Nematodes as bioindicators of irrigated soil health in the Crocodile (West) and Marico catchments

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“The Greatest Threat to Our Planet Is the Belief That

Someone Else Will Save It”

Robert Swan

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ABSTRACT

Globally, irrigated crop production accounts for 40% of produce. However, crop yield and quality is threatened by the deterioration of freshwater resources as a result of anthropogenically induced pollution. The threat of irrigating with low quality water furthermore extends to soil health/quality, which plays an important role in sustainable crop production. In South Africa, the Hartbeespoort and Crocodile (West) irrigation schemes (Crocodile [West] Catchment), representing the experimental sites for this study, are supplied with water from the Crocodile (West) River system. This river system has historically been subjected to pollution (e.g. metals, nutrients, and salts) that originates from urban, industry, and agricultural landscapes. Conversely, water utilized by the Marico-Bosveld Irrigation Scheme (Marico Catchment; reference system) is regarded as minimally impacted. Although the threat posed to crop production can be evaluated using region-specific irrigation water quality guidelines (e.g. *South African Water Quality Guidelines for Agricultural Use: Irrigation*), such guidelines only consider soil health from an abiotic (physico-chemical properties) perspective and disregards biotic attributes. This even though soil fauna play a fundamental role in fulfilling important soil ecosystem functions (e.g. nutrient cycling and pest control). Assessing and monitoring soil health thus requires a holistic approach. Therefore, the soil quality TRIAD approach, which integrates the chemistry, ecology, and ecotoxicology lines of evidence (LOEs) into an ecological risk assessment (ERA) framework, can be applied to assess the health of irrigated soils. A need also exists to expand the toolset for evaluating the toxicity of environmental samples. Subsequently, the aims of this thesis were to:

1) evaluate the quality of irrigation water utilised in selected irrigation schemes associated with the Crocodile (West) and Marico (reference system) catchments,
2) develop a high-throughput assessment method for evaluating the toxicity of spiked and environmental (aqueous) samples, and
3) assess the subsequent threat to the health of irrigated soils following the soil quality TRIAD approach, as part of a site-specific ERA, with nematodes as bioindicators.

Results generated for the first aim confirmed that the Crocodile (West) Catchment has historically been subjected to anthropogenic pollution that posed a risk to crop production. Historical water quality data from 2005 – 2015 showed that the Hartbeespoort and Crocodile (West) irrigation schemes were exposed to calcium sulfate enrichment, while significant differences in water quality parameters occurred between these irrigation schemes and the reference system. Also, specific salt ions and nutrients concentrations exceeded threshold values provided by irrigation water quality guidelines. The Marico Catchment, in turn, was subjected to minimal anthropogenic disturbance. The second aim was also completed successfully, showing that the oxygen consumption rate (OCR) of the bacterivore nematode *Caenorhabditis elegans* can be used as an endpoint of toxicity in high-throughput assessments. The design of this high-throughput protocol facilitated assessments of the toxic effect of specific toxicants or mixtures (aqueous environmental samples) by measuring the OCR inhibition of *C. elegans* after 48 h of exposure. Results produced significant concentration-response relationships following benzylcetyldimethylammonium chloride monohydrate (BAC-C16) and cadmium (Cd) exposure, respectively, allowing the calculation of effective concentration values. Furthermore, a strong, positive correlation was evidenced between *C. elegans* OCR and growth inhibition, validating OCR as a sublethal endpoint of toxicity. The third aim was represented by the soil quality TRIAD for which soil samples were collected from selected farmlands associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes and analysed in line with each LOE. The ecology LOE, represented by terrestrial, non-parasitic (beneficial) nematodes as bioindicators of soil quality, showed that all the studied farmlands presented either disturbed or disrupted ecosystems. Together with data from the chemistry LOE, it was shown that inorganic nitrogen (N) content, likely influenced by the application of fertilizers, presented a strong, positive correlation to the abundance and diversity of beneficial nematodes, which are indicative of enriched soils. For the ecotoxicology LOE, testing the toxicity of selected soil water (capillary water that occupies soil pores) samples was achieved using *C. elegans* reproduction and growth inhibition (ISO 10872), as well as *C. elegans* OCR inhibition using the newly developed high-throughput

protocol. While *C. elegans* growth presented the lowest percentage inhibition/stimulation, a broad range of reproduction and OCR inhibition/stimulation was evidenced for both the study and reference farmlands. Integration of results from the three LOEs into the ERA framework concluded that irrigation water quality posed only a low risk at some of the studied farmlands. This is largely attributed to agricultural activities resulting in soil ecosystem disturbance, enrichment of inorganic N, and soils presenting toxicity at the reference system, which was used for background correction in the calculation of risk numbers. Outcomes of this study ultimately highlighted the impact of anthropogenic activities on irrigation water quality in the Crocodile (West) Catchment. Nonetheless, it remained difficult to elucidate the subsequent effects on irrigated soil health, likely as a result of agricultural activities (e.g. tillage and fertilizer application) causing an even greater disruption. This study concludes that there is a need to address the paucity of information relating to the health of irrigated soils.

Keywords: Crocodile (West) River system; Irrigation water quality; Soil health; Soil quality TRIAD, Ecological risk assessment

PREFACE

This thesis follows the article format style as prescribed by the North-West University. Therefore, articles appear in published format, while manuscripts and other chapters are adjusted according to the instructions to authors of internationally accredited, scientific journals. As an additional requirement by the North-West University, Table A details the contributions of authors for each article/manuscript and provides consent for use as part of this thesis.

The following Chapters were included in this work:

Chapter 1 – Introduction, literature review, and thesis structure: **Applied Soil Ecology (Elsevier)**

Chapter 2 – Article 1 (published): **Environmental Monitoring and Assessment (Springer)**

Chapter 3 – Article 2 (submitted): **Applied Soil Ecology (Elsevier)**

Chapter 4 – Article 3 (prepared): **Ecotoxicology and Environmental Safety (Elsevier)**

Chapter 5 – Article 4 (prepared): **Environmental Research (Elsevier)**

Chapter 6 – Conclusions and future trends: **Applied Soil Ecology (Elsevier)**

Submitted (Chapter 3: Article 2) and unpublished (Chapter 4: Article 3 and Chapter 5: Article 4) manuscripts, as well as Chapter 1 and Chapter 6, were adjusted according to Elsevier's uniform instructions to authors of which an excerpt is provided in Appendix A. Permission was obtained from Springer Nature to present Article 1 as part of this thesis. The licence and associated terms and conditions are available in Appendix B. Also, proof of submission of Article 2 to Applied Soil Ecology is provided in Appendix C. Finally, a declaration of language editing is provided in Appendix D.

