.9	3.8	9.8	7.0
Ba	21.4	118.9	74.5	93.0	80.5	47.0	45.5	96.5	55.2	39.9	33.7	42.8	65.2	73.0	46.2	66.9	62.7	26.6	21.6	57.4	42.1
Be	4.3	1.3	0.7	0.8	0.8	0.3	0.3	1.0	0.4	0.3	0.2	0.3	0.6	0.7	0.3	0.8	0.5	0.2	0.2	0.6	0.4
Bi	2.4	0.0	0.0	0.0	n.d.	0.2	n.d.	0.0	0.2	0.0	n.d.	n.d.	0.3	n.d.	n.d.	0.0	n.d.	n.d.	0.5	0.1	n.d.
Ca	1646.0	15250	16090	13960	14310	11540	14630	14250	13590	13300	13140	13130	12670	13860	13990	12640	13810	13020	10880	12810	12920
Cd	32.8	0.2	0.1	n.d.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	n.d.	0.1	0.0	0.0	0.1	0.1	0.1	0.0	n.d.
Co	8.0	2.9	1.2	1.4	1.2	0.5	0.8	0.8	0.7	0.8	0.9	0.8	1.5	1.4	0.8	0.8	1.0	0.6	0.5	1.4	1.0
Cr	885.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cu	12.2	47.7	32.0	26.5	59.1	18.5	37.8	28.7	21.8	24.2	30.3	34.4	35.2	49.9	55.9	16.8	32.0	26.7	19.2	22.0	43.0
Hg	1096.0	0.9	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.0	0.0	0.1	0.0	0.1	n.d.	0.0	0.0	0.1	0.0	0.0
K	8.5	7846	9375	7677	6482	3851	4238	3733	7389	6269	6896	5594	7310	6361	3739	3296	7254	4649	3986	7109	6851
Mg	3378.0	9794	10140	9428	9187	7022	9389	8621	9201	9539	9756	9439	8527	8053	8922	7973	9210	9356	8236	8653	9133
Mn	29.9	867.5	77.9	112.5	81.4	29.8	43.3	43.5	40.8	37.0	51.5	59.9	84.0	109.3	44.0	48.2	62.7	28.2	18.6	117.1	64.3
Mo	16.3	4.2	4.0	3.6	3.5	2.0	3.1	3.5	4.0	4.5	3.3	3.9	4.5	3.7	3.7	4.5	3.2	3.9	3.4	3.4	3.8
Ni	2.3	16.0	9.2	7.9	6.5	3.1	4.3	6.4	5.9	5.0	5.2	5.1	8.2	7.4	4.5	6.0	6.6	3.0	3.1	5.1	4.3
P	148.0	113.9	n.d.	29.7	n.d.	n.d.	n.d.	139.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	116.2	n.d.	n.d.	n.d.	61.2	n.d.
Pb	67.0	12.0	6.5	9.1	6.7	2.9	5.8	6.3	4.9	3.5	2.9	4.5	6.6	7.7	7.8	5.2	6.2	2.1	5.4	5.5	4.3
Pd	944.1	2.3	0.6	0.6	0.6	0.3	0.3	0.5	0.3	0.3	0.3	0.3	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.3	0.2
Pt	1.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1	0.4	n.d.	n.d.	n.d.	0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Rb	0.9	13.5	9.2	11.8	9.2	5.9	6.2	11.1	7.5	5.9	5.9	6.5	8.4	8.8	5.8	9.4	8.3	4.6	3.8	9.0	6.7
Sb	6.2	40.3	28.8	30.1	29.9	29.1	27.1	28.5	27.2	23.5	26.1	26.8	25.9	28.3	24.4	29.5	28.6	24.5	24.8	25.9	27.2
Se	2.3	4.3	2.6	2.6	2.6	2.5	2.5	3.2	1.8	2.8	1.8	1.8	3.0	2.1	2.5	2.3	2.8	2.7	1.7	2.7	2.3
Sr	4.0	44.6	41.9	40.8	40.4	33.5	41.0	44.3	38.1	36.0	32.8	35.3	35.3	39.2	39.5	37.9	41.1	35.2	27.5	36.4	35.5
Th	107.7	1.6	0.3	0.3	0.3	0.1	n.d.	0.3	0.1	n.d.	n.d.	n.d.	0.1	0.1	0.0	0.3	0.0	n.d.	n.d.	0.0	n.d.
Ti	5.6	3.8	0.9	2.2	1.7	n.d.	n.d.	2.2	0.8	n.d.	n.d.	0.1	5.3	3.8	n.d.	2.0	1.3	n.d.	n.d.	0.8	0.0
Tl	2.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	n.d.	0.0	0.0
U	41.4	1.1	0.7	0.8	0.7	0.2	0.4	0.9	0.4	0.4	0.4	0.4	0.7	0.7	0.4	0.7	0.6	0.3	0.3	0.6	0.4
V	9.3	11.5	6.2	8.1	7.5	2.7	3.5	9.2	4.6	3.1	2.0	3.6	5.2	5.9	2.8	6.9	5.3	1.6	1.1	4.5	2.9
Zn	1.1	1500.0	205.9	83.3	113.2	65.9	104.1	108.1	107.8	81.0	76.5	73.5	107.4	104.5	71.4	89.3	58.5	51.7	112.2	87.6	63.3

4.2 Rainwater parameters

Various physico-chemical parameters of the rainwater were determined on the day of sampling, as part of routine water analysis, also, to evaluate if there are constituents in the water that may influence the cell assay results. The rainwater temperature was 16°C; the pH was 5.73; dissolved oxygen was 16.1%; total dissolved solids 16.9 and alkalinity 10 mg/L. The nutrient results were 0.3 mg/L NO_3–N nitrates, 2 mg/L NO_2^- nitrites, 17 mg/L SO_4^{2-} sulphates, 0.08 mg/L PO_4^{3-} phosphorus, 0.42 mg/L NH_3–N nitrogen ammonia, 4 µg/L S^{2-} sulfides and no free chlorine levels. Two metals were detected: Cd and Pt at 3.2 and 0.5 mg/L respectively.

4.3 Quantification of Cry1Ab, glyphosate and 2,4-D using ELISAs

4.3.1 Quality control (QC)/Quality assurance (QA) for ELISAs

To improve the accuracy of quantification results, QC/QA protocols were incorporated into the method. The samples, a blank, fortified samples and standards to obtain a calibration curve were subjected to ELISA plates in triplicate. The fortified samples were blank soil samples that had been spiked with a high and a low concentration of Cry1Ab, glyphosate and 2,4-D. The recovery of these compounds was determined after the extraction with rainwater. The correlation coefficients (CVs) calculated for each sample, across the glyphosate, 2,4-D and Cry1Ab plates were deemed acceptable with good precision < 20%. The limit of detection (LOD) and limit of quantification (LOQ) were derived from the calibration curve of each target compound from the various ELISA plate tests (Table 4.6).

Table 4:6 The LOD and LOQ values for the Cry1Ab, glyphosate and 2,4-D using ELISA plates

	Cry1Ab (µg/L)	Glyphosate (µg/L)	2,4-D (µg/L)
LOD	0.3	0.4	0.1
LOQ	1.2	1.3	0.5

LOD: Limit of detection; LOQ: limit of quantification

The recovery results for extraction of Cry1Ab from the spiked soil was <LOD for both the low (0.1 µg/L) and high (3 µg/L) concentrations. The low concentrations for glyphosate (0.1 µg/L) and 2,4-D (2 µg/L) were also <LOD, but 51% of the high concentrations for both glyphosate (3.5 µg/L) and 2,4-D (50 µg/L) were extracted. The extraction efficiency of 51% is not high, in an analytical sense, but it is in accordance with what is reported in the literature for the extraction of these compounds from soil (Torstensson, Lundgren & Stenström, 1989; Todorovic *et al*.,

2013). Reporting about the extraction efficiency of the fortified samples is an attempt to get a sense of the extractability of the target compounds. This gives an indication of their real levels in the soil, because if you are able to quantify any of them it means that their real levels are much higher.

4.3.2 Levels after extractions ELISA data

The soil samples obtained from the field trial, at the end of the season, were extracted with rainwater to obtain the water-soluble and bioavailable fraction. These rain extracts were subjected to ELISAs, specific for Cry1Ab, glyphosate and 2,4-D, to measure the concentrations of these compounds after extraction (Table 4.7), and subsequently in the soil (Table 4.8). The water samples hade a four times lower level of the target analytes because 10 g soil was extracted into 40 mL rainwater.

Table 4:7 Concentrations (µg/L) of target compounds glyphosate, 2,4-D and Cry1Ab in the rain extracts after extraction of the soil from the field trial

Herbicides applied	Maize varieties	Glyphosate	2,4-D	Cry1Ab
Plot A: Control	Iso-line	<LOQ	1.97 ± 0.07	1.80 ± 0.07
	Bt	<LOQ	1.71 ± 0.31	1.43 ± 0.10
	Bt and RR	<LOQ	1.79 ± 0.51	1.80 ± 0.05
	RR	<LOQ	1.65 ± 0.34	1.63 ± 0.07
Plot B: Pre- and post emergent Roundup®	Iso-line	3.19 ± 0.00	1.23 ± 0.39	<LOQ
	Bt	2.90 ± 0.44	1.23 ± 0.34	<LOQ
	Bt and RR	3.19 ± 0.00	1.69 ± 0.18	1.75 ± 0.08
	RR	3.19 ± 0.00	2.14 ± 0.27	1.31 ± 0.09
Plot C: Pre- and post emergent Roundup® and 2,4-D	Iso-line	<LOD	55.59 ± 22.14	<LOQ
	Bt	3.19 ± 0.00	58.45 ± 19.27	<LOQ
	Bt and RR	2.94 ± 0.43	64.26 ± 14.27	<LOQ
	RR	3.17 ± 0.04	62.91 ± 15.66	1.45 ± 0.06
Plot D: Post emergent Roundup® and 2,4-D	Iso-line	3.11 ± 0.06	65.20 ± 0.90	1.43 ± 0.13
	Bt	2.31 ± 0.77	55.16 ± 22.40	<LOQ
	Bt and RR	3.19 ± 0.00	67.95 ± 0.94	1.54 ± 0.11
	RR	3.01 ± 0.17	49.43 ± 2.61	1.39 ± 0.02
Plot E: Only 2,4-D	Iso-line	<LOD	55.89 ± 11.28	<LOQ
	Bt	<LOD	62.45 ± 2.79	<LOD
	Bt and RR	<LOQ	25.69 ± 5.71	1.36 ± 0.07
	RR	2.90 ± 0.49	72.67 ± 7.00	<LOQ

No glyphosate was detected in: A- control soil; the iso-line from plot C and the iso-line, Bt and BT& RR from plot E. Glyphosate concentrations ranged from 2.9–3.19 µg/L in plots B, C and D, where Roundup® was sprayed (Table 4.7). In plot E-RR 2.9 µg/L of glyphosate was detected, which may be due to drops that drifted onto another plot from the boom sprayer, even though care was taken to avoid any cross-contamination. 2,4-D was detected throughout the field trial. These levels ranged from 1.23–72.6 µg/L, with the lowest concentrations observed in plots A and B, where no 2,4-D was sprayed and the highest levels in plots C, D and E where the same amount of post-emergent 2.4-D was applied (Table 4.7). Cry1Ab proteins, expressed from Bt maize, were detected in 11 of the 20 treatments, but scattered all over the field. There was no clear pattern. The iso-line and RR varieties were not expected to deliver concentrations of Cry1Ab. Although, the highest levels of Cry1Ab is expressed during pollination, and pollen drift (Strain & Lydy, 2015) which could be the reason for the widespread detections of Cry1Ab (Table 4.7).

Table 4:8 Concentrations (ng/g) of target compounds glyphosate, 2.4-D and Cry1Ab in the soil from field trial

Herbicides applied	Maize varieties	Glyphosate	2,4-D	Cry1Ab
Plot A: Control	Iso-line	<LOQ	7.89 ± 0.07	7.21 ± 0.07
	Bt	<LOQ	6.83 ± 0.31	5.74 ± 0.10
	Bt and RR	<LOQ	7.16 ± 0.51	7.21 ± 0.05
	RR	<LOQ	6.59 ± 0.34	6.51 ± 0.07
Plot B: Pre- and post emergent Roundup®	Iso-line	12.74 ± 0.00	4.91 ± 0.39	<LOQ
	Bt	11.62 ± 0.44	4.93 ± 0.34	<LOQ
	Bt and RR	12.74 ± 0.00	6.75 ± 0.18	7.01 ± 0.08
	RR	12.74 ± 0.00	8.55 ± 0.27	5.24 ± 0.09
Plot C: Pre- and post emergent Roundup® and 2,4-D	Iso-line	<LOD	222.35 ± 22.14	<LOQ
	Bt	12.74 ± 0.00	233.82 ± 19.27	<LOQ
	Bt and RR	11.74 ± 0.43	257.03 ± 14.27	<LOQ
	RR	12.66 ± 0.04	251.65 ± 15.66	5.82 ± 0.06
Plot D: Post emergent Roundup® and 2,4-D	Iso-line	12.45 ± 0.06	260.78 ± 0.90	5.72 ± 0.13
	Bt	9.23 ± 0.77	220.64 ± 22.40	<LOQ
	Bt and RR	12.74 ± 0.00	271.79 ± 0.94	6.14 ± 0.11
	RR	12.04 ± 0.17	197.71 ± 2.61	5.56 ± 0.02
Plot E: Only 2,4-D	Iso-line	<LOD	223.57 ± 11.28	<LOQ
	Bt	<LOD	249.80 ± 2.79	<LOD
	Bt and RR	<LOQ	102.78 ± 5.71	5.43 ± 0.07
	RR	11.62 ± 0.49	290.68 ± 7.00	<LOQ

4.4 Cell results

To determine the endocrine disruptive activity of the single compounds vs mixtures, the reporter-gene assays consisted of exposure to 1) single pure compounds; 2) mixtures of the pure compounds; 3) formulations; 4) mixtures of the formulations and 5) environmental mixtures from the field trial. The two different cells lines used were the H4IIE-*luc* and MDA-kb2 with the AhR and, AR and GR receptors, respectively. The effective concentrations where 50% activity (EC_{50}) occurs, was determined for each positive control. Relative light units (RLU) of the samples below the EC_{10} value, of each assay were considered to be <LOQ. In contrast, for the AR inhibition assay, RLU's above the IC_{90} were not considered to have an inhibitive effect.

4.4.1 Exposure list – from materials and method section

I have included the exposure list from the materials and methods section 3.5 (Table 3.2) as a reminder of the concentrations to which the cells were exposed to (Table 4.9).

Table 4:9 The list of active ingredients (pure compounds) and formulation mixes used in the bio-assays.* Concentrations that remained constant during the entire dose-response exposures. The varying ingredient was diluted twice. Six dilutions were tested.

