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

Agriculture plays a vital role in the South African economy, as well as in the production of maize for food. Genetically modified maize is transformed to encode for crystalline (Cry) proteins found in *Bacillus thuringiensis* (Bt) and is referred to as Bt maize. Ingestion of specific Cry proteins causes the death of target insects that cause harm to maize plants. Bt crops, along with herbicides such as glyphosate and 2,4-dichlorophenoxyacetic acid (2,4-D), are widely adopted as part of the South African farming regime that aims to increase crop yield and reduce costs of production. As chemical compounds used in agriculture often end up in water sources, their presence should be monitored. There are many such monitoring programmes worldwide, but not in South Africa. We screened surface water sources in a maize-dominated agricultural area in the North West Province in South Africa for the presence of Cry1Ab, glyphosate and 2,4-D using enzyme-linked immunosorbent assays (ELISAs). Cry1Ab was not detected at any site; glyphosate was below the limit of detection at most of the sites but one sample had quantifiable traces of glyphosate; and 2,4-D was detected at all the sites. The concentrations of 2,4-D exceeded those for drinking water according to European guidelines, thus highlighting the need for regular monitoring of these compounds. Many people depend on untreated water resources, which may be contaminated by toxic agricultural chemicals. This report is the first on levels of these target compounds in South African water systems.

Significance:

- This report is the first on the presence of glyphosate, 2,4-D and Cry1Ab in the South African aquatic environment.
- Concentrations of 2,4-D in South African surface waters exceed the European guideline for drinking water, indicating a risk to people using these water sources.
- These preliminary results highlight the need to regularly monitor for the presence of glyphosate, 2,4-D and Cry1Ab in water resources in South Africa.

Introduction

In a water-scarce country such as South Africa, water contaminated with chemicals is of even greater concern for residents dependent on untreated surface and groundwater resources because less water causes these compounds to concentrate. One sector of the economy that inadvertently contributes to water pollution is agriculture. A large portion of the South African economy is driven by the agricultural sector; 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.¹ Moreover, maize serves as the staple food for the majority of South Africans. Therefore, meeting the basic needs of the population relies on successful agriculture.²

Globally, there have been major advances in the agricultural sector over the past 40 years which have increased crop yield and reduced pesticide use.³ The genes that encode for crystal (Cry) proteins, which are produced by *Bacillus thuringiensis* (Bt), have been incorporated into maize, thereby creating genetically modified (GM) crops. Ingestion of these proteins can be lethal for specific insect groups; for example, ingestion of Cry1Ab toxin is lethal for lepidopterans. In South Africa, Cry1Ab maize has been used with success against the stem borer *Busseola fusca*.⁴ However, resistance evolution by target pests threatens the sustainability of Bt maize in Africa⁵, in part because of unique challenges, such as a lack of refugia where healthy and susceptible insects can be produced⁶.

Cry proteins are considered to be environmentally benign with little or no effects on non-target organisms.⁷ However, studies on Cry in aquatic ecosystems have been scarce and recent reports indicate negative effects in mussels, some insects and other invertebrates like *Daphnia magna*.⁸ Cry1Ab proteins are not commonly found in water sources but the *Cry1Ab* transgene was detected in river water as far as 82 km away from an area intensively cultivated with Bt maize in Canada.⁹ When Cry1Ab occurs in the aquatic system, it readily partitions to clay and organic materials.¹⁰

Another genetic modification of maize makes plants tolerant to the herbicide glyphosate (the active ingredient in Roundup®). These herbicide-tolerant crops are referred to as Roundup-ready maize and can be sprayed with glyphosate-based herbicides in larger quantities and during the entire period of the growing season without causing damage to the crops.¹¹

Glyphosate [N-(phosphonomethyl)glycine] is the most used herbicide in the world.¹² It is a broad-spectrum, non-selective, post-emergent herbicide used for weed and vegetation control. Glyphosate is known to rapidly degrade and strongly adsorb to the soil.¹³ Glyphosate's mechanism of action is to inhibit the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase 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.¹⁴ Glyphosate is known to be non-toxic to animals and has a low ecotoxicological potential.¹⁵ However, recent evidence of more profound toxicological effects has made the use of glyphosate (Roundup products) more controversial.¹⁶ Moreover, glyphosate has been classified as a probable human carcinogen by the

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International Agency for Research on Cancer¹⁷, but not by the European Food Safety Authority¹⁸.