Table A: Contributions of authors and consent for use.

Author	Article	Contribution	Consent*
GC du Preez	Articles 1 – 4	Principal investigator: Responsible for study design, field sampling, and data analysis and interpretation. Specific responsibilities also included sourcing of data (Article 1), abiotic and biotic assessments (Articles 2 and 4), and setup and execution of experiments (Articles 3 and 4). Served as the first author and was responsible for writing of manuscripts.	
H Fourie	Articles 1 – 4	As promotor, supervised the design and execution of the study. Also provided intellectual input on data analyses and writing of articles and thesis.	
V Wepener	Articles 1 – 4	As co- promotor, supervised the design and execution of the study. Also provided intellectual input on data analyses and writing of articles and thesis.	

Author	Article	Contribution	Consent*
MS Daneel	Articles 1 – 4	As assistant promotor, supervised the design and execution of the study. Also provided intellectual input on data analyses and writing of articles and thesis.	
H Miller	Article 3	Served as the Seahorse XF^{e}96 Extracellular Flux Analyzer operator. Also provided insight into the experimental design.	
S Höss	Article 3	Provided intellectual input relating to the execution of the experimental procedure and data analysis.	
C Ricci	Article 3	Provided intellectual input relating to statistical analyses.	

*I declare that the stated contributions are accurate and have approved the use of this article/manuscript as part of the thesis of Mr. G.C. Du Preez.

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CHAPTER 1

Introduction, literature review, and thesis structure

1.1 Introduction

Since 2000, South Africa's population has increased by 22% and now totals more than 56 million (DAFF, 2017; STATS-SA, 2017a). Furthermore, the country's population is estimated to reach 82 million by the year 2035, which will require agricultural output to double by the same year in order to meet demands (Goldblatt, 2011). Unfortunately, South Africa is faced with serious constraints in terms of arable land and water availability. According to Goga and Pegram (2014) only 12% of the country's surface area is suitable for growing rain-fed crops, while a mere 3% is considered to be high potential arable land. But even this available land is threatened by soil erosion (Le Roux, 2011), pollution (Van den Burg et al., 2012), and climate change (Ray et al., 2015; Ziervogel et al., 2014). South Africa is also listed as a water scarce country and receives only 60% of the world's average rainfall (Goga and Pegram, 2014). For this reason, 30% of crop production by value is cultivated under irrigation (Oelofse and Strydom, 2010; Van der Laan et al., 2017), which comes at the cost of 40% of the country's available runoff (Le Roux et al., 2016). A severe drought during the 2015/2016 growing season, classified as the driest calendar year since data recording started in 1904, exemplified South Africa's insecurities surrounding crop production and food security (Le Roux et al., 2016). In comparison to the previous growing season, yields decreased by 12.7% (DAFF, 2016), which had a severe impact on the economy, as well as on consumer prices (STATS-SA, 2017b). Furthermore, current estimates predict that water demand will exceed South Africa's total supply by the year 2025 (Van der Laan et al., 2017). This while approximately 20% of the population already experiences food insecurity (Goga and Pegram, 2014).

Irrigated crop production in South Africa is further threatened by anthropogenic activities that adversely affect the country's freshwater systems. Pollution sourced from urban, industrial, and agricultural runoff, sewage effluent, as well as wastewater discharge, enter freshwater and groundwater systems that are utilized for the production of crops (Ballot et al., 2014;

DEAT, 2005; Malan et al., 2015). This is particularly prevalent in the Crocodile (West) Marico Water Management Area (WMA) as the water quality of one of its major freshwater systems, the Crocodile (West) River system, was classified as poor by the River-Health-Program (DEAT, 2005). Furthermore, one of this river system's largest and most important freshwater bodies, the Hartbeespoort Dam, has been impacted by pollution ever since bacterial blooms were first recorded in the 1950s (Ballot et al., 2014). According to Ballot et al. (2014) the Hartbeespoort Dam frequently suffers from severe cyanobacteria blooms, including the potential toxin producing *Microcystis aeruginosa*. This is as a result of nutrient loading following the influx of treated and untreated sewage effluent discharged from various upstream waste water works (Rimayi et al., 2018). Informal settlements along the irrigation canal systems also contribute to the nutrient loading (DWAF, 2013). Despite government intervention, these conditions still prevail today as Matthews and Bernard (2015) classified the Hartbeespoort Dam as hypertrophic, as well as the most impacted water body in South Africa. Other pollutants present in the Crocodile (West) River system include metals (Almécija et al., 2017), persistent organic pollutants (Amdany et al., 2014), pesticides (Ansara-Ross et al., 2012), pharmaceuticals (Rimayi et al., 2018), and salts (DWAF, 2004a; Walsh and Wepener, 2009).

Associated with this river system is the Hartbeespoort Dam and Crocodile (West) Irrigation Schemes, which utilize water directly from the Hartbeespoort Dam and Crocodile (West) River, respectively. In total, more than 65 000 ha of land are irrigated within this catchment on which crops including citrus (e.g. orange [*Citrus sinensis* L. Osbeck] and tangerine [*Citrus reticulate* Blanco]), fodder (e.g. lucerne [*Medicago sativa* L.]), maize (*Zea mays* L.), soybean (*Glycine max* L. Merrill), various vegetables (e.g. carrot [*Daucus carota* L.] and beetroot [*Beta vulgaris* L.]), and wheat (*Triticum aestivum* L.) are produced (DWAF, 2004a). The Marico-Bosveld Irrigation Scheme, in turn, utilizes water from the Marico River system, which is associated with the Marico Catchment (also part of the Crocodile [West] Marico WMA). This river system is regarded as minimally impacted by anthropogenic activities (DEAT, 2005; Kemp et al., 2016; Wolmarans et al., 2017).

The threat that irrigation water quality poses to crop production can be assessed using the *South African Guidelines for Agricultural Use: Irrigation* (DWAF, 1996). Guidelines such as these typically consider the impact of water quality on 1) crop yield and quality as influenced by, for example, salt and trace element concentrations, 2) soil suitability as influenced by the degradation of soils, and 3) irrigation equipment following corrosion and/or encrustation (DWAF, 1996). Soil suitability, as listed in these guidelines, refers to a soil's physical and chemical (physico-chemical) properties, i.e. the abiotic component of soil health. However, holistic soil health assessments also consider ecosystem health (ecological or biotic component) as a measured endpoint (Stirling et al., 2016; Turmel et al., 2015). It is widely agreed that the integrity of soil ecosystems play a fundamental role in fostering healthy environments by fulfilling important soil ecosystem functions (e.g. nutrient cycling, carbon transformation, and pest control) (Kibblewhite et al., 2008; Lehman et al., 2015). Therefore, it is reasonable to argue that the threat posed by water quality to soil health in irrigated farmlands should be considered from both an abiotic (physico-chemical) perspective, as well as a biotic perspective.