Compounds	Concentrations (µg/L)		
	Glyphosate	2,4-D	Cry1Ab
	Active ingredients		
Cry1Ab	0	0	7
2,4-D	0	220	0
Glyphosate	13	0	0
AI Mix 1	13	220*	4*
AI Mix 2	13*	220*	4
AI Mix 3	13*	220	4*
AI Mix 4	13	220	4
AI Mix 5	1.2	220	3

AI: active ingredient

Table 4:9 continued:10 The list of formulation mixes used in the bio-assays.
*** Concentrations that remained constant during the entire dose-response exposures. The varying ingredient was diluted twice. Six dilutions were tested.**

Compounds	Concentrations (µg/L)		
Formulations			
	Glyposhate (from Roundup® PowerMax)	2,4-D (from 2,4-D amine 480 SL)	Cry1Ab (pure compound)
Roundup®Powermax	13	0	0
2,4-D amine 480 SL	0	220	0
Formulation mix 1	13	220*	4*
Formulation mix 2	13*	220*	4
Formulation mix 3	13*	220	4*
Formulation mix 4	13	220	4
Formulation mix 5	1.2	220	3

Table 4:9 continued: The list of environmental extracts used in the bio-assays.

Compounds	Concentrations (µg/L)		
	Glyphosate	2,4-D	Cry1Ab
Environmental extracts			
A: Iso-line	0	8	7
A: Bt	3	7	6
A: Bt + RR	5	7	7
A: RR	3	7	7
B: Iso-line	13	5	4
B: Bt	12	5	5
B: Bt + RR	13	7	7
B: RR	13	9	5
C: Iso-line	0	171	4
C: Bt	13	189	3
C: Bt + RR	12	224	4
C: RR	13	216	6
D: Iso-line	12	261	6
D: Bt	9	169	4
D: Bt + RR	13	272	6
D: RR	12	198	6
E: Iso-line	1	221	3
E: Bt	1	250	1
E: Bt + RR	2	101	5

4.4.2 Aryl-hydrocarbon (AhR) activation

The H4IIE-*luc* cells have an AhR receptor, of which the activation was investigated in this study. The cells were exposed to the compounds listed in the exposure list (Table 4.9) for 72 h. The positive control, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was also included in the exposures as the AhR reference agonist (Fig. 4.1). The responses obtained from the samples were expressed in terms of the %TCDDmax of the positive control and converted into TCDD equivalents (TCDD-EQs). The EC_{50} of the TCDD dose-response curve was 2.86 µg/L and the maximum fold induction of luciferase between assays varied from 6–25.

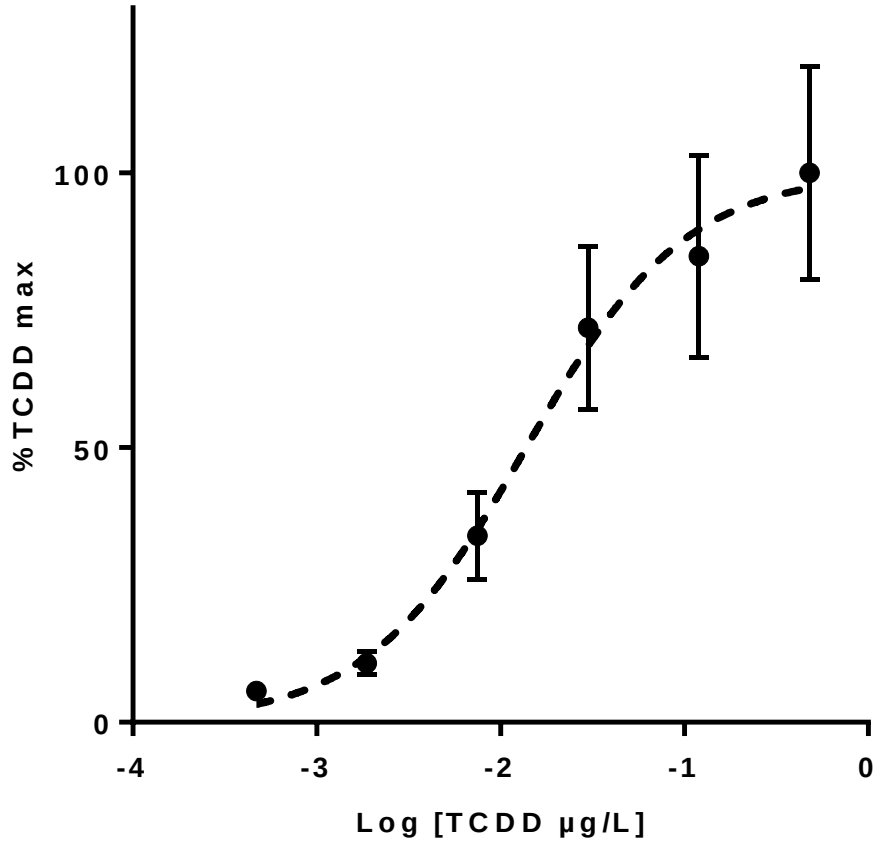

Figure 4:1 The %TCDDmax of the AhR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the H4IIE-*luc* cells (the bars indicate standard deviation).

The viability of the cells was determined by use of the MTT assay. These assays were run in parallel to the luminescence assays to ensure that no effects are due to the exposure compounds and not dead or dying cells. None of the compounds was cytotoxic at the concentrations they were tested and viability ranged from 93–151% for the H4IIE-*luc* cells (Table 4.10). The maximum %TCDD elicited by any of the test analytes and mixtures was 6.1% (Table 4.10). These responses were all below the limit of quantification (LOQ) of the assay and therefore no toxic equivalents could be determined.

Table 4:110 AhR activity (%TCDD max) and percentage viability of the cells after exposure to various compounds

Exposures	%TCDD max	%viability	TCDD-EQ
Active ingredients			
Cry1Ab	11.8 ± 0.8	*151 ± 0.09	<LOQ
2,4-D	12.4 ± 1.3	*127 ± 0.07	<LOQ
Glyphosate	13.7 ± 1.1	*132 ± 0.14	<LOQ
AI mix 1	12.5 ± 0.9	107 ± 0.12	<LOQ
AI mix 2	12.1 ± 0.7	102 ± 0.1	<LOQ
AI mix 3	12.4 ± 0.6	105 ± 0.12	<LOQ
AI mix 4	13.1 ± 0.1	*123 ± 0.1	<LOQ
AI mix 5	11.4 ± 1.3	117 ± 0.1	<LOQ
Formulations			
Roundup®	16.9 ± 0.2	102 ± 0.1	<LOQ
2,4-D amine SL	17.2 ± 1.5	93 ± 0.08	<LOQ
Formulation mix 1	11.2 ± 1.3	108 ± 0.1	<LOQ
Formulation mix 2	10.8 ± 0.5	*130 ± 0.1	<LOQ
Formulation mix 3	11.3 ± 0.7	*134 ± 0.13	<LOQ
Formulation mix 4	16.8 ± 0.5	*118 ± 0.05	<LOQ
Formulation mix 5	11.2 ± 0.4	114 ± 0.04	<LOQ
Environmental extracts			
A: Iso-line	4.1 ± 0.4	110 ± 0.1	<LOQ
A: Bt	3.8 ± 0.1	96 ± 0.1	<LOQ
A: Bt + RR	5.2 ± 0.1	*142 ± 0.2	<LOQ
A: RR	6.0 ± 0.2	108 ± 0.3	<LOQ
B: Iso-line	6.1 ± 0.1	125 ± 0.2	<LOQ
B: Bt	3.4 ± 0.2	126 ± 0.2	<LOQ
B: Bt + RR	3.8 ± 0.1	90 ± 0.2	<LOQ
B: RR	3.5 ± 0.2	109 ± 0.2	<LOQ
C: Iso-line	3.4 ± 0.2	99 ± 0.2	<LOQ
C: Bt	3.8 ± 0.3	96 ± 0.1	<LOQ
C: Bt + RR	2.5 ± 0.2	105 ± 0.2	<LOQ
C: RR	2.6 ± 0.3	86 ± 0.1	<LOQ
D: Iso-line	5.8* ± 0.2	93 ± 0.1	<LOQ
D: Bt	5.4 ± 0.4	93 ± 0.1	<LOQ
D: Bt + RR	5.3 ± 1.1	*84 ± 0.1	<LOQ
D: RR	3.0 ± 0.1	93 ± 0.1	<LOQ
E: Iso-line	4.0 ± 1.3	106 ± 0.1	<LOQ
E: Bt	3.8 ± 0.2	92 ± 0.1	<LOQ
E: Bt + RR	3.7 ± 0.2	*80 ± 0.1	<LOQ
E: RR	3.9 ± 0.3	86 ± 0.2	<LOQ

AI: active ingredient; * statistically significantly different from control ($p < 0.05$)

4.4.3 Androgen receptor (AR) activation

The MDA-kb2 cell line was used to determine androgen receptor (AR) activation. To be able to compare the responses from this cell line, the assay was first validated. Testosterone was used as the agonist for the AR and a dose-response curve was created (Fig. 4.2) The maximum fold induction of luciferase at the highest testosterone concentration and the solvent control varied from 6 to 11 across experiments. The EC_{50} of the testosterone dose-response curve was 0.04 µg/L. These parameters were in line with previously published values using the same cellular model (with slight differences in the assay procedures), that makes our results comparable to other studies (Wilson *et al.*, 2002; Aït-Aïssa *et al.*, 2010). None of the extracts was cytotoxic with the percentage viability of the cells varying from 67–192% (Table 4.11).

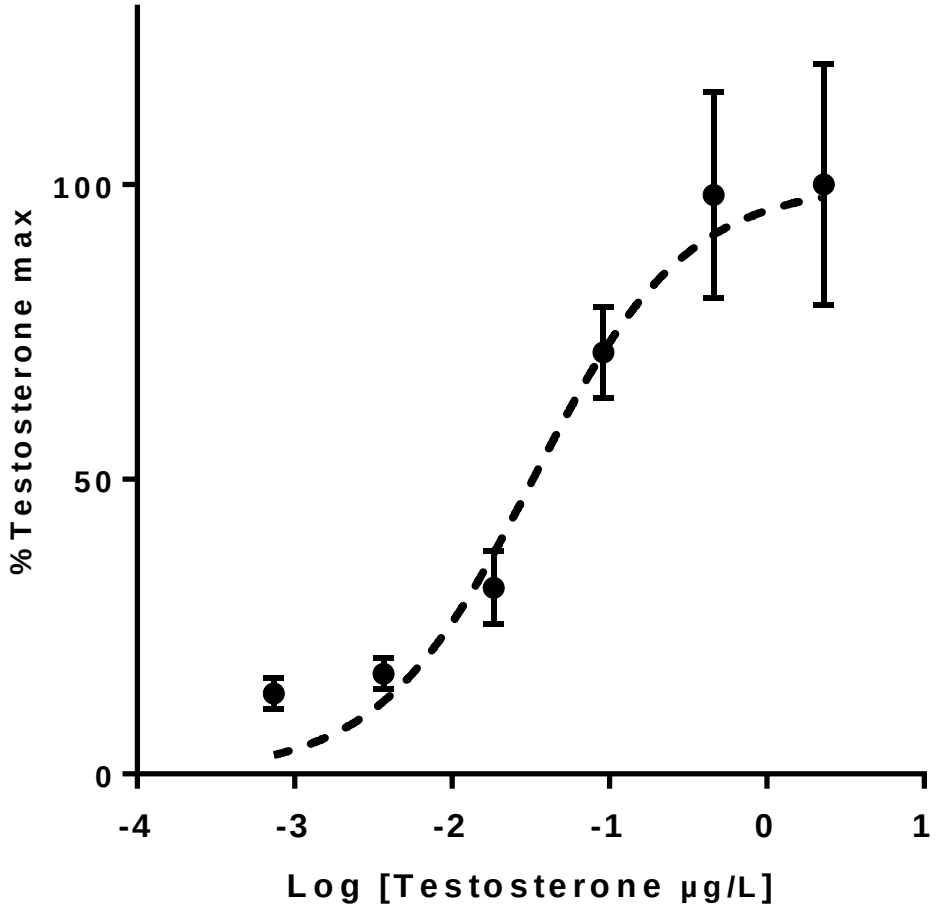

Figure 4:2 Luciferase induction by the AR agonist, testosterone, expressed as %Testosterone max (the bars indicate standard deviation).

Table 4:12 AR activity (%Testosterone max) and percentage viability of the cells after exposure to various compounds

Exposures	%Testosterone max	%viability
Active ingredients		
Cry1Ab	7.4 ± 0.7	*126 ± 0.03
2,4-D	7.9 ± 0.9	*117 ± 0.05
Glyphosate	9.3 ± 0.4	111 ± 0.3
AI mix 1	10 ± 1.1	82 ± 0.06
AI mix 2	13 ± 1	*67 ± 0.11
AI mix 3	13 ± 1.9	*41 ± 0.17
AI mix 4	16 ± 0.9	96 ± 0.04
AI mix 5	11 ± 1.7	123 ± 0.23
Formulations		
Roundup®	5.6 ± 0.6	*91 ± 0.11
2,4-D amine SL	8.2 ± 0.6	97 ± 0.09
Formulation mix 1	4.9 ± 0.7	103 ± 0.21
Formulation mix 2	8.0 ± 1.1	118 ± 0.1
Formulation mix 3	10 ± 0.5	*128 ± 0.07
Formulation mix 4	9.1 ± 0.4	98 ± 0.05
Formulation mix 5	9.8 ± 1	99 ± 0.05
Environmental extracts		
A: Iso-line	1.6 ± 0.9	*146 ± 0.3
A: Bt	52 ± 4.2	*119 ± 0.1
A: Bt + RR	101 ± 6.4	104 ± 0.15
A: RR	47 ± 12	*129 ± 0.11
B: Iso-line	176 ± 13	*126 ± 0.1
B: Bt	177 ± 3.7	111 ± 0.1
B: Bt + RR	226 ± 8.3	107 ± 0.2
B: RR	117 ± 5.8	110 ± 0.17
C: Iso-line	0 ± 0	130 ± 0.24
C: Bt	52 ± 13	133 ± 0.52
C: Bt + RR	86 ± 18	*142 ± 0.22
C: RR	171 ± 18	121 ± 0.27
D: Iso-line	8.3 ± 3.4	*137 ± 0.24
D: Bt	0 ± 0	98 ± 0.24
D: Bt + RR	3 ± 1.9	*127 ± 0.08
D: RR	1 ± 2.18	*120 ± 0.02
E: Iso-line	23 ± 1.7	*128 ± 0.07
E: Bt	0.3 ± 0.9	*192 ± 0.35
E: Bt + RR	0.2 ± 0.8	140 ± 0.09
E: RR	16 ± 1.2	98 ± 0.2

AI: active ingredient; * statistically significantly different from control ($p < 0.05$)