Insufficient crop management has led to glyphosate-resistant weeds.¹⁹ To address the tolerance of weeds towards glyphosate, farmers use herbicides with different mechanisms of action.²⁰ One of the herbicides used in South Africa, against which fewer weeds have developed resistance, is 2,4-dichloro-phenoxyacetic acid (2,4-D).^{21,22} 2,4-D is a post-emergent auxin herbicide and has been used for 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.²³ The use of herbicides on GM maize – of which 80% is the Roundup-ready version – has increased drastically over past years, and further increases are expected to occur in the next few years.²² Glyphosate-based herbicides are the most used herbicides in South Africa, with an estimated 23 million litres sold in 2012. The amount of herbicides used in South Africa (with a maize production of 2 million ha) is far less than that by the top producers such as the USA (40 million ha maize production), Brazil (13 million ha maize production) and China (7 million ha maize production).²⁴ Generally, pesticides are developed to target specific pests and to be immobile. However, run-off, leaching and spray drift occur and spread the compounds into unintended sections of the environment, and to water sources. These compounds generally occur at low concentrations and it is assumed that they would not have detrimental effects on non-target organisms. However, exposure to low levels of pesticides poses a chronic risk to human health, including endocrine disruption, immune impacts, neurotoxicity, genotoxicity, carcinogenesis and mutagenicity.²⁵

This report is the first on the presence of the herbicides glyphosate and 2,4-D as well as Cry proteins in water sources in South Africa. In this study, the aforementioned herbicides were applied to GM maize expressing Cry1Ab proteins on two farms in South Africa. Because this was a screening survey, further studies are needed to determine how these contaminants reach the water; how long after application they remain in the aquatic environment; and how their concentrations change within and between seasons. These compounds are not regularly

monitored in South Africa. However, South Africa has a target water quality guideline level for 2,4-D of 20 µg/L of water used for livestock.²⁶ The persistence of glyphosate, 2,4-D and Cry proteins in the environment and their toxicity are still under scientific discussion worldwide. To the best of our knowledge there are no data published on environmental concentrations of these compounds for South Africa.

Materials and methods

Study area

The sampling sites were located on two farms in close proximity to the Renoster and Vaal Rivers in South Africa. Farm A is in the Free State Province and Farm B is on the border between the North West and Free State Provinces (Figure 1). Fields on Farm A were planted with Bt and Roundup-ready maize and those on Farm B were planted with Roundup-ready maize only. Farm A employed rainfed farming practices whereas Farm B used an irrigation system. On both farms, the pesticide spraying regime consisted of pre-emergent Roundup® and post-emergent Roundup® as well as 2,4-D. It was assumed that the farmers applied the herbicides according to the manufacturer's guidelines. Climatic conditions, such as rainfall, are one of the mechanisms that move these compounds from the point of application to water sources. Rainfall during the month of the sampling periods was 10–25 mm for the pre-herbicide application (October 2014), 100–200 mm for the post-herbicide application (November 2014) and 50–100 mm after the harvest (March 2015).²⁷

Sampling

Water was sampled at different intervals during the planting season of 2014/2015 (October–May): (1) pre- and (2) post-herbicide application, as well as (3) after the harvest (Table 1). Water was sampled on Farm A from the Renoster River (A1) and from a dam on the farm (A2) and on Farm B from the Vaal River (B1), from an inflow dam on the farm where water is recycled from run-off after rainfall and irrigation (B2) and used again for irrigation, and from a dam on the farm used for recreational activities (B3). Surface water at a 30-cm depth was sampled in 250-mL high-density polyethylene bottles (Nalgene™, Rochester, NY, USA), protected from UV radiation and kept at 4 °C during transportation.