Since DWAF (1996) currently do not take into account the ecological component of soil health, the threat that water quality poses can be investigated with site-specific assessments following a TRIAD approach. This approach was recently adapted and standardised as part of a framework for ecological risk assessments (ERA) of contaminated soils (ISO, 2017). By using a weight-of-evidence method, the TRIAD approach incorporates data generated from three lines of evidence (LOEs), namely, chemistry, ecology, and ecotoxicology (Gutiérrez et al., 2015; Ribé et al., 2012). It should be noted that often a tiered structure is adopted and lower tiers, with the benefit of being cost-effective, represent more basic and broader assessments of the respective LOEs. With a sediment TRIAD assessment, for example, preliminary chemical data can be used to determine whether any constituents occur at concentrations that threaten aquatic ecosystem health (Väänänen et al., 2018). But as mentioned before, current irrigation water quality guidelines lack ecological perspective, thus limiting lower tier assessments. Furthermore, soil screening values for toxicity mostly consider the total

concentration of pollutants (e.g. metals) and not the bioavailable fraction (Jensen et al., 2006). This study, however, is focused on pollutants present in soil water (capillary water that occupies soil pores), which represents a fraction that is bioavailable to soil fauna (e.g. earthworms [phylum Annelida], mites [phylum Arthropoda], and nematodes [phylum Nematoda]). Lower tier assessments were thus not applied in this study.

In the soil quality TRIAD framework, the chemistry LOE is typically represented by the concentration of the constituent(s) of concern (ISO, 2017), while the ecotoxicology LOE can be assessed using, for example, *Caenorhabditis elegans* Maupas, 1900 toxicity assays (ISO, 2010). Such assays typically utilize sublethal endpoints (e.g. growth, fertility, and reproduction) of toxicity, which are more sensitive than, for example, lethality assessments (ISO, 2010; Schertzinger et al., 2017). Nematode oxygen consumption rate (OCR) has, although infrequently, also been used as an endpoint of toxicity (Fourie et al., 2014; Kohra et al., 2002; Lau et al., 1997). Recently, however, renewed interest was generated by studies which have shown that a state-of-the-art platform, i.e. the Seahorse XF^{e}96 Extracellular Flux Analyzer (respirometer), can potentially be used as an alternative high-throughput assessment method (Koopman et al., 2016; Van Aardt et al., 2016). Until now only stage four larvae and older *C. elegans* life stadia have been used (Kohra et al., 2002; Koopman et al., 2016; Luz et al., 2015a; Luz et al., 2015b). Stage one larvae, however, is generally more sensitive to toxicants (Avila et al., 2011).

The ecology line of evidence, in turn, is generally represented by community structure characterisation or group-specific assessments. Nematode-specific indices such as the Maturity Index, for example, can be effectively used to measure ecosystem disturbance as a result of anthropogenic activities (Ferris and Bongers, 2009; Gutiérrez et al., 2016) and is often used in soil ERAs (Jensen et al., 2006). Following, data generated by the three LOEs are scaled, weighted (if necessary), and incorporated into a final risk number, which together with a decision on how to proceed, serve as the output of the ERA (ISO, 2017).

1.2 Research aims and objectives

1.2.1 General aims

The aims of this thesis were to 1) evaluate the quality of irrigation water utilised in selected irrigation schemes associated with the Crocodile (West) and Marico catchments, 2) develop a high-throughput assessment method for evaluating the toxicity of spiked and environmental (aqueous) samples, and 3) assess the subsequent threat to the health of irrigated soils following the TRIAD approach, as part of a site-specific ERA, with nematodes as bioindicators.

1.2.2 Objectives

The specific objectives of this study included:

I. Comparing water quality data (of selected parameters) sourced from South Africa's Department of Water and Sanitation against irrigation water quality guidelines in order to assess the historical threat posed to crop production in the Crocodile (West) and Marico catchments.

II. Studying historical spatial and temporal variation in selected irrigation water quality parameters, as well as natural and anthropogenic factors influencing it.

III. Developing and testing a protocol for using *C. elegans* OCR as an endpoint of toxicity and applying this technique in the ecotoxicology LOE (objective VIII).

IV. Collecting irrigation water and soil samples from selected farmlands associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld (reference system) irrigation schemes.

V. Acquiring historical information on crop production and agricultural practices (e.g. tillage and application of fertilizers) for farmlands selected for investigations.

VI. Soil quality TRIAD: Quantifying specific soil quality parameters (electrical conductivity, organic content, particle size distribution, and pH), as well as the concentration of nutrients, salts, and trace elements, in collected samples.

VII. Soil quality TRIAD: Studying the nematode community structure of collected soil samples in order to assess the ecological impact of anthropogenic activities. This also includes the potential influence of agricultural practices.

VIII. Soil quality TRIAD: Determining the toxicity of collected soil samples using *C. elegans* bioassays with growth, fertility, and reproduction as measured endpoints of toxicity.

IX. Conducting an ERA with data generated during the soil quality TRIAD in order to evaluate the threat posed by irrigation water quality to the health of soils in selected farmlands associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes.

1.2.3 Hypotheses

The following hypotheses were postulated:

I. The Crocodile (West) Catchment has historically been subjected to anthropogenic pollution that posed a risk to crop production (yield and quality, and sustainability). The Marico Catchment, in turn, was subjected to minimal anthropogenic disturbance.

II. The OCR of the bacterivore nematode *C. elegans* can be used as an endpoint of toxicity in high-throughput assessments.

III. Farmlands in the Crocodile (West) Catchment are at risk of soil health degradation as a result of being subjected to low quality irrigation water.

1.3 Literature review

1.3.1 The Crocodile (West) and Marico catchments

The Crocodile (West) and Marico catchments (Fig. 1.1) form part of the Crocodile (West) Marico WMA, which is considered to be one of South Africa's most developed regions (DEAT, 2005). The Crocodile (West) Catchment consists of the north-western, north-eastern, and south-western (partly) sections of the Gauteng, North-West, and Limpopo provinces, respectively. The Marico Catchment, in turn, is represented by the north-western and remaining south-eastern sections of the North-West and Limpopo provinces, respectively (DWAF, 2004a).