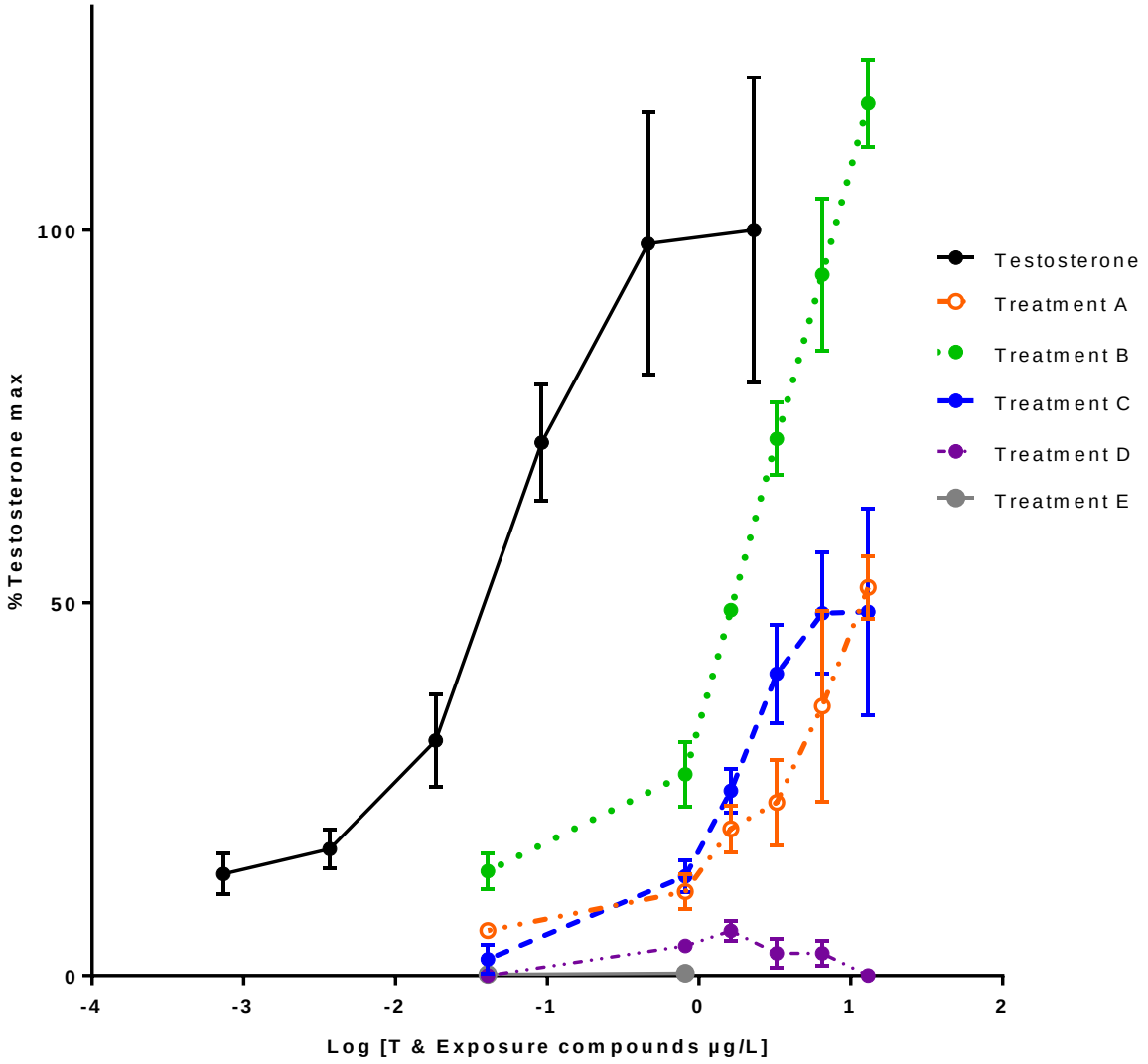

Figure 4:3 Dose-response curve of the highest %Testosterone max obtained by each environmental plot, A to E, compared to the AR reference agonist, testosterone (T).

The pure compounds (active ingredients) and their formulations did not cause activation of the AR and their responses were <LOQ. The highest AR activity related to testosterone equivalents (TTEQ) was caused by extracts from plots A, B and C (Fig 4.3 and Fig 4.6). Although plot A did not receive herbicides, the effects could be due to other environmental chemicals present in the extract (Fig. 4.4). Plots B and C both received pre-and post-emergent Roundup® applications which could have contributed to the effects observed from these plots. Plot D also received Roundup®, but only one post-emergent application and caused no effects. Plot E that only received 2,4-D also caused no AR activation (Fig. 4.5 and Fig. 4.6).

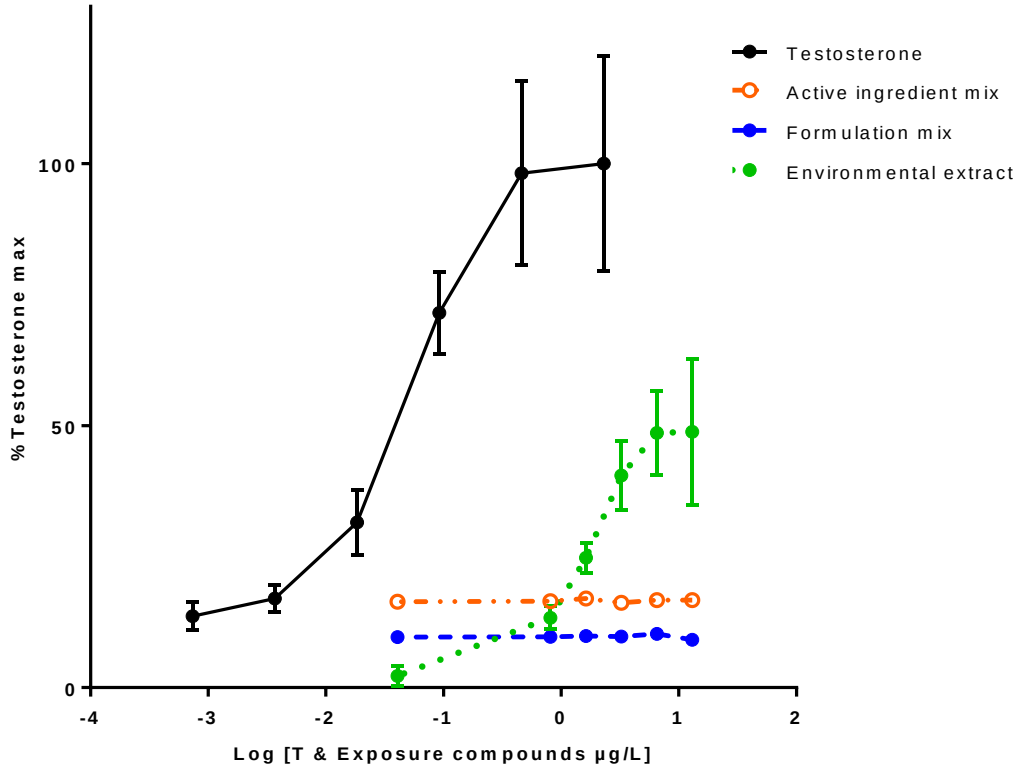

Figure 4:4 Dose-response curve of AR agonist, Testosterone (T), active ingredient mix, formulation mix and plot C-Bt & RR treatment

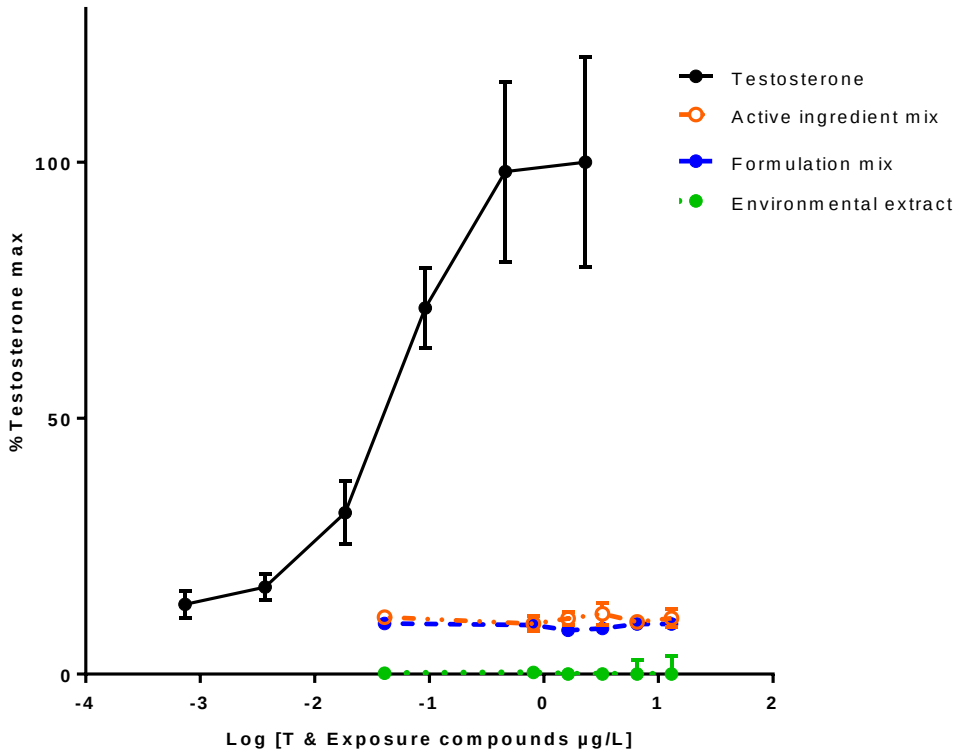

Figure 4:5 Dose-response curve of AR agonist, Testosterone (T), active ingredient mix, formulation mix and plot E-Bt & RR treatment.

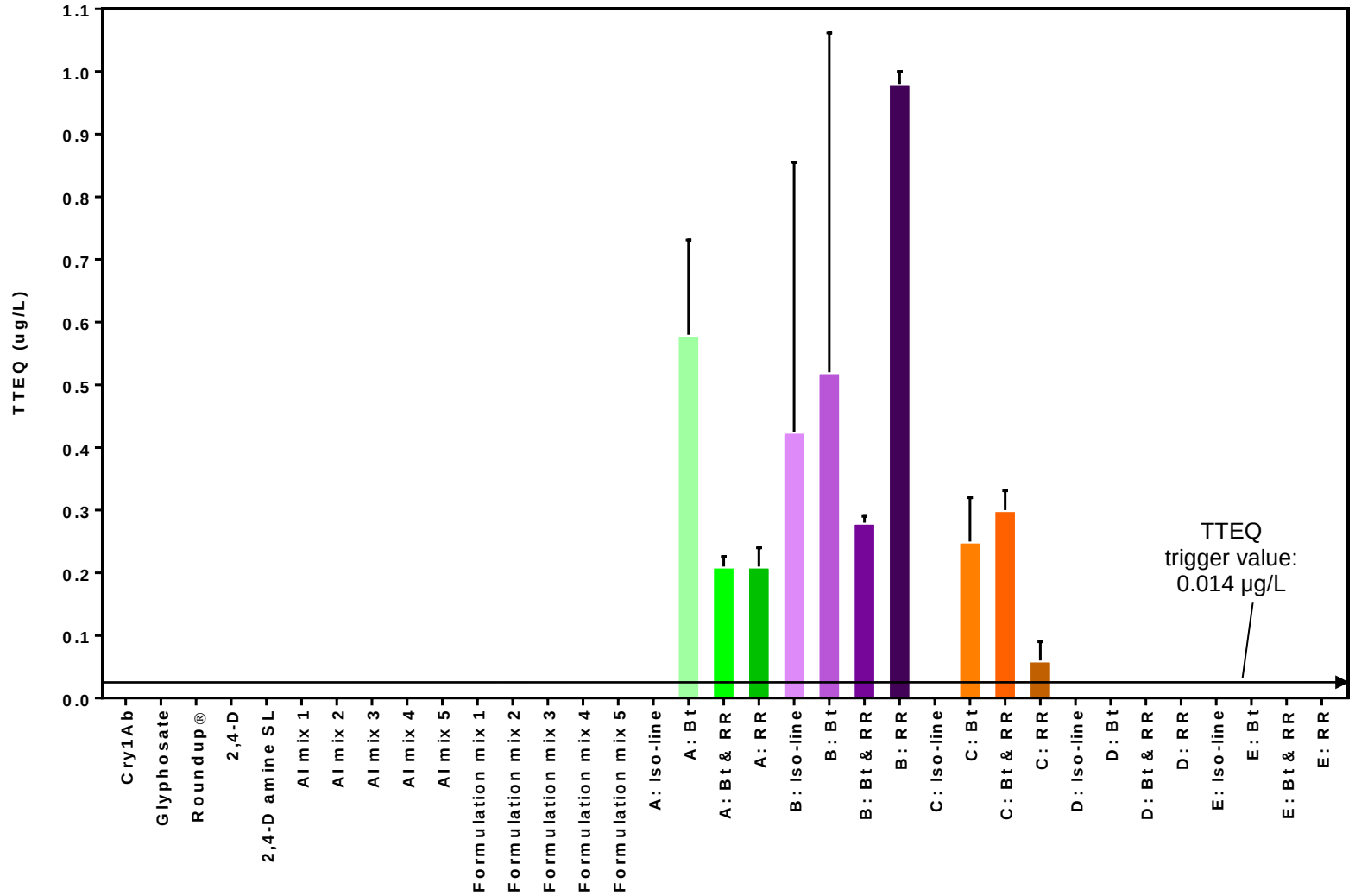

Figure 4:6 Testosterone equivalents (TTEQ) obtained after exposure to the MDA-kb2 cells. Active ingredients, formulations and the 20 environmental treatments were included. The cultivars were Iso-line, Bt, Bt & RR and RR and the 5 spraying regimes: A: Control, B: Roundup® 2x, C: Roundup® 2x and 2,4-D, D: Roundup® 1x and 2,4-D and treatment E were only sprayed with 2,4-D (the bars indicate standard deviation).

4.4.4 Glucocorticoid receptor (GR) activation

The MDA-kb2 cell line has androgen and glucocorticoid receptors. During the AR activation assay, it is impossible to distinguish to which receptor the ligands have bound, to cause light. If any light was emitted by the reaction between luciferase and its substrate luciferin, then the assay must be repeated to identify which of the two available receptors was activated. This is done by repeating the assay but then block one of the receptors. In this case, the AR was blocked, by incubating the cells with 0.2 µg/mL flutamide. Any light produced would then come from GR activation. Dexamethasone was used as the reference compound for the GR and a dose-response curve was obtained (Fig. 4.7). The GR agonist, dexamethasone, had an EC_{50} of 6.6 µg/L and induced luciferase 40–45 times compared to the solvent control cells which coincided with Aït-Aïssa *et al*., (2010). The cells were all viable at the end of the exposures according to the MTT results (Table 4.12).