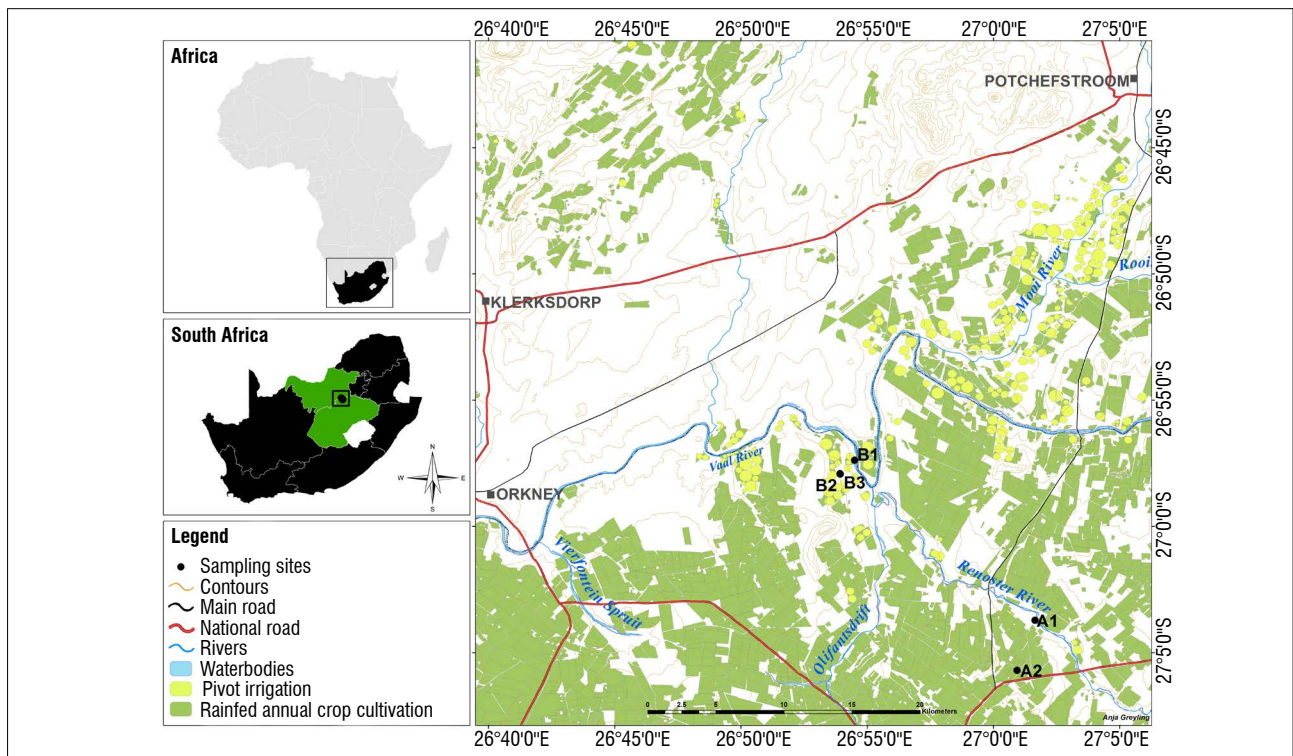


Figure 1: Map of the sampling sites situated on Farms A and B. A1: Renoster River; A2: water from a farm dam; B1: Vaal River; B2: inflow dam; B3: water from a farm dam.