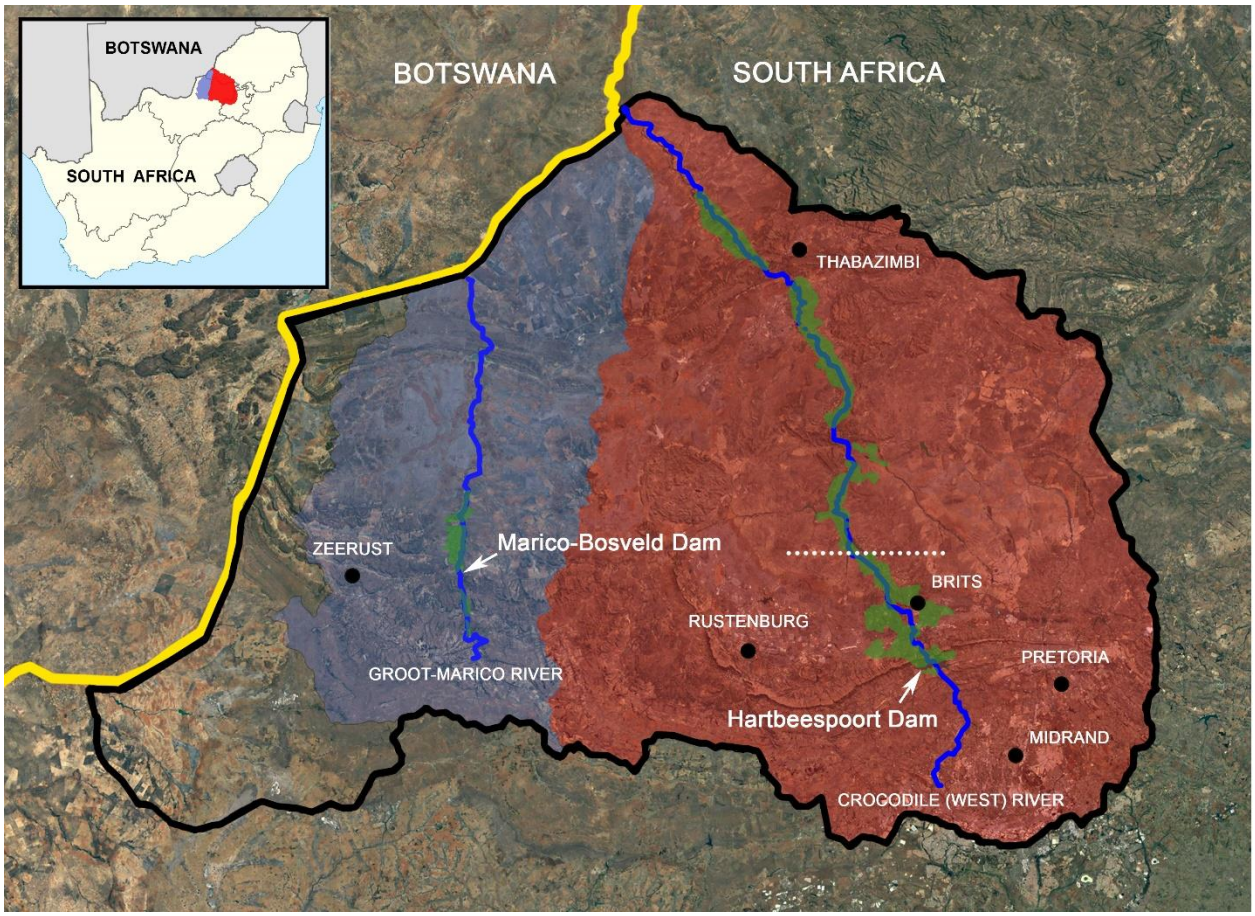

Fig. 1.1. The Crocodile (West) (red polygon) and Marico (blue polygon) catchments form part of the Crocodile (West) Marico Water Management Area (outlined in black). Associated with these catchments are the Crocodile (West) and Marico river systems, respectively, which provide water to extensive irrigation schemes (green polygons). The Hartbeespoort (below dotted line) and Crocodile (West) (above dotted line) irrigation schemes are associated with the Crocodile (West) Catchment, while the Marico-Bosveld Irrigation Scheme is associated with the Marico Catchment. Modified from Du Preez et al. (2018).

The Crocodile (West) Marico WMA hosts large metropolitans including Pretoria (South Africa's capital), Rustenburg, and part of Johannesburg, which is considered to be the country's economic hub. Subsequently, this WMA contributes substantially to the country's gross domestic product (approximately 25%) by hosting financial services, government sectors, and industry, as well as large scale mining and agricultural activities (DWAF, 2004a).

Mean annual precipitation in the Crocodile (West) Catchment ranges from 500-600 mm in the northern and western sections, to 800 mm in the southern and eastern sections (DWAF, 2013). From a geological perspective, the Crocodile (West) Catchment's main feature is the Bushveld Igneous Complex, which is a mineral rich, volcanic intrusive rock (DWAF, 2004a). Subsequently, extensive mining operations of especially platinum group metals are associated with Rustenburg, Brits, and the surrounding areas (Almécija et al., 2017; Van der Walt et al., 2012; Walsh and Wepener, 2009). Soils associated with this catchment are primarily classified as moderate to deep clayey loams (DWAF, 2013). The Marico Catchment, in turn, receives on average between 600 and 800 mm rain per year, while its geology is mainly represented by dolomitic and other sedimentary rocks, which have a large water storage capacity (DWAF, 2004b).

The Crocodile (West) and Marico rivers (Fig. 1.1) are the main surface water systems associated with the respective catchments, while their confluence represents the origins of the Limpopo River system. The Crocodile (West) River system is subjected to an influx of water from the Apies, Elands, Hennops, Jukskei, Magalies, Moretele, and Pienaars rivers and hosts large water bodies including the Hartbeespoort, Roodekopjes, Roodeplaat, and Vaalkop dams (DWAF, 2004a; 2013). The Marico River, in turn, receives water from the Klein and Groot Marico rivers, while major water bodies include the Marico-Bosveld and Molatedi dams (DEAT, 2005).