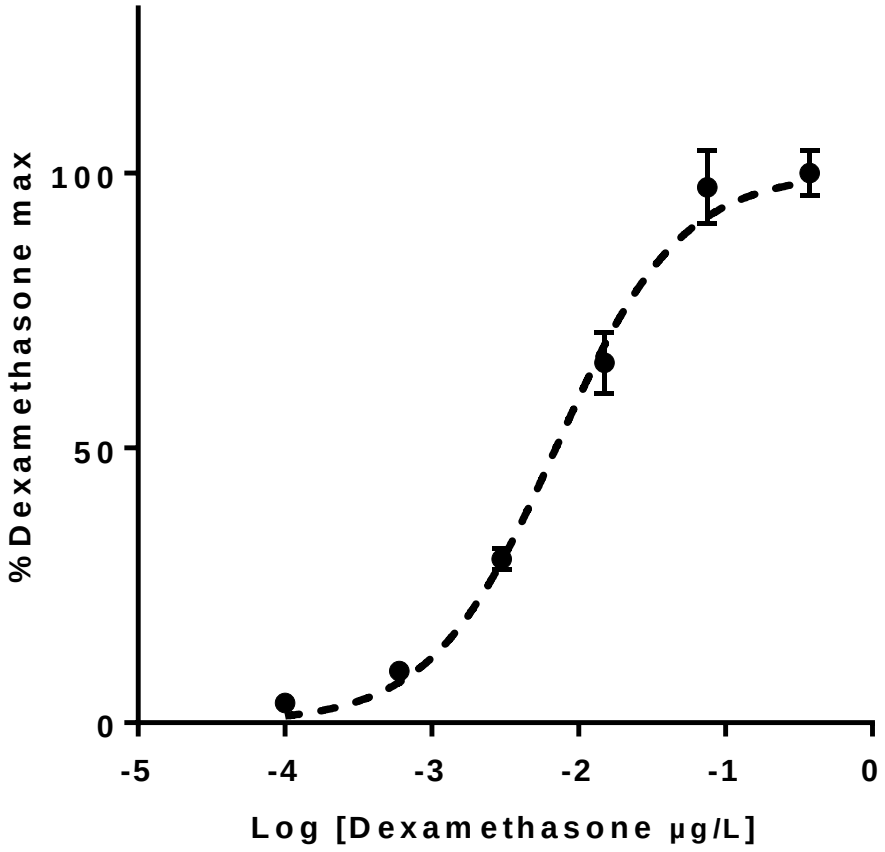

Figure 4:7 Dose-response curve of dexamethasone, as the GR agonist, expressed as % Dexamethasone (the bars indicate standard deviation).

Table 4:132 GR activity (%Dexamethasone max) and percentage viability of the cells after exposure to various compounds

Exposures	%Dexamethasone max	%viability	DexaEQ (µg/L)
Active ingredients			
Cy1Ab	0.8 ± 0.2	*126 ± 0.03	<LOQ
2,4-D	1.6 ± 0.3	*117 ± 0.05	<LOQ
Glyphosate	2.2 ± 0.1	111 ± 0.3	<LOQ
AI mix 1	1.6 ± 0.1	82 ± 0.06	<LOQ
AI mix 2	1.8 ± 0.2	*67 ± 0.11	<LOQ
AI mix 3	1.4 ± 0.02	*41 ± 0.17	<LOQ
AI mix 4	1.5 ± 0.1	96 ± 0.04	<LOQ
AI mix 5	1.6 ± 0.04	123 ± 0.23	<LOQ
Formulations			
Roundup®	0.8 ± 0.1	*91 ± 0.11	<LOQ
2,4-D amine SL	1.0 ± 0.1	97 ± 0.09	<LOQ
Formulation mix 1	1.3 ± 0.3	103 ± 0.21	<LOQ
Formulation mix 2	1.6 ± 0.4	118 ± 0.1	<LOQ
Formulation mix 3	2.0 ± 0.2	*128 ± 0.07	<LOQ
Formulation mix 4	2.0 ± 0.1	98 ± 0.05	<LOQ
Formulation mix 5	2.0 ± 0.1	99 ± 0.05	<LOQ
Environmental extracts			
A: Iso-line	4.3 ± 0.6	*146 ± 0.3	<LOQ
A: Bt	11.3 ± 2.0	*119 ± 0.1	<LOQ
A: Bt + RR	0.2 ± 0.3	104 ± 0.15	<LOQ
A: RR	0.2 ± 0.3	*129 ± 0.11	<LOQ
B: Iso-line	4.4 ± 0.3	*126 ± 0.1	<LOQ
B: Bt	16.5 ± 2.0	111 ± 0.1	14.1 ± 0.6
B: Bt + RR	6.7 ± 0.8	107 ± 0.2	<LOQ
B: RR	10.7 ± 2.2	110 ± 0.17	<LOQ
C: Iso-line	13 ± 2.2	130 ± 0.24	<LOQ
C: Bt	17.8 ± 0.9	133 ± 0.52	13.8 ± 3.5
C: Bt + RR	3.5 ± 2.1	*142 ± 0.22	<LOQ
C: RR	0.4 ± 0.5	121 ± 0.27	<LOQ
D: Iso-line	1.5 ± 0.3	*137 ± 0.24	<LOQ
D: Bt	9.6 ± 1.1	98 ± 0.24	<LOQ
D: Bt + RR	0.4 ± 0.2	*127 ± 0.08	<LOQ
D: RR	4 ± 0.7	*120 ± 0.02	<LOQ
E: Iso-line	14.2 ± 2.1	*128 ± 0.07	<LOQ
E: Bt	13.1 ± 1.4	*192 ± 0.35	<LOQ
E: Bt + RR	4.9 ± 0.6	140 ± 0.09	<LOQ
E: RR	5.5 ± 0.2	98 ± 0.2	<LOQ

AI: active ingredient; DexaEQ: Dexamethasone equivalents; * statistically significantly different from control ($p < 0.05$)

The GR activation of the active ingredients and formulations, as well as their mixtures, were all <LOQ. In contrast, the environmental samples, expressed in terms of dexamethasone, had values between 3.7 and 30.7% dexamethasone max. An EC_{20} concentration was derived for the samples that had responses high enough to quantify, subsequently dexamethasone equivalents (DexaEQ) were determined and only 2 environmental extracts, B: Bt and C: Bt, caused and activation of the GR: 14.1 and 13.8 µg/L DexaEQs respectively.

4.4.5 Androgen receptor (AR) inhibition

To determine the anti-androgenic ability of the samples, they were co-incubated with an AR agonist, testosterone (at 0.02 ng/mL that creates 100% induction of the luciferase activity) (Pop *et al*., 2016), and all the samples (Table 4.9). The antagonist reference compound used in this study was flutamide and a dose-response curve was obtained (Fig. 4.8). The IC_{50} concentration was estimated at 5.2 µg/mL with a fold inhibition value of 0.1. Different approaches to determine the anti-androgenic potential of samples had been reported in literature: ideally, the IC_{50} values of the antagonist and the samples should be determined and the sample responses expressed in terms of the IC_{50} of the antagonist (Aït-Aïssa *et al*., 2010). For the anti-androgenic results acquired in this study, the samples did not respond in a dose-response manner (Table 4.13) and accurate IC_{50} values could not be determined based on a dose-response curve. It was for this reason that I determined the fold change (FC) of luciferase induction of the exposure compounds in terms of the luciferase induction by the solvent control (water). A fold change <1 indicates inhibition and a FC >1 shows possible activation (which can be compared to the activation results).

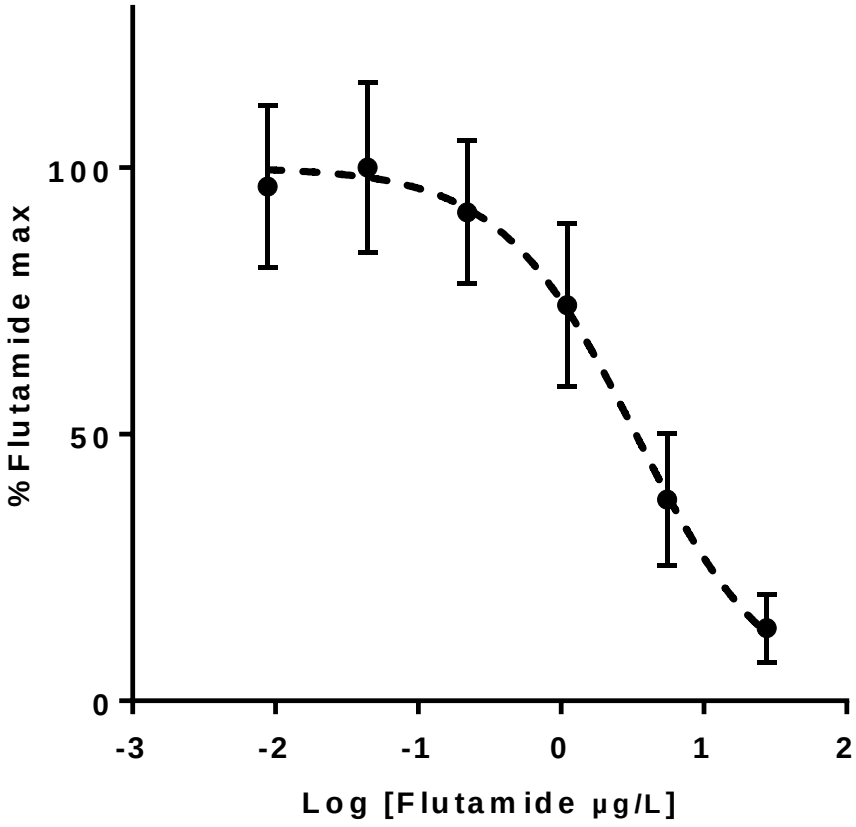

Figure 4:8 Luciferase inhibition caused by AR antagonist, flutamide, in the MDA-kb2 cells. These cells were co-incubated with 0.02 ng/mL testosterone for 48 h (the bars indicate standard deviation).

The MTT viability assay results indicated that none of the compounds or compound mixtures was cytotoxic at the exposure concentrations. (Table 4.13). These results are very important when it comes to the interpretation of the inhibition results. The viability of the cells act as a quality control to prove that low-luciferase activity is due to inhibition of the AR, and not dead or dying cells.

The active ingredients and formulations had an effect and caused inhibition of the AR with fold change values lower than 1 (Fig. 4.9). The black circles indicate statistical significant ($p < 0.05$) differences between the samples' response and the solvent control. Many compounds also had fold change values >1, which can be ascribed to activation and were tested for in AR and GR activation assays.

Table 4:13 AR antagonistic activity (%Flutamide max) and percentage viability of the cells after exposure to various compounds

Exposures	%Flutamide max	%viability
Active ingredients		
Cy1Ab	36 ± 4.4	*126 ± 0.03
2,4-D	46 ± 2.7	*117 ± 0.05
Glyphosate	63 ± 7	111 ± 0.3
AI mix 1	32 ± 6	82 ± 0.06
AI mix 2	54 ± 10	*67 ± 0.11
AI mix 3	75 ± 5	*41 ± 0.17
AI mix 4	78 ± 6	96 ± 0.04
AI mix 5	45 ± 11	123 ± 0.23
Formulations		
Roundup®	30 ± 8.8	*91 ± 0.11
2,4-D amine SL	60 ± 14	97 ± 0.09
Formulation mix 1	54 ± 14	103 ± 0.21
Formulation mix 2	81 ± 12	118 ± 0.1
Formulation mix 3	71 ± 11	*128 ± 0.07
Formulation mix 4	76 ± 14	98 ± 0.05
Formulation mix 5	85 ± 7	99 ± 0.05
Environmental extracts		
A: Iso-line	104 ± 12	*146 ± 0.3
A: Bt	124 ± 12	*119 ± 0.1
A: Bt + RR	216 ± 19	104 ± 0.15
A: RR	172 ± 2.3	*129 ± 0.11
B: Iso-line	103* ± 3.3	*126 ± 0.1
B: Bt	138 ± 16	111 ± 0.1
B: Bt + RR	328 ± 16	107 ± 0.2
B: RR	225 ± 28	110 ± 0.17
C: Iso-line	127 ± 4.9	130 ± 0.24
C: Bt	185 ± 19	133 ± 0.52
C: Bt + RR	162 ± 13	*142 ± 0.22
C: RR	217 ± 3.1	121 ± 0.27
D: Iso-line	226 ± 33	*137 ± 0.24
D: Bt	125 ± 8.7	98 ± 0.24
D: Bt + RR	141 ± 20	*127 ± 0.08
D: RR	92 ± 9.1	*120 ± 0.02
E: Iso-line	73 ± 1.1	*128 ± 0.07
E: Bt	96 ± 7.6	*192 ± 0.35
E: Bt + RR	112 ± 9.9	140 ± 0.09
E: RR	136 ± 12.2	98 ± 0.2

AI: active ingredient; * $p < 0.05$

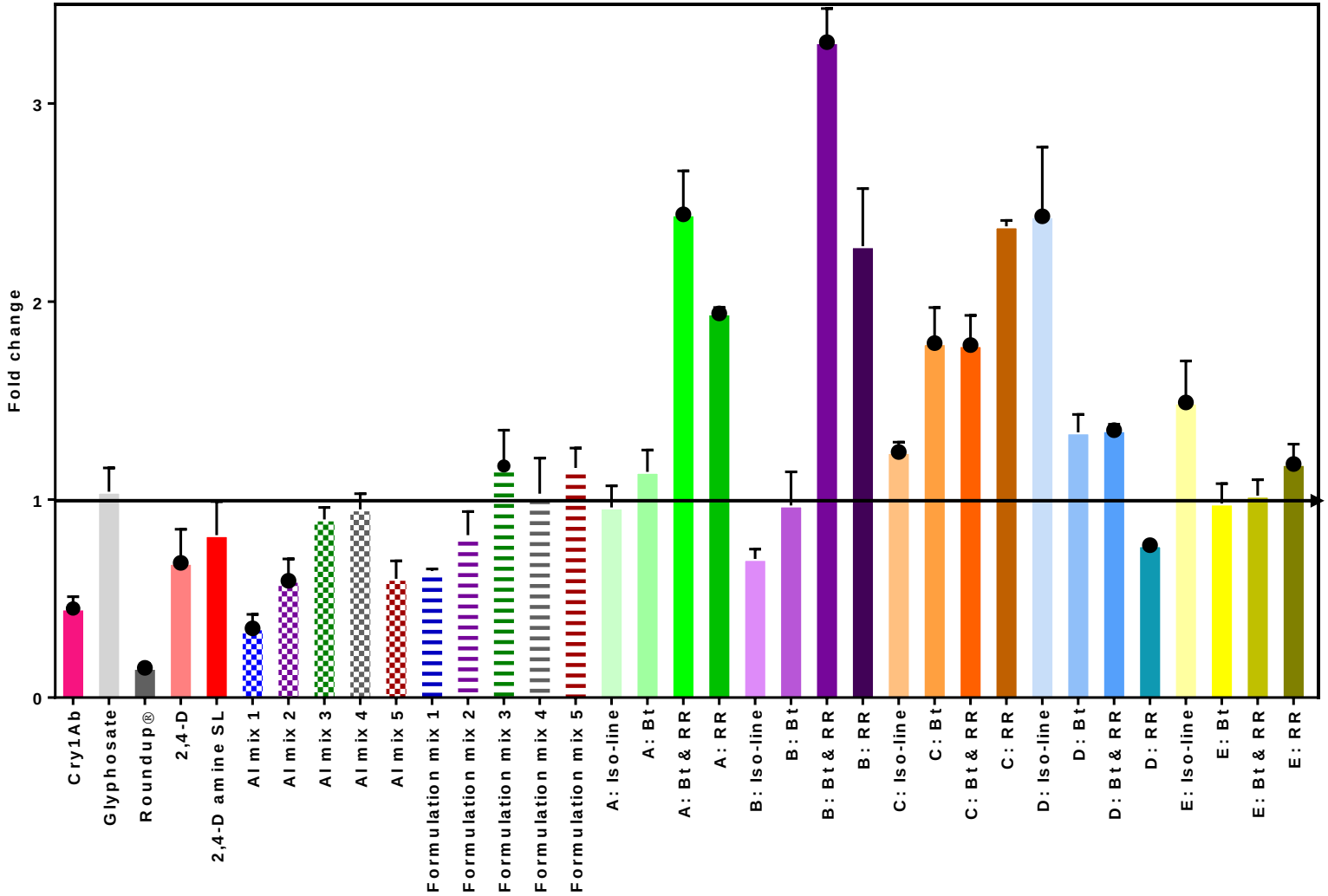

Figure 4:9 Fold change (FC) values from the anti-androgenic assay. The responses of all the exposure compounds were compared to the solvent control and FC >1= activation and FC<1=inhibition. Cultivars and the 5 spraying regimes: A: Control, B: Roundup® 2x, C: Roundup® 2x and 2,4-D, D: Roundup® 1x and 2,4-D and E were only sprayed with 2,4-D (● indicates $p < 0.05$) (the bars indicate standard deviation).