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

Site	Sampling event	Sampling date	Cry1Ab ($\mu\text{g/L}$)	Glyphosate ($\mu\text{g/L}$)	2,4-D ($\mu\text{g/L}$)
Farm A					
River (A1)	Before planting	6 October 2014	<LOD	<LOD	<LOQ
	After spraying	26 November 2014	<LOD	<LOD	0.93±0.08
	End of season	9 March 2015	<LOD	<LOD	<LOQ
Dam (A2)	Before planting	6 October 2014	<LOD	<LOD	<LOQ
	After spraying	26 November 2014	<LOD	<LOD	0.72±0.02
	End of season	9 March 2015	<LOD	<LOD	0.72±0.07
Farm B					
River (B1)	Before planting	6 October 2014	<LOD	<LOD	<LOQ
	After spraying	26 November 2014	<LOD	<LOD	1.02±0.03
	End of season	9 March 2015	<LOD	<LOD	0.96±0.16
Inflow (B2)	Before planting	6 October 2014	<LOD	<LOD	0.83±0.10
	After spraying	26 November 2014	<LOD	0.42±0.04	1.08±0.04
	End of season	9 March 2015	<LOD	<LOD	0.99±0.03
Dam (B3)	Before planting	6 October 2014	<LOD	<LOD	0.74±0.02
	After spraying	26 November 2014	<LOD	<LOQ	0.90±0.08
	End of season	9 March 2015	<LOD	<LOD	0.92±0.08

LOD, limit of detection; LOQ, limit of quantification

Concentrating Cry1Ab proteins from water samples

Each water sample was concentrated using an Amicon® ultracentrifugation 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 another 15 mL was added and again centrifuged at 870 *g* for 30 min. The Amicon® tubes were subjected to a third centrifugation cycle whereafter the Cry proteins were rinsed off the membrane with 1 mL phosphate-buffered saline and Tween assay buffer. This concentrate of the samples was stored at 4 °C and quantified within 24 h.

Enzyme-linked immunosorbent assays

Over the past few years, enzyme-linked immunosorbent assays (ELISAs) have demonstrated results comparable with those of instrumental analytical methods for the quantification of contaminants in water sources. ELISA assays are therefore reliable and good substitutes for screening and monitoring such systems.²⁸

Cry1Ab

The commercially available ELISA kit used for quantification of Cry1Ab in the water samples was obtained from Envirologix (Portland, ME, USA) (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 from elsewhere. Lyophilised activated Cry1Ab toxin prepared from Cry1Ab protoxin was acquired from Marianne Pusztai-Carey at the Department of Biochemistry, Case Western University (Cleveland, OH, USA).²⁹ The lyophilised protein was re-suspended in 10 mM CAPS buffer at pH 10.5 at a concentration of 100 $\mu\text{g/mL}$ and frozen at -80 °C until use.³⁰ The quantification of the Cry1Ab protein was determined by including two independent 12-point standard curves ranging from 0 to 3.5 $\mu\text{g/L}$. 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 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 was measured at 450 nm and 650 nm (reference) using a multimode microplate reader (TriStar LB 941, Berthold, Bad Wildbad, Germany).³¹

Glyphosate

Glyphosate was quantified through the 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 to 4 $\mu\text{g/L}$ 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 the plates were 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.^{28,32}

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 curve ranged from 0 to 80 $\mu\text{g/L}$. The water samples, standards and blanks were added to the wells on the test plate. 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 wash buffer. After the washing step, a colour substrate was added and incubated for 30 min, after which a stop solution was added and absorbance was read 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 was determined for each sample, requiring a

coefficient of variation of <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 (Table 2).³³ 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.

Table 2: Limit of detection (LOD) and limit of quantification (LOQ) values for each of the target compounds

		2,4-D	Glyphosate	Cry1Ab
LOD	($\mu\text{g/L}$)	0.2	0.2	0.1
LOQ	($\mu\text{g/L}$)	0.7	0.4	0.5

Results and discussion

Concentration of the compounds in water sources

Cry1Ab

Although the water samples were concentrated 30 times, there were no detectable levels of Cry1Ab proteins in any of the water samples. 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). Cry1Ab proteins break down when exposed to high temperatures (24–33 °C), thus resulting in microbial degradation. Soil type influences adsorption, making these proteins more persistent, but also decreasing their extractability. Cry1Ab has high conformational stability and retains its activity when absorbed to polar, charged surfaces in soils, which is important when assessing its potential adverse effects in agricultural systems.³⁴ There is a lack of evidence on the bioactivity and potential health risks of Cry1Ab fragments that may be present in the environment.