Associated with both of these river systems are large irrigation schemes. The Hartbeespoort Irrigation Scheme receives water via an extensive canal system originating from the Hartbeespoort Dam. With a total volume of 205 million m^3, this freshwater body provides

farmers with water via the eastern (78 km) and western (58 km) canals, each capable of transporting 6.8 m^3/s (DWAF, 2013). According to the latter report, a total area of 13 911 hectares (ha) are under irrigation on which crops including wheat (29%), vegetables (e.g. beetroot) (27%), soybean (20%), and maize (7%) are produced. The Hartbeespoort Irrigation Scheme utilizes an average of 62.36 million m^3 of water per annum for irrigation purposes (DWAF, 2013). The Crocodile (West) Irrigation Scheme, in turn, is not supplied by a canal system. Instead, farmers abstract water directly from the Crocodile (West) River and temporarily holds this water in irrigation dams (DWAF, 2004a). Lastly, the Marico-Bosveld Irrigation Scheme is provided with water via a canal system from the Marico-Bosveld Dam, which has a storage capacity of 27 million m^3 (Förster et al., 2017).

1.3.2 Sources of pollution entering the Crocodile (West) River system

As mentioned before, the Crocodile (West) River system is subjected to pollutants including metals (Almécija et al., 2017), nutrients (DEAT, 2005), persistent organic pollutants (Amdany et al., 2014), pesticides (Ansara-Ross et al., 2012), pharmaceuticals (Rimayi et al., 2018), and salts (DWAF, 2004a; Walsh & Wepener, 2009). These pollutants originate from different anthropogenic activities in the Crocodile (West) Catchment. However, one of the biggest factors contributing to the deterioration of water quality is the mismanagement of sewage. According to Oelofse et al. (2012) there are several sewage works that discharge directly into the Crocodile (West) River and its tributaries. These include the Northern Works, Olifantsfontein, and Sunderland Ridge waste water treatment plants associated with the Jukskei, Crocodile (West), and Hennops rivers, respectively. However, the discharge of even raw or untreated sewage into the Crocodile (West) River system is not uncommon (Nkosi, 2016; Van Dyk et al., 2012). According to Nkosi (2016) a large spill of untreated sewage from the Northern Works Waste Water Treatment Plant, near Fourways and Diepsloot (Johannesburg, South Africa), occurred in November 2016. This untreated sewage spill, only one of many, was transported via the Jukskei River directly into the Hartbeespoort Dam.

Similar reports of wastewater discharge and industry discharge into the Hennops River have also been published (Milford, 2017). Furthermore, untreated sewage is also discharged from Brits (Madibeng) Sewage Works into the Crocodile (West) River a few kilometres downstream of the Hartbeespoort Dam (BritsPos, 2017). Video footage of this recent untreated sewage spill can be viewed on Youtube using the search parameters: “Britspos Madibeng sewage spill”. Furthermore, a number of informal settlements associated with the banks of the Crocodile (West) River System, as well as the irrigation canals (Fig. 1.2) of the Hartbeespoort Irrigation Scheme, also result in untreated sewage entering this freshwater system (DWAF, 2013). Untreated sewage effluent likely contains high concentrations of nutrients (Yan et al., 2016), harmful pathogens (Castro-Rosas et al., 2012; DWAF, 1996), elevated levels of toxic metals (Balkhair and Ashraf, 2016; Chary et al., 2008; Qadir et al., 2000; Sikka and Nayyar, 2016; Zia et al., 2017), as well as salts (Hanjra et al., 2012; Kunhikrishnan et al., 2012).

Metals and other pollutants (e.g. salts) are also transported with wastewater that originate as industrial effluent and urban runoff (DEAT, 2005). According to DEAT (2005) wastewater from Centurion, Diepsloot, Johannesburg, and Krugersdorp are transported to the Hartbeespoort Dam primarily via the Crocodile, Hennops, and Jukskei rivers. Water from Pretoria and the surrounding region, in turn, are transported via the Apies and Pienaars rivers, which join the Crocodile (West) River system north of Roodekoppies Dam (DEAT, 2005). According to Hanjra et al. (2012) studies in Mexico have shown that irrigating for extended periods with mixed waste and river water may be responsible for up to 31% of metal accumulation (cadmium [Cd], cobalt [Co], chromium [Cr], lead [Pb], and nickel [Ni]) in surface soils.

Furthermore, mining activities in especially the Rustenburg, Brits, and surrounding areas result in pollutants entering the associated freshwater systems (Almécija et al., 2017; DEAT, 2005; Somerset et al., 2015). According to Almécija et al. (2017) the Hex river is subjected to platinum group element pollution with the highest concentrations recorded closest to mines. This supports findings by DEAT (2005) that high salinity levels in the same river system is as a result of mining activities. Also, Somerset et al. (2015) reported bio-accumulated metals (Cd,

palladium [Pd], platinum [Pt], and rhodium [Rh]) in tissue samples of freshwater crabs (*Potamonautes warren* Calman, 1918) collected in the Hex river. The Hex River is a tributary of the Elands River, which flows into the Crocodile (West) River downstream of Roodekoppies Dam (DEAT, 2005).

Fig. 1.2. Informal settlement along a canal as part of the Hartbeespoort Irrigation Scheme. Image sourced from DWAF (2013).

Lastly, runoff from extensive irrigated farmlands along the Crocodile (West) River system also pose a threat to environmental health (Ansara-Ross et al., 2008; 2012). Ansara-Ross et al. (2008) created a risk assessment model based on agricultural practices, pesticide characteristics, physical environmental properties, and ecotoxicological data, which predicted that several pesticides (e.g. deltamethrin and cypermethrin) posed probable risks. Deltamethrin and cypermethrin are insecticides that contain pyrethroids as active ingredients (Ansara-Ross et al., 2008).

1.3.3 Risk of pollution to irrigated crop production

Pollution levels present in the Crocodile (West) River system not only pose a serious risk to crop production, but also to aquatic ecosystem health, as well as animal and human health. A study by Chary et al. (2008), which investigated crops cultivated on sewage irrigated farmlands, found that Cr, Pb, Ni, and zinc (Zn) exceeded permissible limits in the soil, while Cr, Pb, and Zn concentrations in crops also presented a human health hazard. Furthermore, Cd has been shown to accumulate in especially leafy vegetables (Qadir et al., 2000), while Pb concentrations in crops irrigated with contaminated water may exceed acceptable levels set by the World Health Organization (Sikka and Nayyar, 2016). The accumulation of metals in crops as a result of irrigating with polluted water is well documented and poses an important risk to consumers (Khan et al., 2013; Singh et al., 2010; Van Oort et al., 2017).

Nutrients in excess of plant nutritional requirements can result in excessive vegetable growth, lodging, delayed plant maturity, and low quality produce (ANZECC, 2000a; DWAF, 1996). This is especially true when irrigation water contains high nitrogen (N) concentrations, which can, similarly to the overuse of fertilizer, result in reduced crop yield and quality. Therefore, if high nutrient levels are recorded in irrigation water, the application of fertilizer should be adjusted accordingly (DWAF, 1996).