5. DISCUSSION

One of the most significant tasks confronted by industrial agriculture is to feed a growing human population. Increasing the efficiency of crop production is necessary, but also maintaining high quality products. To achieve production efficiency many chemical compounds are introduced into the fields: fertilisers to enhance growth, and pesticides to protect the crop plants from a variety of organisms that cause harm with. Generally, pesticides are toxic to target organisms and supposedly safe for non-target organisms. Pesticides are only subjected to acute toxicity testing before entering the market. These chemicals enter the environment through agricultural processes and thereby contaminate environmental matrices. Humans and wildlife are repeatedly exposed to mixtures of chemicals through consumption, inhalation and dermal contact. The levels of these chemicals are usually low, but humans are chronically exposed to these and other toxicants, which may lead to chronic health issues such as endocrine disruption.

This study was set out with the aim to assess the endocrine disruptive potential of current–use agricultural chemicals and compounds by using *in vitro* assays to test for activation and/or inhibition of one or more of the nuclear receptors (AhR, AR, GR). The risks associated with consumption or exposure to water and soil containing Cry1Ab, and formulations of 2,4-D, and glyphosate were investigated in the current study. The focus was on environmental water because South Africa has many citizens—who depend on these contaminated water sources—that are immune-compromised because of human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS). Immuno-compromised individuals are more vulnerable to unexpected harmful effects of these chemicals. The results obtained in this study may inform on more effective use of these chemicals and encourage regular monitoring of agro-chemicals or effects presented by water sources in an agricultural driven country such as South Africa.

5.1 Activation of the AR receptor

The most prominent finding of the study was caused by the environmental extracts of the field trial. Three out of the five spraying regimes from the field trial caused significant activation of the androgen receptor (AR). The three spraying regimes included the A-Control; B-Roundup® 2x (pre- and post-emergent) and C-Roundup® 2x (pre- and post-emergent) plus 2,4-D (See Section 4.4; Fig. 4.6). However, neither the active ingredients of the formulations nor the

formulations themselves, elicited any AR response. Moreover, two of the field trial spray regimes (environmental extracts from plots D and E) also did not have any AR activation.

5.1.1 Single compounds and formulations

Prior studies reported that the toxicity of the herbicides to non-target organisms is not due to the active ingredients, but rather the claimed inert compounds (such as surfactants) in the formulations (Defarge *et al*., 2016). In this study, not only were the pure active ingredients and formulations tested, but also environmentally relevant concentrations of mixtures of the pure active ingredients and mixtures of the formulations (Section 3.5; Table 3.2). None of these activated the AR at the concentrations that were tested (Section 4.4; Fig. 4.6).

The absence of AR activity in the presence of the pure compounds and formulations may be explained by the target compounds having low-affinity for the AR, or they were not bio-available and thus unable to enter through the cell membrane. These results are supported by previous studies: No activation of the AR by glyphosate and 2,4-D pure chemicals are consistent with the results published by Kojima, *et al*., (2004) where they tested the potential androgenic effects of 200 pesticides at low concentrations ($<10^{-5}$ M) using hAR Chinese Hamster Ovary (CHO) cells. Also, 2,4-D did not bind to a recombinant androgen receptor (AR) in a competitive binding assay (Fang *et al*., 2003) and Westlund and Yargeau (2017) reported no AR agnostic activity for 2,4-D in the yeast androgen screen (YAS) assay.

5.1.2 Environmental extracts from the field trial

The correlation between concentration of 2,4-D, glyphosate, and Cry1Ab in the environmental extracts to the TTEQs, were investigated. Because of the non-parametric nature of the data Spearman's correlation was conducted. The correlation strength was defined according to these categories: $|r| = 0.9–1$ (very strong correlation), $|r| = 0.7–0.9$ (strong correlation), $|r| = 0.5–0.7$ (moderate correlation), $|r| = 0.3–0.5$ (weak correlation), and $|r| = 0.0–0.3$ (no correlation) (Mukaka, 2012).

There was a moderate but statistically significant negative correlation between the 2,4-D concentrations and TTEQs from the same environmental extracts: As the TTEQs increased, the 2,4-D concentrations were at their lowest, and as the 2,4-D concentration increased, the TTEQs decreased ($r = -0.614$; $p < 0.05$) (Fig. 5.1). Both plot B and plot C had quantifiable TTEQ levels, although for three of the four cultivars, the TTEQ levels in plot B were significantly higher ($p < 0.05$). Both plots received Roundup® 2x but only plot C received 2,4-D as well, which might explain why the TTEQ levels of plot C were not as high as those in plot B (Section 4.4; Fig. 4.6). Both plots D and E had no AR activation (Section 4.4; Fig. 4.6), and both received 2,4-D. Plot D

received Roundup® but only once, which might explain the lack of any AR activation. This apparent inhibitive effect of 2,4-D will be revisited in the following section (Section 5.2).

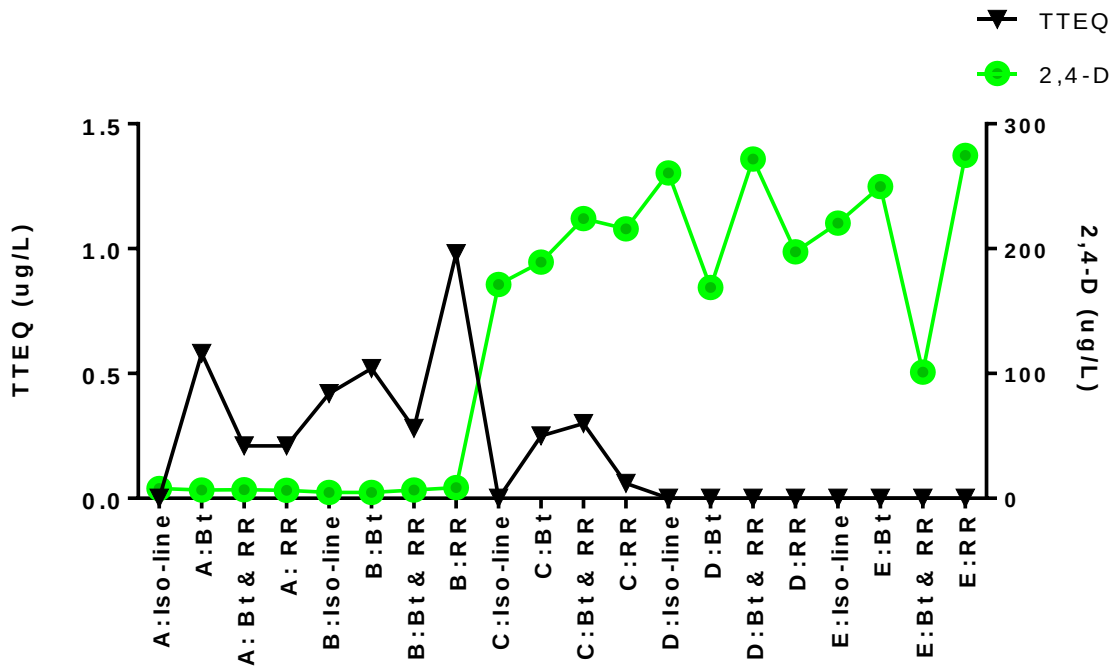

Figure 5:1 Trend between TTEQs concentration—derived from AR activation—and the concentrations of 2,4-D in the extracts (correlation: r = -0.614; p < 0.05).

Glyphosate levels showed a positive and weak, but statistically significant correlation with the TTEQs ($r = 0.45$; $p < 0.05$) (Fig. 5.2). This is contradictory to the results from the single exposures (Section 4.4, Fig. 4.6). The reason for this effect might be found in the ability of compounds in mixtures to potentiate each other's response: separately the compounds do not elicit any response, but when together, they enable a response. This effect was reported for the relationship between glyphosate and oestradiol (Williams, Watson & Desesso, 2012).

The Cry1Ab concentrations did not have a significant correlation with the TTEQs (graph not shown) ($r = 0.15$; $p > 0.05$). The lack of correlation between Cry1Ab and TTEQ is evident in the Cry1Ab concentrations in the soil irrespective of the presence of the nature of the maize in its proximity, i.e. sometimes no Cry1AB could be detected from soil close to a Bt-containing cultivar and sometimes Cry1Ab was quantified in soil from a non-Bt containing cultivar. For instance there were Cry1Ab quantified in the non-Bt containing (iso-line and RR) soil (Section 4.3; Table 4.8). Strain and Lydy (2015) also detected Cry concentrations in fields planted with maize that does not contain the Bt-gene. These results may be attributed to Cry1Ab-containing pollen that could have drifted to neighbouring plots during pollination.

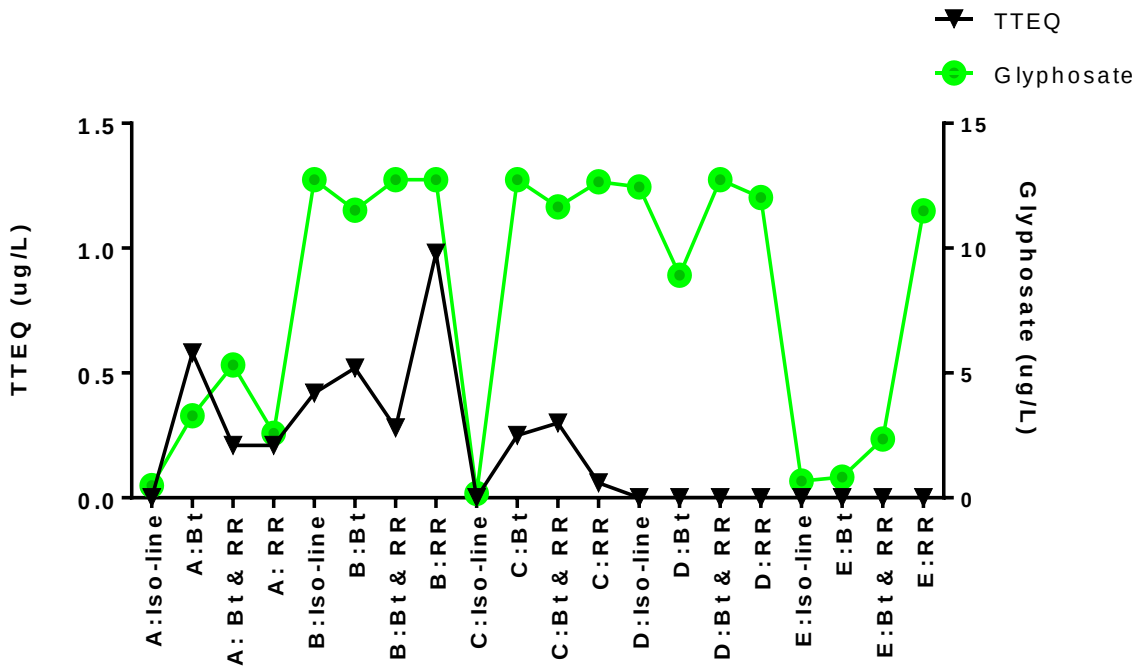

Figure 5:2 Trend observed between TTEQs concentration—derived from AR activation—and the concentrations of glyphosate in the environmental extracts (correlation r = 0.45; p < 0.05).

From the preceding correlations, it is clear that the active ingredients, on their own, are not responsible for activation of the AR but rather the mixtures of other compounds in the water-soluble fraction of the environmental extract. This result is also reflected by Fig. 4.4 containing the dose-response curves of the environmental extract from plot C (Bt and RR variety planted), the mixture of the active ingredients and formulations at the same environmental concentration (AI mix 4 formulation mix 4; Section 4.4; table 4.9) in relation to the AR agonist, testosterone.

Although the different spraying regimes might explain the differences in AR responses between plots B and C, it is more difficult to explain the AR activation by samples from plot A. Plot A was the control plot and not sprayed with any herbicides. In the preceding paragraphs I made a case for glyphosate (or at least the Roundup® formulation) to have contributed to the AR activation once in the environment, and if this is true, then it seems that plot A must have received some Roundup® despite all the precautions. When looking at the field trial layout (Section 3; Figure 3.2) plots A and C were next to one another and some droplets from the boom sprayer might have contaminated plot A, but this is unlikely because the distance between the plots was wide enough. A more likely source of contamination could have been run-off during irrigation or rainfall during the field trial. If no rain was predicted for that week, the field was irrigated, but then it would rain, resulting in higher run-off rates. The irrigation pipe-liner was placed on the

border between plot A and C. A slight decline from plot C to A might have resulted in run-off and soil particle movement from plot C to A containing compounds responsible for the AR activation. According to literature glyphosate residues are still present the following year (Duke *et al*., 2018) which could also have contributed to the contamination, however, to our knowledge no Roundup® was used on the field trial farms for the past 5 years. Soil extracts from the corresponding three cultivars caused AR activation in plots A and C but since the same cultivars did not create any AR activation in the other plots it is likely not the cultivars causing this effect. However, these findings need further investigation to determine the compounds in the extracts that could have caused the effect. None of the other variables that were quantified, such as the metal levels, soil characteristics, TOC, water holding capacity, and CEC could explain this phenomenon. These did not differ between the plots.