In contrast to our results, Tank et al.³⁵ detected Cry1Ab proteins in 23% of 215 water samples taken from streams near agricultural fields 6 months after harvest. They reported a mean concentration of 14 ng/L and a maximum of 32 ng/L. Whiting et al.³⁶ detected no Cry1Ab in groundwater samples, but found 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, a maximum mean concentration of only 9 ng/g was detected in soil during the pollination stage of the maize plants. Cry1Ab was detected in run-off water from a non-Bt maize field with levels from below LOD to 42 ng/L, whilst higher levels (maximum concentration of 130 ng/L) were detected from a Bt maize field.³⁷ It should be noted that the concentrations of Cry1Ab detected in other studies were below the LOD of the current study. The ELISA method used could therefore have missed the presence of Cry1Ab at lower levels. The presence of Cry1Ab proteins in water, although at low levels, highlights the importance of investigating the potential long-term effects of these proteins on non-target organisms.

Glyphosate

The levels of glyphosate were below the LOD at most of the sites (Table 1). 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 $\mu\text{g/L}$ were detected at the in-flow dam on Farm B (B2) after the spraying event. These levels decreased to <LOD at the end of the season (Table 1). 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 low detection. Some studies ascribe the lower than detection limit levels of glyphosate and its quick metabolising capability to its main metabolite aminomethylphosphonic acid (AMPA).^{38,39} AMPA was, however, not quantified within the scope of this study. Glyphosate concentrations are also highly influenced by precipitation and can change from year to year.⁴⁰

In contrast to the current study, in other studies from all over the world, glyphosate has been detected in water sources. Sanchis et al.⁴¹ analysed 140 groundwater samples from Spain and found quantifiable levels for 41% of the samples. The mean concentration of glyphosate in Sanchis et al.'s study was 200 ng/L and the maximum concentration was 2.5 $\mu\text{g/L}$. Glyphosate concentrations of 663 ng/L were found in the Nottawasaga River watershed in Canada.⁴² According to Smith et al.⁴³, 45 $\mu\text{g/L}$ of glyphosate was detected in well water at the Massey Drive substation in the USA 7 weeks after spraying. This station is built on a limestone bed that has high permeability, thus emphasising that glyphosate is very mobile in water sources. In the USA, glyphosate was detected in a stream and wastewater treatment plant effluent samples in a study by Kolpin et al.⁴⁴ The maximum concentration they reported was 2.2 $\mu\text{g/L}$. Also in the USA, an extensive study by Battaglin et al.³⁹ reported glyphosate levels for different environmental matrices: 73 $\mu\text{g/L}$ in streams; 2.03 $\mu\text{g/L}$ in groundwater; 427 $\mu\text{g/L}$ in ditches and drains; 3.08 $\mu\text{g/L}$ in large rivers; 1 $\mu\text{g/L}$ in soil water; 301 $\mu\text{g/L}$ in wetlands, lakes, and ponds; 2.5 $\mu\text{g/L}$ in precipitation; 476 $\mu\text{g/L}$ in soil and sediment; and 0.3 $\mu\text{g/L}$ in wastewater treatment outfall. It is evident that glyphosate ends up in water sources.

2,4-D

Most of the samples in the current study contained quantifiable levels of 2,4-D with a minimum of 0.72 $\mu\text{g/L}$ and a maximum of 1.08 $\mu\text{g/L}$. Before planting, the concentrations of 2,4-D were below the LOD in both river samples and the dam on Farm A. It was also detected at low quantifiable levels before planting in both dams on Farm B. The highest concentration was detected after the spraying event and decreased towards the end of the season (Table 1).

According to Wilson et al.⁴⁵, 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 readily moves into water resources – a finding confirmed by Mountassif et al.⁴⁶ who reported that 91.7% of the applied 2,4-D eventually ends up in water, thus explaining the high levels detected in various countries.