An increase in irrigation water salinity resulting from anthropogenic activities also poses a threat to crop production (Amini et al., 2016; Grattan, 2002; Rengasamy, 2010). According to Grattan (2002) the most common salts in irrigation water include sodium chloride (NaCl), calcium sulfate ($CaSO_4$), magnesium sulfate ($MgSO_4$), and sodium bicarbonate ($NaHCO_3$). Ions including potassium (K), carbonate (CO_3), and nitrate (NO_3) are also common, while boron (B) can occur at levels toxic to sensitive crops (Grattan, 2002). Depending on the amount of water applied and the leaching fraction, these salts can accumulate in the soil and result in salinity-induced water stress, which occurs when the dissolved salts influence the physiological availability of water (DWAF, 1996). In this regard, sodium (Na) is especially relevant as it is adsorbed by soil particles, which adversely affects soil structure and hydraulic

properties (Rengasamy, 2010). Therefore, the sodium adsorption ratio (SAR) was developed as a measure of the sodicity of irrigation water and soils of which the influence on crop production can then be predicted using irrigation water quality guidelines (DWAF, 1996; Rengasamy, 2010). Some of the more salt-sensitive crops include bean (*Phaseolus vulgaris* L.), tomato (*Solanum lycopersicum* L.), onion (*Allium cepa* L.), and carrot (DWAF, 1996). Specific elements (e.g. Na, chloride [Cl], and B) can also be toxic at high concentrations (Grattan, 2002). Although perineal crops tend to be more sensitive, annual crops can be subjected to leaf injury under sprinkler (pivot) irrigation (DWAF, 1996; Grattan, 2002). The *South African Water Quality Guidelines for Agricultural Use: Irrigation* lists the water quality target for Na, Cl, and B as 0.5 mg/L, 100 mg/L, and 70 mg/L, respectively (DWAF, 1996).

The presence of pathogens in irrigation water (from especially untreated sewage) poses the greatest threat to humans if unprocessed crops (e.g. raw vegetables) are consumed. Castro-Rosas et al. (2012) reported that of the 130 salads purchased from different restaurants in Pachuca-City (Mexico), 99% harboured faecal coliforms and 85% *Escherichia coli*. Kirk et al. (2015) stated that while ingesting food contaminated with *E. coli* can result in diarrhoea, stomach cramps, and vomiting, some strains may even cause kidney failure. Other pathogens that may be present in untreated sewage include bacteria (e.g. *Campylobacter* and *Salmonella* spp.), protozoa, viruses, and helminths (Melloul et al., 2001; Steele and Odumeru, 2004).

1.3.4 Risk of pollution to the health of irrigated soils

Soil health is broadly defined as 'the continued capacity of the soil to function as a vital living ecosystem that sustains plants, animals, and humans' (Doran and Zeiss, 2000; Haney et al., 2018; Stott and Moebius-Clune, 2017; Turmel et al., 2015). Although soil quality also refers to the fitness of soil for a specific use, soil health and quality are often used synonymously (Doran and Zeiss, 2000). Soil health is generally considered to consist of three components, namely, physical, chemical, and biological (Lal, 2015; Magdoff, 2001; Stirling et al., 2016; Turmel et al., 2015). According to Stirling et al. (2016) the physical component represents the soil structure, i.e. the distribution of sand, silt, and clay particles, while the chemical component is typically measured as pH, electrical conductivity (EC), soil organic matter, nutrients, and cation exchange capacity. The biological component, in turn, is represented by the soil food web status and ecosystem health that plays an important role in providing sustainable ecosystem functions (e.g. nutrient cycling and pest control) and services (Stirling et al., 2016; Zhang et al., 2017). Each of these components ultimately influence crop yield and quality (Turmel et al., 2015).

Although these components are intricately linked, the effect of irrigation water quality on soil health is primarily considered from a physico-chemical perspective. This is especially relevant in the formulation of region-specific irrigation water quality guidelines since information on, for example, the toxic effect of specific ions (e.g. Cl) on soil fauna remains insufficient (ANZECC, 2000b). Nonetheless, studies have shown that irrigation water quality can adversely affect soil ecosystems (Becerra-Castro et al., 2015; Hu et al., 2014; Ma et al., 2015). Relevant to this study is the presence of nutrients, salts, and trace elements in the soil water, which is bioavailable to soil fauna (e.g. nematodes).

Metal pollution as a result of wastewater irrigation has been shown to pose a threat to soil ecosystems (Hanjra et al., 2012; Hu et al., 2014; Lamy et al., 2006; Ma et al., 2015). According to Hu et al. (2014) and Ma et al. (2015) soil enzymatic activity is highly inhibited by metal contamination, while Hedde et al. (2012) found that invertebrate trait-based indices can also

be influenced by enrichment. Although nematodes have rarely been used to study the health of farmland ecosystems subjected to contaminated irrigation water, Yeates (1995) showed that nematode specific-indices (e.g. Maturity Index) were sensitive to irrigating with sewage effluent in a pine tree plantation.

In general, metal accumulation in soils pose a serious toxicity threat to nematodes (Gutiérrez et al., 2016; Park et al., 2011; Šalamún et al., 2012) and other soil fauna (Hagner et al., 2018; Visioli et al., 2013). High concentrations of salts in soils also pose an important threat to soil health (Rath et al., 2016; Šalamún et al., 2014; Yuan et al., 2007). Šalamún et al. (2014) investigated the toxic effect of magnesium (Mg) in soils contaminated by a Mg ore processing plant and concluded that the associated nematode communities were adversely affected, which was indicated by the absence of sensitive species. Rath et al. (2016), in turn, demonstrated that saline soils inhibited microbial growth, while respiration was mostly inhibited by Cl salts. Other studies have also illustrated the deleterious effects of soil salinity, typically measured as EC, on microbial communities and soil ecosystems in general (Ibekwe et al., 2010; Yuan et al., 2007).