From the field trial, plots B and C caused the highest activation of the AR. The only coinciding factor between these two plots is that both received a pre-and post-emergent (2x) Roundup® application, with C also receiving a 2,4-D post-emergent application. Plot D received Roundup® only once, but despite this fact, it had the same glyphosate trace levels as plots B and C which received more Roundup® (Section 4.5, Table 4.8). The glyphosate levels in the soil between these three plots only ranged from 3.4–5.4 ng/g (Table 4.8). According to some authors it is not the glyphosate *per se* that is toxic but rather another ingredient of the formulation most likely a surfactant such as polyethoxylated tallow amine (POEA) (Defarge *et al*., 2016; Benachour & Séralini, 2009). It is speculated that the AR agonistic effects from plots B and C may be attributed to the surfactant in the Roundup® sprayed twice. The two plots (B and C) would contain a higher concentration of the persistent surfactant than in plot D, that was only sprayed once with Roundup® and which also did not activate the AR. Tallow amines cause non-specific damage to biological membranes, enhancing glyphosate penetration into the cells, increasing toxicity to the weeds (Chłopecka *et al*., 2017). POEA is known to cause membrane pores, which might have heightened permeability of the cell membranes of non-target organisms (Benachour *et al*., 2007; Székács *et al*., 2014) and facilitated the movement of other compounds into the cells that could have activated the AR. These compounds would be unidentified at this stage because the active ingredients (Cry1Ab, glyphosate and 2,4-D) are eliminated as candidate AR agonists by the results of the current study. In support of our speculation, Richard *et al*. (2005) reported that it is the formulation of Roundup® that facilitates glyphosate penetration of human placental cells. Once inside the glyphosate disrupted the aromatase enzyme responsible for oestrogen synthesis in the steroidogenesis pathway, an example of an endocrine disruption effect because it would change the ratio of oestrogen to testosterone inside a body.

The rainwater extracts are likely to contain multiple compounds from the soil that was water soluble. These would include compounds such as the applied herbicides, but also fertilisers, antifungal seed coatings and metals inherently part of the soil. These mixtures may have additive or combinatorial effects and interact to enhance or inhibit each other's properties (Chłopecka *et al*., 2017). To my knowledge, there have been no published studies on the occurrence, fate, and transport of POEA in the environment, but POEA strongly adsorb to agricultural soils and may persist for two years or more after application (Tush, Maksimowicz & Meyer, 2018). POEA has a larger adsorption constant than glyphosate and thus it is anticipated that POEA persists longer in the soil than glyphosate. POEA can however be transported to another location by means of erosion or soil-related run-off (dispersion) (Tush, Maksimowicz & Meyer, 2018). This characteristic of POEA could be the reason that only the surfactant (POEA) was transported to plot A, explaining the lack of detection of glyphosate in plot A. The surfactant itself, might not only contribute to increasing permeability of the cell membranes, but possibly also be able to activate the AR which in turn leads to endocrine disruptive effects. This assumption is supported by previous studies in which the co-formulants—POEA and alkyl polyglucoside (APG) previously decreased aromatase activity (Defarge *et al*., 2016; Mesnage, Bernay & Séralini, 2012). Aromatase activity is not directly related to AR activation, but it is responsible for the balance of sex hormones and highlights the endocrine disruptive potential of surfactants. More evidence of endocrine disruption caused by the surfactants present in Roundup® was observed by other researchers: increase/decrease of sexual steroid biosynthesis of a human placental cell line (Benachour & Séralini, 2009) and *in vitro* disturbances by Roundup® on testosterone and aromatase in rat testicular cells (Clair *et al*., 2012).

5.1.3 Multivariate statistical analysis

A principle component analysis was done to establish whether the concentrations of the target compounds Cry1Ab, glyphosate and 2,4-D, and the metals and salts in the rainwater extracts were associated with the AR activation (TTEQs). The ordination explains 77% of the variance in the data with 55.5% by factor/axis 1 and 21.5% by factor/axis 2.

2,4-D levels are grouped on the positive side of factor 1 together with Pt and plots C:RR, C:Iso, D:Iso, D:Bt, D:RR, E:Iso, E: Bt, E:RR C:Bt, and C:Bt&RR (Fig. 5.3). The sites B:Iso, B:Bt, B:RR, A:Bt and A:RR all loosely associated with TTEQ on the negative side of factor 1. These preceding deductions were already confirmed by the investigation into correlations between effects and levels. Most of the metals and salts grouped at a 90° angle to TTEQs and thereby showing that the metals and salts are not responsible for the AR activation (Fig. 5.3). 2,4-D is almost at a 180° angle to TTEQs (Figs. 5.1 and 5.3).

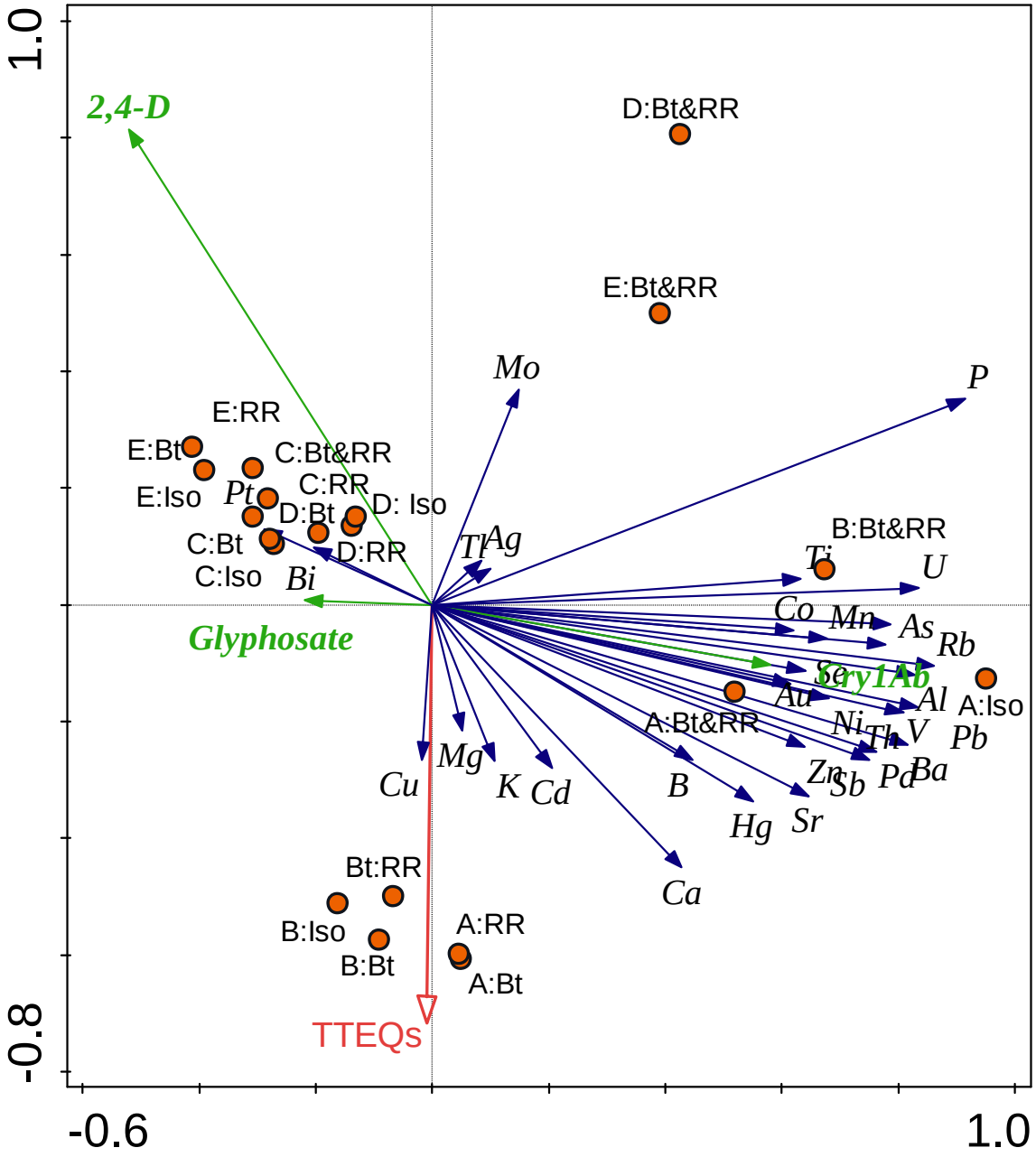

Figure 5:3: A principle component analysis (PCA) bi-plot of testosterone equivalents (TTEQs) and environmental variables—metals and salts, Cry1Ab, glyphosate and 2,4-D concentrations—among the 20 different environmental treatments (sample sites).

5.1.4 Seed coatings

Up to this section, there has been little evidence, but many suggestions as to which compounds caused activation of the androgen receptor. Here I include an extensive discussion of possible agonists to also investigate for androgenicity and why these could be the source of the androgen activity observed in this study. These include fungicides and insecticides that are used for seed coatings. The development of these seed coatings coincided with precision

agriculture and the drive to replace broadcast application (Hladik, Kolpin & Kuivila, 2014) with a more targeted method of pesticide application. The usage of treated seeds has tripled in the last decade now leading to the increased presence of these newly formulated pesticides in the environment (Hladik, Kolpin & Kuivila, 2014). The seeds planted in the field trial of this study were coated with a combination of Poncho® containing the active ingredient: clothianidin (insecticide) and Celest®XL with fludioxonil and mefenoxam (fungicides) as active ingredients.

The levels of these insecticides and fungicides were not quantified in this study, as it was not part of the initial hypothesis. In order to evaluate the potential influences clothianidin, fludioxonil and mefenoxam might have on the AR activation (Section 5.1), the following section contains some background information about the nature of these compounds.

Clothianidin has a half-life of 545 days in soil and is extremely water soluble. It is highly likely that clothianidin might be transported from the application area to surface- and groundwater via run-off from rainfall or irrigation (Hladik, Kolpin & Kuivila, 2014). From studies in the USA (Hladik, Kolpin & Kuivila, 2014) and Germany (Reemtsma, Alder, & Banasiak, 2013) clothianidin was detected in water samples near intensively farmed areas. Clothianidin forms part of the insecticide class, neonicotinoids. The uptake of the active ingredient of neonicotinoids by the target crop is only 16–20% and the remainder ends up in the soil. These compounds however, do not adsorb to the soil particles but readily leaches to water sources, especially where the soil organic content is low, and the field is on steep slopes (Goulson, 2013). This information regarding the nature of this insecticide links to our previous statement that compounds might have moved between plots because of irrigation of rainfall during the field trial. The high water solubility of the compounds, the low organic content and steep slopes mentioned by Goulson (2013) directly support our statement that the contamination of plot A might be due to possible run-off from plot C. However, this is still a speculation and the real AR agonist(s) still need to be identified by a thorough instrumental investigation. Compared to the target compounds of the present study, monitoring data of seed coating compounds is limited, but this group recently have received more attention due to potential toxic effects to non-target organisms (Sadaria, Supowit & Halden, 2016).

The second possible agonist present in the seed coatings is fludioxonil [4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile]. This antifungal agent has low water solubility and a high affinity for soil (Pose-Juan *et al*., 2011). A study done by Medjakovic *et al*. (2013), reported that fludioxonil (100 µM) caused weak AR binding in the yeast androgen screen (yAs). These results should however be interpreted with caution, as the exposure concentration inhibited yeast growth, indicating that a higher AR activation response might have been expected if the

yeast growth had been optimal. This preceding study might reveal a compound responsible for AR activation. Although fludioxonil is not highly water soluble, the surfactants or interactions with compounds present in the soil could have influenced fludioxinol's properties and make it more polar.

Mefenoxam is the third ingredient of the seed coating treatment on the seeds planted in the current study. Mefenoxam is a fungicide and highly water soluble at 26 g/L. It does not bind to soil particles and irrigation and rainfall are likely to have a significant effect on leaching of mefenoxam. The half-life of mefexonam is, however, only eight days (Gardner, Branham & Lickfeldt, 2000). The short half-life of mefenoxam makes it highly unlikely to have persisted through the whole planting season of 109 days in this study and it is fair to assume that it will not be present in the rainwater extracts from the soil collected at the end of the season. Mefenoxam is the biologically active stereoisomer of metalaxyl, on which a considerable amount of research has been conducted, not so much on mefenoxam itself. Metalaxyl did not bind to the hAR when tested in an immune-immobilised androgen receptor assay (IRA) binding study (Bauer *et al*., 2002). Mefenoxam is therefore probably not a likely candidate to have contributed to the AR activity found in the current study.

5.2 The role of the androgen receptor and the consequences of inappropriate binding to it

The AR is mainly expressed in androgen target tissues with the highest expression level in the prostate, adrenal gland, and epididymis and acts as a ligand-dependent transcription factor for the expression of specific genes. Natural androgens, such as testosterone (T) and dihydrotestosterone (DHT), play a vital role at several stages of male development and in the maintenance of the male phenotype. Testosterone is responsible for the increase in muscle mass, and cause penis, scrotum and vocal cord enlargement, support spermatogenesis, the male sex drive and performance. DHT promotes the development of facial and body hair, acne, scalp hair recession, and prostate enlargement (Davey and Grossmann, 2016; Gao, Bohl and Dalton, 2007). Reduced or excessive production of androgens is a major problem with regards to prostate cancer, male pattern baldness and spinal bulbar muscular atrophy (Gao, Bohl & Dalton, 2007).

Humans and wildlife are continuously exposed to contaminants to some degree, either through food or water consumption or even through the use of personal care products and pharmaceuticals (Kortenkamp, 2008) in the case of humans. These compounds are considered xenobiotics and when they enter the vertebrate body, may cause a hormonal imbalance of the

natural occurring hormone levels. These mixtures have the ability to act as receptor-specific agonists and/or antagonists. Overactivation of the AR, due to unintentional exposure to environmental contaminants, such as the compounds responsible for the high AR activation in the current study, might cause serious health effects such as prostate cancer (Davey & Grossmann, 2016). Prostate cancer is the most common organ cancer in men and a major cause of death. It is known that the responsiveness of prostate cancer is dependent on AR activation for growth and survival (Davey & Grossmann, 2016). Despite androgen deprivation therapy (ADT), i.e. reducing androgen levels which are the general treatment for advanced prostate cancer, it is not curative and the cancer returns (Gao, Bohl & Dalton, 2007). Advanced prostate cancer can become androgen-insensitive, i.e. the cancer is no longer sustained by a response to AR activation but by alternative steroid receptors such as the GR (Davey & Grossmann, 2016).

On the other hand, inhibition of the AR causes male androgen deficiency. Androgen deficiency is a multi-system syndrome, diagnosed based on symptoms and signs, and confirmed by abnormally low serum testosterone levels (Davey & Grossmann, 2016). This disease has various outcomes that cause symptoms like reduced sexual desire, lethargy and fatigue, breast development (gynecomastia), reduced muscle mass and strength, depression and obesity, to name a few. The exposure to any contaminants interfering with hormone levels should, therefore, be regulated as they pose serious health risks to the human population and wildlife leading to reproductive problems.