Hernandez et al.⁴⁷ detected 0.05 $\mu\text{g/L}$ 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 as those in two European studies: Rodil et al.⁴⁸ detected levels of 0.062–0.2 $\mu\text{g/L}$ 2,4-D in drinking and surface water in Spain and Tsaboula et al.⁴⁹ reported 1.16 $\mu\text{g/L}$ 2,4-D in the Pinios River Basin, Greece. A few US studies by Serrano and DeLorenzo⁵⁰, Ensminger et al.⁵¹ and Wijnja et al.⁵², reported 2,4-D levels in surface water, urban run-off, a freshwater pond and Kushiwah Creek, Charleston, of 0.1 $\mu\text{g/L}$ to 11.5 $\mu\text{g/L}$. Rodil et al.⁴⁸ reported 2,4-D detected in drinking and surface water in Spain at concentrations ranging between 62 ng/L and 207 ng/L. The estimated recent environmental concentrations of 2,4-D in US water sources ranged from 4 $\mu\text{g/L}$ to 24 $\mu\text{g/L}$.⁵³ These concentrations are much higher than the levels obtained in the current study.

The Canadian guideline for the maximum residue limit (MRL) for any pesticide in drinking water is 280 $\mu\text{g/L}$, and for freshwater aquatic life is 65 $\mu\text{g/L}$.⁵⁴ In the USA, the MRL for pesticides in drinking water is 700 $\mu\text{g/L}$ ⁵⁴ and the maximum contaminant level – specifically for 2,4-D – is 70 $\mu\text{g/L}$.⁵⁵ In the European Union (EU), the MRL for pesticides in drinking water is less than 0.1 $\mu\text{g/L}$ ⁵⁴ – a level exceeded by the 2,4-D concentrations found in the current study (Figure 2). Some of the levels of 2,4-D were an order of magnitude higher than the EU guideline (Figure 2), which could mean possible effects on human health. A Canadian study found a significantly increased risk of cancer (non-Hodgkins' disease) in men exposed to 2,4-D.⁵⁶ 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.⁵⁷ In contrast, other studies examined the systemic toxicity, developmental neurotoxicity, developmental immunotoxicity, reproductive toxicity, endocrine modulation and thyroid effects in humans, and found that 2,4-D is unlikely to pose a significant health risk.^{58,59} The debate on the safety of herbicides continues as there may be unknown long-term effects on human health and the environment.⁶⁰