1.3.5 Nematodes as bioindicators of soil health

Nematodes represent the most abundant multicellular organisms on earth (Ferris and Bongers, 2009; Renčo and Baležentienė, 2015). With an ubiquitous distribution, they even occupy extreme environments, which include the depths (3.6 km below surface) of a gold mine in South Africa (Borgonie et al., 2011) and an isolated, chemoautotrophic based cave ecosystem (Movile Cave, Romania) (Muschiol et al., 2015; Poinar and Sarbu, 1994). In soils, nematodes typically occur in numbers of several million individuals per cubic meter where a distinction is made between plant-parasitic (phytophagous or herbivorous) and beneficial (non-parasitic or free-living) nematodes (Renčo and Baležentienė, 2015; Sánchez-Moreno et al., 2018). Although different nematode classification systems have been proposed, De Ley and Blaxter (2002) provided a Linnean classification system (Table 1.1) based on phylogenetic

relationships. Many of the orders (e.g. Mononchida Jairajpuri, 1969, Monhysterida Filipjev, 1929, Plectida Malakhov, 1982, and Rhabditida Chitwood, 1933) listed in Table 1.1 are representative of beneficial nematodes commonly found in soils. Plant-parasitic nematodes, in turn, are mainly represented by the infraorder Tylenchomorpha (order: Rhabditida), as well as other groups including Longidoridae Thorne, 1935 and Trichodoridae Thorne, 1935. Relevant to this study is the beneficial nematodes in soil environments, which are aquatic organisms that occupy and migrate through water films (25-100 µm) in the soil (Neher, 2010). According to Renčo and Baležentienė (2015) approximately 27 000 species have been described, however, there may be close to a million that remain unknown to science.

Nematodes influence important ecosystem functions such as the control of pests, carbon transformation, and nutrient cycling (Neher, 2010; Sánchez-Moreno et al., 2018). Predatory nematodes help regulate pest densities by feeding on, for example, plant-parasitic nematodes (Neher, 2010). Different species have also been studied for their potential use as alternative biological control agents (Kim, 2015). Carbon cycling, in turn, is promoted by the ingestion of organic molecules followed by the release of carbon dioxide across the cuticle (Yeates et al., 2009). According to Yeates et al. (2009) as much as 40% of the ingested carbon can be released in this way. Similarly, excess N (as ammonium) is also released by nematodes, which is then available for plant uptake. It is estimated that bacterivore and predatory nematodes contribute between 8 and 19% of N mineralization in farming systems (Neher, 2010). Since nematodes occupy several trophic groups and play an active role in ecosystem functioning, they are considered ideal indicators of soil ecosystem health (Hu et al., 2017).

Table 1.1. Classification of nematodes (Phylum Nematoda) up to family level as reported by De Ley and Blaxter (2002).

PHYLUM NEMATODA Potts, 1932
***Incertae sedis*:**
ORDER BENTHIMERMITHIDA Tchesunov, 1995
Family Benthimermithidae Petter, 1980
***Incertae sedis*:**
ORDER RHAPTOTHYREIDA Tchesunov, 1995
Family Rhaptothyreidae Hope and Murphy, 1969

CLASS ENOPLEA Inglis, 1983

SUBCLASS ENOPLIA Pearse, 1942

ORDER ENOPLIDA Filipjev, 1929
***Incertae sedis*:** Family Andrassyidae Tchesunov and Gagarin, 1999

Suborder Enoplina Chitwood and Chitwood, 1937
Superfamily Enoploidea Dujardin, 1845
Family Enoplidae Dujardin, 1845
Family Thoracostomopsidae Filipjev, 1927
Family Anoplostomatidae Gerlach and Riemann, 1974
Family Phanodermatidae Filipjev, 1927
Family Anticomidae Filipjev, 1918

Suborder Oncholaimina De Coninck, 1965
Superfamily Oncholaimoidea Filipjev, 1916
Family Oncholaimidae Filipjev, 1916
Family Enchelidiidae Filipjev, 1918

Suborder Ironina Siddiqi, 1983
Superfamily Ironoidea de Man, 1876
Family Ironidae de Man, 1876
Family Leptosomatidae Filipjev, 1916
Family Oxystominidae Chitwood, 1935

Suborder Tripyloidina De Coninck, 1965
Superfamily Tripyloidoidea Filipjev, 1928
Family Tripyloididae Filipjev, 1928

Suborder Alaimina Clark, 1961
Superfamily Alaimoidea Micoletzky, 1922
Family Alaimidae Micoletzky, 1922

ORDER TRIPLONCHIDA Cobb, 1920

Suborder Diphtherophorina Coomans and Loof, 1970
Superfamily Diphtherophoroidea Micoletzky, 1922
Family Diphtherophoridae Micoletzky, 1922
Family Trichodoridae Thorne, 1935

Suborder Tobrilina Tsalolikhin, 1976
Superfamily Tobriloidea De Coninck, 1965
Family Tobrilidae De Coninck, 1965
Family Triodontolaimidae De Coninck, 1965
Family Rhabdodemaniidae Filipjev, 1934
Family Pandolaimidae Belogurov, 1980
Superfamily Prismatolaimoidea Micoletzky, 1922
Family Prismatolaimidae Micoletzky, 1922

Suborder Tripylina Andrássy, 1974
Superfamily Tripyloidea de Man, 1876
Family Tripylidae de Man, 1876
Family Onchulidae Andrássy, 1963

ORDER TREFUSIIDA Lorenzen, 1981
Superfamily Trefusioidea Gerlach, 1966
Family Simpliconematidae Blome and Schrage, 1985
Family Trefusiidae Gerlach, 1966
Family Laurathonematidae Gerlach, 1953
Family Xenellidae De Coninck, 1965

SUBCLASS DORYLAIMIA Inglis, 1983

ORDER DORYLAIMIDA Pearse, 1942

Suborder Dorylaimina Pearse, 1942
Superfamily Dorylaimoidea de Man, 1876
Family Dorylaimidae de Man, 1876
Family Aporcelaimidae Heyns, 1965
Family Qudsianematidae Jairajpuri, 1965
Family Nordiidae Jairajpuri and Siddiqi, 1964
Family Longidoridae Thorne, 1935
Family Actinolaimidae Thorne, 1939
Superfamily Belondiroidea Thorne, 1939
Family Belondiridae Thorne, 1939
Superfamily Tylencholaimoidea Filipjev, 1934
Family Leptonchidae Thorne, 1935
Family Tylencholaimidae Filipjev, 1934
Family Aulolaimoididae Jairajpuri, 1964
Family Mydonomidae Thorne, 1964

Suborder Nygolaimina Thorne, 1935
Superfamily Nygolaimoidea Thorne, 1935
Family Nygolaimidae Thorne, 1935
Family Nygellidae Andrássy, 1958
Family Aetholaimidae Jairajpuri, 1965
Family Nygolaimellidae Clark, 1961

Suborder Campydorina Jairajpuri, 1983
Superfamily Campydoroidea Thorne, 1935
Family Campydoridae Thorne, 1935

ORDER MONONCHIDA Jairajpuri, 1969

Suborder Bathyodontina Siddiqi, 1983
Superfamily Cryptonchoidea Chitwood, 1937
Family Bathyodontidae Clark, 1961
Family Cryptonchidae Chitwood, 1937
Superfamily Mononchuloidea De Coninck, 1965
Family Mononchulidae De Coninck, 1965

Suborder Mononchina Kirjanova and Krall, 1969
Superfamily Anatonchoidea Jairajpuri, 1969
Family Anatonchidae Jairajpuri, 1969
Superfamily Mononchoidea Chitwood, 1937
Family Mononchidae Chitwood, 1937
Family Mylonchulidae Jairajpuri, 1969

Table 1.1. Continued.