5.3 Inhibition of the AR receptor

The anti-androgenic potential of the active ingredients, formulations, mixtures of the two and the rainwater environmental extracts from the field trial, were determined. In contrast to the AR activation assay, when testing for AR inhibition the cells were co-exposed to the exposure compounds and the AR agonist, testosterone. The presence of testosterone caused binding to the AR, except if exposure compounds have the ability to prevent/inhibit the testosterone, from activating the AR. This response is quantified in terms of light production between control and exposed cells. Some of the exposure compounds caused inhibition of the AR, but this did not happen in a dose-dependent manner. It was therefore impossible to generate IC_{50} concentrations for each sample in relation to the AR antagonist, flutamide. There seems to be an overall lack of confidence in the anti-androgen assays because in a publication by Escher *et al*. (2018) where effect-based trigger values for these reporter gene assays were developed, none could be developed for the AR antagonistic effects. These authors suggested that more research, with appropriate quality control, be conducted to generate meaningful answers about the anti-androgenic effects of environmental samples. Antagonistic effects should be

represented by truly competitive action and not only a non-specific suppression of an AR signal before guidelines can be adopted and interpreted.

Despite this shortcoming, we obtained statistically significant AR inhibition results which were used to arrange the response from strong to weak antagonistic effects. The following four samples (either active ingredient, formulation, combinations thereof, or environmental extracts) statistically significantly inhibited the androgen receptor: Roundup® > Cry1Ab > 2,4-D > D:RR. Active ingredient mix 1 and 2 showed significant inhibition, but these compounds were cytotoxic and excluded from the AR inhibition data interpretation.

In the present study Roundup® was the strongest inhibitor of the AR (Section 4.4, Fig 4.9). The inhibition potential of Roundup® detected in our study was in agreement with Gasnier *et al*.'s (2009) findings which also showed that Roundup® inhibited the AR of the MDA-MB453-kb2 breast cancer cell line. However, we tested a lower, more environmentally relevant concentration and Gasnier *et al*. (2009) tested application concentrations of Roundup®. Their results distinguished between different inhibition efficiencies of various Roundup® formulations. The inhibition caused by Roundup® in the current study was not caused by glyphosate but is likely attributed to another constituent in the formulation we used such as a surfactant (Section 4.4, Fig 4.9). A study by Kojima, *et al*., (2004) support this finding in which glyphosate did not inhibit the AR. Glyphosate correlated moderately ($r = 0.51$; $p < 0.05$) with the FC values from the environmental extracts. The study by Gasnier *et al*. (2009) did show antagonistic AR activity but at much higher concentrations (500 µg/L) than the low 13 µg/L used in the present study. Gasnier *et al*. (2009) were one of the publications in which they proved that the glyphosate was less detrimental (AR inhibition) than the Roundup® formulations and concluded that the formulations were more toxic because of the nature of the adjuvants.

The 2,4-D concentration that caused inhibition was 220 µg/L and the environmental extract, D:RR, had 198 µg/L 2,4-D. These results indicate a trend in which inhibition was driven by the 2,4-D concentration. Westlund & Yargeau (2017) concluded that 2,4-D is responsible for moderate anti-androgenic activity in the YAS assay and Kojima *et al*., (2004) also showed anti-androgenic effects of 2,4-D at sub-agricultural concentrations ($< 10^{-5}$ M) using hAR CHO cells). Therefore, there is enough evidence in the literature and in the present study that 2,4-D has AR inhibitive potential, even though different cell lines were used in the respective studies from literature and here: the mechanism of action is the same in them all.

Correlation results discussed in section 5.1 between the levels of 2,4-D to TTEQs (Fig. 5.1) indicated a negative and statistically significant correlation. From this correlation result it was

assumed that the negative correlation was due to antagonistic effects caused by the presence of 2,4-D in plots D and E, and the absence of the compound which caused activation of the AR from plot C. Contrary to expectations, 2,4-D from treatment D and E did not cause quantifiable inhibition of the AR (Section 4.4; Fig. 4.9) probably due to other unexplained compound interactions. 2,4-D correlated negatively and not significantly ($r = -0.05$; $p > 0.05$) with the FC.

The Cry1Ab (pure compound), active ingredient mix 1 and 2 (containing Cry1Ab) caused statistically significant inhibition of the AR. This contrasts with what happens when Cry1Ab is an ingredient in the environmental extracts: there seem to have been a slight activation of the AR due to the Cry1Ab presence, because the FC was mostly greater than 1 (Fig 5.3). This slight activation was only visible after the cells were treated with the testosterone, which is the process for the inhibition assay. This effect was a statistically significant and positive correlation ($r = 0.5$; $p < 0.05$) (Table 4.7; Fig. 5.3). Even so this might be a coincidence effect and needs further confirmation.

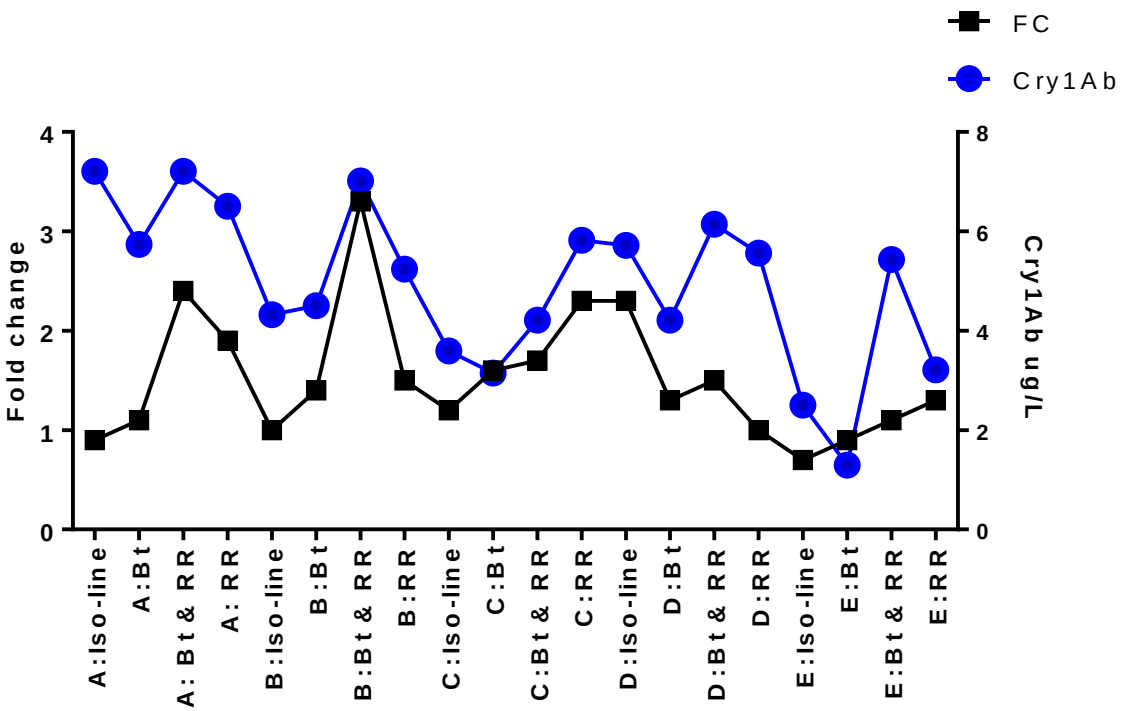

Figure 5:4 Visual representations of fold change obtained in the AR inhibition assay compared to the Cry1Ab concentrations.

5.4 Activation of the GR receptor

The MDA-kb2 cells' glucocorticoid receptor (GR) was also activated by some of the environmental extracts. These activation results were however low and DexaEQs were calculated for only two samples. The samples that elicited a significant response were plot B: Bt with 14.1 µg/L DexaEQ and plot C: Bt with 13.8 µg/L DexaEQ. These two samples were also

responsible for AR agonist activity. As for all the other compounds used in the exposures, none of the single compounds, formulations, mixtures of these and the other rainwater extracts activated the GR. The MDA-kb2 cells have both AR and GR receptors. The lack of literature on GR activation results regarding environmental exposures, might be contributed to the fact that if no activation of the AR/GR was found, researchers do not usually report on the GR activity.

However, the GR plays a vital role in the human body and is expressed in almost every cell in the body. The GR is mediated by glucocorticoids and is responsible for the regulation of genes controlling development, metabolism, and immune responses (Wei *et al*., 2007).

The activation of the GR in the current study indicated that some of the compounds in the rainwater extract could mimic glucocorticoids and bind to the GR. These compounds/xenobiotics might then occupy the GR causing an increase of naturally occurring glucocorticoids circulating in the body. Chronic exposure to elevated glucocorticoids can lead to chronic stress, altered expression/activity of enzymes involved in glucocorticoid biosynthesis or abnormalities at receptor level. These effects have been related with suppression of the immune system, hyperglycemia, hypertension, osteoporosis, visceral obesity, insulin resistance, dyslipidemia, atherosclerosis and cardiovascular diseases (Chrousos & Gold, 1992; Feelders *et al*., 2012). On the other hand, overexpression of the GR has been proven to lead to increased environmental reactivity and emotional lability which mimic some aspects of bipolar illness (Wei *et al*., 2007).

It is the fields of clinical research and development of new medication that mainly focuses on glucocorticoid and GR research, although the GR is used to indicate environmental contamination, it hasn't received as much attention as the other nuclear receptors yet.

5.5 H4IIE-luc assay: AhR activity

An activated AhR initiates detoxification of the xenobiotics by expression of the *CYP1A1* gene leading to the production of P450 enzymes. As mentioned in the literature review, activation of this receptor could also contribute to endocrine disruption effects. Any activation of the AhR may lead to potential adverse effects (Takeuchi *et al*., 2006). The compounds and mixtures tested in the current study did not cause quantifiable activation of the AhR. This finding corroborates the Takeuchi *et al*. (2006) study that used DR-EcoScreen cells (mouse hepatoma) and found no AhR activation by 2,4-D or glyphosate. Kojima, Takeuchi and Nagai (2010) also reported no AhR activity caused by 2,4-D or glyphosate when exposing Hepa1c1c7 cells that were stably transfected with the xenobiotic response element.

The lack of AhR activation was therefore expected for the active ingredients tested in this study. However, the results from the mixtures, formulations and the environmental extracts should be further investigated because of the unique combination of compounds. It is usually, but not always, hydrophobic aromatic hydrocarbons with very specific chemical characteristics that would rather activate the AhR, binding to it with high affinity. The compounds and environmental extracts tested in the current study are mostly water soluble and are hydrophilic by nature. Despite this rule of thumb, Takeuchi *et al*., (2006) divided AhR ligands into two categories: 1) a classical group having high affinity for AhR for example environmental contaminants such as halogenated aromatic hydrocarbons (HAHs) including 2,3,7,8-TCDD, polycyclic aromatic hydrocarbons (PAHs) and 2) a non-classical group, containing structurally diverse chemicals that are weak inducers of CYP1A1 and/or have a lower affinity for AhR than TCDD. This latter category of AhR ligands widely exists in the environment and include current-use pesticides used during crop production (Takeuchi *et al*., 2006). Although they are weak AhR inducers, these are important because pesticides are released in large quantities into the environment, also distributing to unintended areas.

Possible reasons that might explain why the environmental samples did not activate the AhR, apart from not being able to fit into the receptor, would include the inability to be transported across the cell membrane, and affinity for other receptors and other transcription co-factors that might be present in the H4IIE-*luc* cells. Takeuchi *et al*. (2006) tested 200 pesticides for AhR agonistic activity. Only 11 of the 200 pesticides activated the AhR and they concluded that the current-use pesticides are more likely to elicit an effect via the oestrogen receptor and AR than the AhR (Takeuchi *et al*., 2006).

5.6 Cell viability

The MTT viability assay in this study had two purposes: i) as a form of control to prevent false interpretation of the luminescence assays and ii) viability or cytotoxicity is an endpoint in its own right. The overall viability of H4IIE-*luc* and MDA-kb2 cells ranged between 67 and 192% at the end of the exposures (Section 4.4; Tables 4.10–4.13). The high percentage (>70%) viability of the cells after exposures verifies that any deduction from the luminescence assays, i.e. either increase in light emission or decrease in light emissions, are due to the compounds' effects and not dead or dying cells, except for AI mix 2 and 3 (Section 4.4; Tables 4.10–4.13).

An increase in viability is also an indication of an increase in the number of cells and this principle is used in proliferation assays to determine the effect of a compound on for example cancer cells (Haneef *et al*., 2012). And the immortalised cell lines used in this study are cancer cells, which means that a proliferation assay would indicate if a compound would suppress cell

growth (possible cancer treatment) or enhance cell growth (enhance cancer growth). Lin and Garry (2000) reported that 2,4-D (1–10 µg/mL), glyphosate (0.228–2.28 µg/mL) and Roundup® (1–10 µg/mL) significantly induced MCF-7 breast cancer cell proliferation of 149%, 135% and, 126% respectively. Despite the fact that they tested much higher concentrations than in the current study, the viabilities were comparable to Roundup® containing samples. The improved viability seen in almost all of the samples in this study, not only the Roundup® containing ones, is an indication that there are many more compounds capable of enhancing cancer cell growth.

5.7 Concentrations

Soil samples collected at the end of the field trial, were extracted with rainwater to target the water-soluble fraction and use these mixtures in bio-assay exposures. The levels of the target compounds in the water fraction were quantified to: 1) determine their contribution to receptor activation/inhibition; and 2) compare their concentrations in the rainwater to other water resources (Section 4.3; table 4.7). The herbicides and Cry1Ab investigated in this study are very mobile in water and highly likely to be transported to water sources during rainfall or irrigation.

The rainwater extracts were prepared according to ratios reported in literature (1:2; soil:water). Eventually this mixture of compounds would move to rivers and nearby water resources. When entering these sources, the concentration will dilute. There might be risks to humans that use and consume water from a river source. Some of the effects caused by glyphosate and 2,4-D in the laboratory included endocrine disruptive effects on rats (Beuret, Zirulnik & Giménez, 2005), dairy cows (Kruger *et al*., 2013), ducks (Oliveira *et al*., 2007) and human cell-lines (Daruich, Zirulnik & Sofía Gimenez, 2001; Dallegrave *et al*., 2003; Richard *et al*., 2005; Benachour, Moslemi *et al*., 2007; Benachour & Séralini, 2009; Paganelli *et al*., 2010; Samsel & Seneff, 2013). Roundup® and 2,4-D have genotoxic effects on thale cress Filkowski *et al*. (2003); caused reduced growth rates on animals and microorganisms (Gervais *et al*., 2008) and created oxidative stress in yeast (Teixeira *et al*., 2004). But all of these experiments were done using application concentrations of the herbicides, which are higher than environmental levels. The results obtained after exposure to these high concentrations may miss effects caused at the lower environmental levels, to which humans and wildlife are realistically exposed to. Although the (anti-)androgenic effects identified in this study is not solely associated with Cry1Ab, glyphosate and 2,4-D levels, the concentrations reported for the rainwater extracts in the present study were compared with what had been reported for these compounds in the environment. By comparing my results to that found in literature, I can extrapolate and estimate the AR activity for those studies in the literature, likely related to these levels. This can be done because the glyphosate and 2,4-D levels would have come from similar herbicide, insecticide and fungicide combinations as well as their surfactants, that co-extracted in the rainwater

samples. Thus, the glyphosate and 2,4-D concentrations can be used as a rough indication of androgen activity caused by agricultural practices that use a similar spray regime.