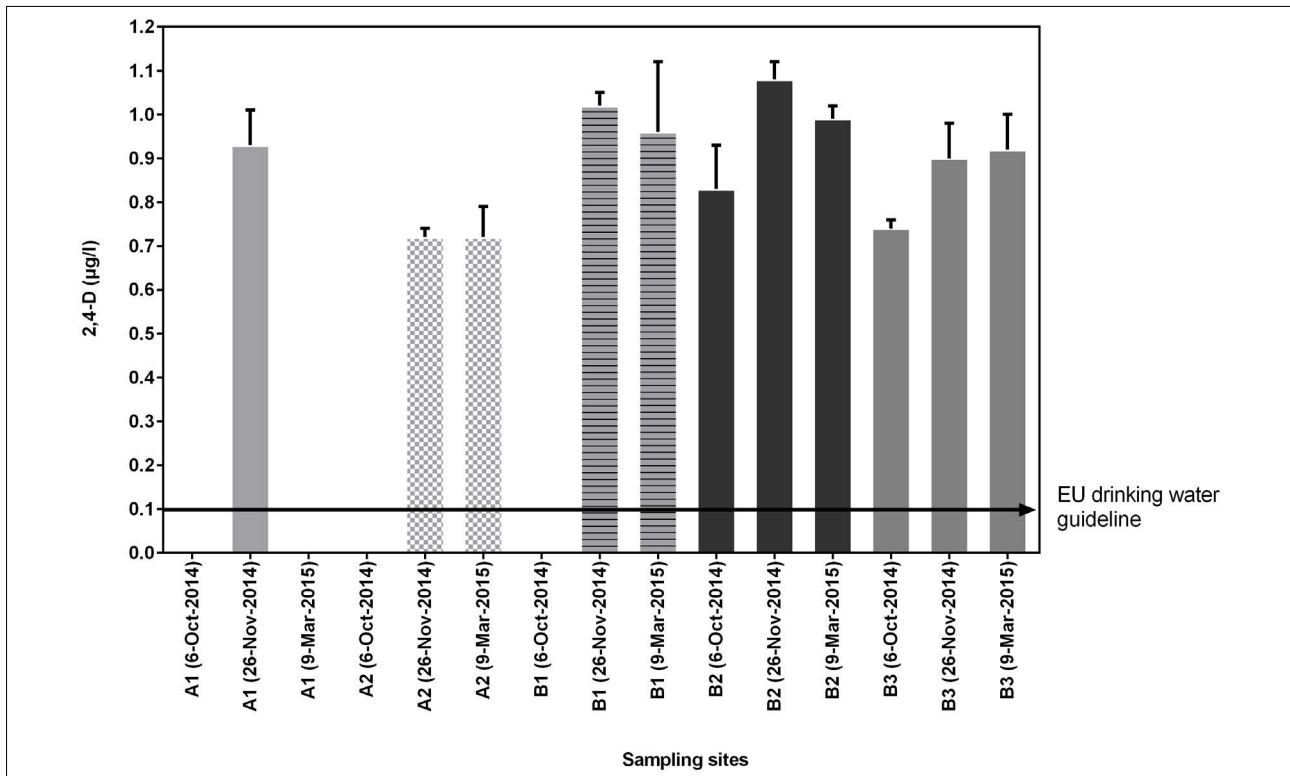


Figure 2: Concentrations of 2,4-D ($\mu\text{g/L}$) found across all sites and exceedance of the EU drinking water guideline ($0.1 \mu\text{g/L}$).

Conclusion

South Africa relies on agriculture to supply food to the majority of its people and is the 10th largest maize producer in the world. Both small-scale subsistence farming and modern agriculture are important in the country and both sectors use transgenic insect toxins and may experience development of tolerance to herbicides. Modern agriculture increases food production but may involve excessive use of herbicides and toxins for pest control. Ideally, herbicidal compounds are developed to have a specific mechanism or mode of action to avoid toxic effects in non-target organisms. However, non-target effects need to be investigated and the risk assessed for each chemical substance in use. The first step is to monitor and determine whether herbicides and agricultural toxins used by farmers can be found in the environment. To our knowledge, this has not been done previously for Cry1Ab toxin, glyphosate and 2,4-D in South Africa, although these are dominant agrochemicals in modern South African agriculture. Thus, this report is the first investigation of the presence and concentrations of these substances in water sources in South Africa.

As Cry1Ab, glyphosate and 2,4-D are highly mobile once released into the environment, increased use will elevate the levels in the environment. We did not find Cry1Ab proteins at quantifiable levels and only one sample contained glyphosate. 2,4-D was present at quantifiable levels in more than 70% of the samples and all of these concentrations exceeded the EU guideline for drinking water. Recently, research has revealed adverse health effects of Cry1Ab, glyphosate and 2,4-D exposure to non-target organisms. These effects could also influence biodiversity; therefore, water sources should be monitored to ensure both healthy aquatic ecosystems as well as safe drinking water.

Recommendations

From the results of this first survey conducted over a single maize growing season it is recommended that follow-up studies be done which include more sampling locations across larger geographical regions in South Africa. Also, monitoring should be performed over longer periods to cover variability over seasons and between years. We recommend the use of ELISAs as a screening tool followed by confirmation of positive results using other analytical methods.

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Authors' contributions

S.H. was responsible for conceptualisation; data collection; sample analysis; data analysis and validation; and writing of the initial draft. R.P. contributed to the conceptualisation and was responsible for sample collection; student supervision; funding; and writing revisions. T.B. contributed to the conceptualisation and was responsible for project leadership; funding; and writing revisions.

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