The glyphosate levels present in the rainwater extract were 2.9–3.2 µg/L (Section 4.3; Table 4.7). Van Stempvoort *et al*. (2016) reported diluted levels of glyphosate in Canadian groundwater of 0.6 µg/L. Battaglin *et al*. (2003) published levels of glyphosate from water sources in the USA, which were in the same range and one order of magnitude lower than our results. In a more recent study by the same authors, Battaglin *et al*. (2014), the following levels were reported: 3.08 µg/L in large rivers, 2.5 µg/Lin precipitation run-off, 2.03 µg/L in groundwater and 1 µg/L in soil water. These levels were reported for samples at the end of the growing season and are in the same order of magnitude as the rainwater extracts of the current study.

The concentrations of 2,4-D in the rainwater was 1.2–72.6 µg/L (Section 4.3; Table 4.7). The estimated current environmental concentrations of 2,4-D in water sources in the USA ranges from 4–24 µg/L (Serrano & DeLorenzo, 2008; Ensminger *et al*., 2013; Wijnja, Doherty & Safie, 2014), again in the same order of magnitude as the concentrations in our rainwater extracts. Lower levels of 2,4-D (0.06 and 0.20 µg/L) were found in drinking water in Spain (Rodil *et al*., 2012). Low levels of 2,4-D, 0.05 µg/L, were also published by Hernandez *et al*. (2011) in Lake Chapala, Mexico, and Tsaboula *et al*. (2016) reported 1.16 µg/L in the Pinios River Basin, Greece.

Low levels of Cry1Ab protein were detected in the rainwater extract and ranged from 1.3–1.8 µg/L. The Cry1Ab levels from streams near agricultural fields published by Tank *et al*. (2010) were an order of magnitude higher when compared to my study and could be ascribed to higher acreage used for planting and pollen drift (Li *et al*., 2013). Run-off water from a Bt field had a maximum concentration of 130 ng/L Cry1Ab (Strain & Lydy, 2015). Their reported concentration was a 100-fold higher than the levels detected in the current study. Pollen containing Cry1Ab from Bt crops, have been measured at concentrations of 10.3 µg/g (Li, Meissle & Romeis, 2008) and 22 µg/g (Liu et al., 2016). These concentrations are 10 000 to 20 000 times larger than the levels detected in the soil from the current study., these could be sources of Cry. Pollen from GM crops of the plot used for this study, as well as neighbouring trail plots could have contributed to the Cry residues detected in the soils where no Bt crops had been grown. It is also possible that because of historical use of the trial plot, there were traces of Cry already present in the soil: Baumgarte & Tebbe (2005) reported Cry protein in soil months after harvest. The varying decomposition rate of both pollen and vegetation containing Cry proteins, would explain the Cry levels in the non-Bt plots.

However, none of the referenced papers reported on androgen activity. In the cases where the concentrations levels are similar to, or even much higher than in our samples, it would be interesting to see whether their samples would have had AR activity.

5.8 Perspectives on human health risks

We, as toxicologists, are tasked to assess the risks associated with exposure to synthetic chemicals and help decide if the use of these outweigh their benefits. A critical view of the results obtained in this study is needed to provide society with up-to-date knowledge for making the necessary regulatory decisions. The goal of guideline levels is to have a reference to which results can be compared to in order to evaluate the potential risks to humans and/or wildlife.

Two types of guidelines will be discussed in this section: 1) guidelines to compare levels of the single compounds, i.e. based solely on their concentration, and 2) trigger values developed to compare the effects—obtained from bioassay—which reflects the effect of mixtures.

The drinking water guideline for glyphosate (over a life-time of exposure) in the Unites States of America (USA) is 700 µg/L (USEPA) and 1 000 µg/L in Australia (London *et al*., 2005). The levels of glyphosate in the rainwater extracts of the present study were all below this guideline. For 2,4-D, these levels are 70 µg/L in the USA and the WHO set a level of 30 µg/L which was adopted by Australia as well (London *et al*., 2005). Some of the concentrations of 2,4-D in the rainwater extracts of the current study ranging from 25 to 72 µg/L exceeded the WHO guidelines. In contrast to the above-mentioned guidelines, the maximum residue limit (MRL) for pesticides in drinking water, set by the European Union, is 0.1 µg/L (Rubio *et al*., 2003). All three pesticides tested in this study, Cry1Ab, glyphosate and 2,4-D, exceeded the EU guideline level (Table 4.7), indicating cause for concern when the levels reported in our study are present in the environment. It is important to keep in mind that these guidelines are specifically developed based on a lifetime of consumption of treated water. Consumption of this water contaminated by 2,4-D will not cause acute toxic effects to an individual exposed to these extracts. However, if 2,4-D survives the treatment of drinking water, the guideline predicts the possibility that chronic exposure may cause adverse health effects.

Guideline levels based on concentrations only, do not take the effects of the mixture of compounds into consideration. Multiple compounds mediating a similar mode of action might occur together in the environment, but instrumental analysis of environmental matrices cannot quantify them all, because the chemist is unaware of their presence. Because of this limitation, bio-assays were developed that would detect and quantify a similar mode of action caused by the mixture of compounds. The reporter gene assays employed in the current study may serve

as an example of this. The result is that we are able to evaluate a health risk without identifying (all) the compounds responsible for it. Trigger values were developed for endocrine disruption effects through exposure to the respective endocrine associated reference compounds, to which samples can be compared to in a suite of *in vitro* assays (Kase *et al*., 2018). These trigger values were derived based on acceptable daily intake (ADI) values for some well-known endocrine reference compounds, combined with pharmacokinetic factors representing the adsorption, distribution, metabolism, excretion as well as exposure assumptions. There are some considerations when interpreting the exceedance of the trigger values: 1) children are more at risk because of their smaller body mass; 2) volumes of water consumed may influence exposure; 3) susceptibility to EDCs are different at specific stages of development (Vandenberg *et al*., 2012). Another factor to consider is that populations with weakened immune systems (in reference to the high HIV/AIDS prevalence in South Africa), are more susceptible to EDCs. The most significant advantage of *in vitro* assays in comparison to instrumental analysis is the fact that they can detect the whole effect of the mixture present in realistic environmental samples.

In my study, there were 10 environmental rainwater extracts that significantly activated the AR and their TTEQ values exceeded the drinking water trigger values (14 ng/L TTEQ) from the Netherlands (Section 4.4, Fig. 4.6) (Escher *et al*., 2015). Two environmental rainwater extracts (B:Bt and C:Bt) activated the GR and both of these samples', DexaEQ concentrations exceeded the GR trigger value of 21 ng dexamethasone-eq/L (Section 4.4, Table 4.12). The information on the exceedance of the drinking water trigger value should act as a warning signal. It would therefore be prudent to extend the sampling to drinking water prepared from river water that would have received run-off from intense agricultural areas.

The exceedance of guideline levels by environmental extracts from this study indicates potential harmful effects. Since the rainwater extracts represent the water-soluble fraction, the target compounds will likely end up in water sources due to run-off from irrigation or rainfall. Humans, dependent on river or other sources of water, might be exposed to these compounds through consumption and use of water. The average human body (depending on age and mass of the individual) consists of approximately 60% water, which makes uptake of the water-soluble xenobiotics—as these targeted in the current study—easier (Wang, 1999). The water solubility of xenobiotics enables them to be easily distributed through the human body and enter the cells (if they have the innate ability to cross the cell-membranes or is facilitated by a surfactant) and bind to nuclear receptors; but polar compounds are also quickly metabolised in the human body (Kampa & Castanas, 2008).

The rainwater mixtures from the field trial caused (anti-)androgenic and glucocorticoid effects. Even though the culprits responsible for the effects are unknown and only suspects were identified a definitive answer regarding a specific mode of action could be given. If the compounds can pass through the cell membrane like in the *in vitro* assays, and bind to a hormonal receptor, the same can happen in the human body. However, it should be kept in mind that effects from *in vitro* assays, cannot be applied to an *in vivo* situation directly. The *in vitro* assays are performed using cells, that grow in a tissue culture dish, without an intact immune system that can act as a defence mechanism, neither do they have a skin to act as an initial barrier. Nevertheless, the over/under expression of hormones could disturb pathways dependent on hormone production and lead to hormonal imbalances that might cause a sequence of events with serious repercussions in an intact body.

Furthermore, this information can be used to inform risk managers and communicate these findings to the regulatory level with responsibility for the use of herbicides. Finally, it is my hope that my research will inspire further investigations on related topics.

6. CONCLUSIONS

The world currently faces the need for large-scale cultivation of food plants to feed the increasing population. The use of pesticides is an integral part of this dominant industrial agriculture. Regardless of the benefits of these pesticides, their widespread use contaminates environmental matrices, possibly posing health risks to non-target organisms. Exposure to several groups of pesticides has been linked with various reproductive and developmental disorders as well as cancer (McDuffie *et al*., 2001) in the human population. Consequently, it is of great interest to investigate the mechanisms by which pesticides could interfere with developmental processes and hormone pathways. In this study, it was the receptor-mediated mechanisms of binding to the androgen, glucocorticoid and aryl-hydrocarbon receptors (AR, GR and AhR) that were used to investigate potential endocrine disrupting effects of some of the most commonly used agricultural herbicides.

Accordingly, the aim was to assess if there are health risks, as well as, where does the toxicity levels lie with regards to mixtures of Cry1Ab toxin, present in Bt maize, and the herbicides Roundup® and 2,4-D that are used in the same system.

When samples were collected from farms where both Roundup® and 2,4-D were used, only 2,4-D were detected in water from nearby rivers and dams. No Cry1Ab or glyphosate levels could be quantified, but they might be detectable in highly intensive agricultural areas. These compounds are not regularly tested for in South Africa, and even if they occur at non-detectable levels, adjuvants or surfactants of the formulations might be present and facilitate mixture toxicity.

However, when the farm situation was simulated under controlled field conditions, Cry1Ab, glyphosate and 2,4-D were detected at the end of the season. Thus, we show that these target compounds (and others) responsible for (anti-)androgenic effects may persist in the soil for the whole season. Most of these chemicals are highly water soluble and are therefore likely to move to water sources or aquatic ecosystems where they may form even more complex mixtures, with unpredictable effects on human or ecosystem health.

An unexpected result from the cell viability assay, that was included as a quality control measure, revealed that all of the single active ingredients and some of the formulation mixes caused increased proliferation of the cells. This means that some of these compounds have the ability to cause increased growth of cancer cells at environmentally relevant concentrations, in

other words, enhance cancer growth when an individual with cancer is exposed. Such possible effects are expected after chronic exposure to low-levels, as tested in this study.

Another and crucial aspect of the field trial was to obtain a bioavailable fraction by extracting the soil with rainwater and not to follow the harsh extraction protocols conventionally done for instrumental analysis. Interestingly, exposure to these extracts caused activation of the AR and GR receptors. The active ingredients of Cry1Ab, glyphosate and 2,4-D were not directly responsible for the endocrine-related toxicity. They did however contribute to the effects caused by other unidentified compounds in the environmental mixtures. One explanation for the AR activation caused by the environmental extracts is the likely presence of adjuvants/surfactants. These surfactants would be a part of, or come from the formulations investigated in this study, but may also originate from the formulations of seed coating fungicides and/or insecticides.

The most significant finding in my study is that environmental cocktails are the most potent trigger of the test model, more potent than both the used formulations and the active ingredients in isolation.

The responses from the test model from some samples that caused an exceedance of the bio-assay derived drinking water trigger values by some samples, underlines the need for an in-depth investigation into the identification of the substances responsible for the activity. The 2,4-D concentrations in the extracts correlated negatively with AR activation, and it therefore has AR inhibitive properties although this effect was so slight that it was not supported by the inhibition assay. Roundup, Cry1Ab and the active ingredient 2,4-D caused significant inhibition of the AR receptor.

At the start of the study, we hoped to predict the mixture effects by using the effects caused by the single compounds and apply the concentration addition (CA) and independent action (IA) prediction models. But for these to work, the single compounds must have caused a full-dose response curve with a wide range of concentrations. Unfortunately, the single substances in the current study did not produce any effects at the concentrations they were tested, l*et a*lone complete dose-response curves. Thus, the mathematical models were useless in this particular instance. However, the bioassays were adequate to screen and indicate that mixture AR toxicity did occur.

Pesticides are well-developed and formulated to be water soluble for practical reasons and to reach target systems successfully. My study's results suggest that non-target organisms are definitely at risk to be exposed to the compounds, especially those in the water-soluble fraction.

This fraction is the most mobile and ends up in the water resources. This is an important finding because observed effects were caused by environmentally relevant concentrations, but not the application concentrations. Aquatic life, as well as humans consuming these water resources, are exposed to chemical mixtures that possess endocrine disrupting potential. Especially individuals or populations already battling other health issues or stress factors would be more at risk.

The combinations of chemicals investigated in this study as well as the evaluation approach, i.e. testing the water-soluble fraction with our model systems and/exposure procedures, have, to my best knowledge, never been published. It is impossible to eliminate pesticides from agricultural activities in the short term, but they should be used with care and caution together with integrated pest management regimes, and they should be tested with rigorous risk assessment. I, therefore conclude with a list of recommendations to expand the findings of my study.

7. RECOMMENDATIONS

- Chemical identification of the compounds responsible for the AR effects is necessary. A broad screening of chemicals using forensic and toxicology libraries of analytical instrumentation such as LC and GC MS would help identify possible agonists/antagonists, but their effects would need confirmation in a bio-assay. For this to be successful, the assistance of experienced analytical chemists would be crucial as well as access to affordable, yet valid reference compounds.

- Other factors that would influence the behaviour of the target compounds in the environment, such as time and type of soil, need to be investigated. Temporal changes would involve the role of rainfall, UV radiation, day-length, microbial activity and many more factors. The type of soil including its mineralogy, organic content and grain size composition would all influence the mobility and bioavailability of the target compounds.

- It is also essential to regularly monitor water resources (rivers and groundwater) for these compounds on a country-wide basis to determine the environmental levels during growing seasons and resting seasons. This monitoring should not only be based on instrumental analysis, because, as we have shown, even when a compound's concentration that does not exceed a guideline level, but it may still be harmful. Monitoring should also include high throughput screening with bioassays, looking for effects. A broad suite of assays aimed at different mechanisms of action should ideally be included. To this end, there is a global drive to develop trigger values for some of the reporter gene assay used in this study, enabling risk management based on effects, and not only concentrations of single toxicants.

- In the research field of ecotoxicology, there exists a gap between *in vitro* and *in vivo* assays, and the balance between a quick, cost-effective determination of a compound's effect and how applicable and relevant the information is to what is happening in the environment. A recent development is the use of three-dimensional cellular models (Aucamp *et al.*, 2017) which is another method to explore the effects of toxicants and their combinations in the laboratory.

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