ORDER ISOLAIMIDA Cobb, 1920
Superfamily Isolaimoidea Timm, 1969
Family Isolaimiidae Timm, 1969

ORDER DIOCTOPHYMATIDA Baylis and Daubney, 1926

Suborder Dioctophymatina Skrjabin, 1927
Family Dioctophymatidae Castellani and Chalmers, 1910
Family Soboliphymatidae Petrov, 1930

ORDER MUSPICEIDA Bain and Chabaud, 1959

Suborder Muspiceina Bain and Chabaud, 1959
Family Muspiceidae Sambon, 1925
Family Robertdollfusiidae Chabaud and Campana, 1950

ORDER MARIMERMITHIDA Rubtzov, 1980
Family Marimermithidae Rubtzov and Platonova, 1974

ORDER MERMITHIDA Hyman, 1951

Suborder Mermithina Andrássy, 1974
Superfamily Mermithoidea Braun, 1883
Family Mermithidae Braun, 1883
Family Tetradonematidae Cobb, 1919

ORDER TRICHINELLIDA Hall, 1916
Superfamily Trichinelloidea Ward, 1907
Family Anatrichosomatidae Yamaguti, 1961
Family Capillariidae Railliet, 1915
Family Cystoopsidae Skrjabin, 1923
Family Trichinellidae Ward, 1907
Family Trichosomoididae Hall, 1916
Family Trichuridae Ransom, 1911

CLASS CHROMADOREA Inglis, 1983
SUBCLASS CHROMADORIA Pearse, 1942
ORDER DESMOSCOLECIDA Filipjev, 1929
Suborder Desmoscolecina Filipjev, 1934
Superfamily Desmoscolecoidea Shipley, 1896
Family Desmoscolecidae Shipley, 1896
Family Meyliidae De Coninck, 1965
Family Cyartonematidae Tchesunov, 1990

ORDER CHROMADORIDA Chitwood, 1933
Suborder Chromadorina Filipjev, 1929
Superfamily Chromadoroidea Filipjev, 1917
Family Chromadoridae Filipjev, 1917
Family Ethmolaimidae Filipjev and Schuurmans Stekhoven, 1941
Family Neotonchidae Wieser and Hopper, 1966
Family Achromadoridae Gerlach and Riemann, 1973
Family Cyatholaimidae Filipjev, 1918

ORDER DESMODORIDA De Coninck, 1965

Suborder Desmodorina De Coninck, 1965
Superfamily Desmodoroidea Filipjev, 1922
Family Desmodoridae Filipjev, 1922
Family Epsilonematidae Steiner, 1927
Family Draconematidae Filipjev, 1918
Superfamily Microlaimoidea Micoletzky, 1922
Family Microlaimidae Micoletzky, 1922
Family Aponchiidae Gerlach, 1963
Family Monoposthiidae Filipjev, 1934

ORDER MONHYSTERIDA Filipjev, 1929

Suborder Monhysterina De Coninck and Schuurmans Stekhoven, 1933
Superfamily Monhysteroidea de Man, 1876
Family Monhysteridae de Man, 1876
Superfamily Sphaerolaimoidea Filipjev, 1918
Family Xyalidae Chitwood, 1951
Family Sphaerolaimidae Filipjev, 1918

Suborder Linhomoeina Andrássy, 1974
Superfamily Siphonolaimoidea Filipjev, 1918
Family Siphonolaimidae Filipjev, 1918
Family Linhomoeidae Filipjev, 1922
Family Fusivermidae Tchesunov, 1996

ORDER ARAEOLAIMIDA De Coninck and Schuurmans Stekhoven, 1933
Superfamily Axonolaimoidea Filipjev, 1918
Family Axonolaimidae Filipjev, 1918
Family Comesomatidae Filipjev, 1918
Family Diplopeltidae Filipjev, 1918
Family Coninckiidae Lorenzen, 1981

ORDER PLECTIDA Malakhov, 1982
Superfamily Leptolaimoidea Örley, 1880
Family Leptolaimidae Örley, 1880
Family Rhadinematidae Lorenzen, 1981
Family Aegialoalaimidae Lorenzen, 1981
Family Diplopeltoididae Tchesunov, 1990
Family Paramicrolaimidae Lorenzen, 1981
Family Ohridiidae Andrássy, 1976
Family Bastianiidae De Coninck, 1935
Family Odontolaimidae Gerlach and Riemann, 1974
Family Rhabdolaimidae Chitwood, 1951
Superfamily Ceramonematoidea Cobb, 1933
Family Tarvaiidae Lorenzen, 1981
Family Ceramonematidae Cobb, 1933
Family Tubolaimoididae Lorenzen, 1981
Superfamily Plectoidea Örley, 1880
Family Plectidae Örley, 1880
Family Chronogasteridae Gagarin, 1975
Family Metateratocephalidae Eroshenko, 1973
Superfamily Haliplectoidea Chitwood, 1951
Family Peresianidae Vitiello and De Coninck, 1968
Family Haliplectidae Chitwood, 1951
Family Aulolaimidae Jairajpuri and Hooper, 1968

Table 1.1. Continued.

ORDER RHABDITIDA Chitwood, 1933
***Incertae sedis*:** Family Teratocephalidae Andrássy, 1958
***Incertae sedis*:** Family Chambersiellidae Thorne, 1937
***Incertae sedis*:** Family Brevibuccidae Paramonov, 1956

Suborder Spirurina
***Incertae sedis*:** Superfamily Dracunculoidea Stiles, 1907
Family Dracunculidae Stiles, 1907
Family Philometridae Baylis and Daubney, 1926
Family Phlyctainophoridae Roman, 1965
Family Skrjabillanidae Schigin and Schigina, 1958
Family Anguillicolidae Yamaguti, 1935
Family Guyanemidae Petter, 1975
Family Micropleuridae Baylis and Daubney, 1926

INFRAORDER GNATHOSTOMATOMORPHA
Superfamily Gnathostomatoidea Railliet, 1895
Family Gnathostomatidae Railliet, 1895

INFRAORDER OXYURIDOMORPHA
Superfamily Thelastomatoidea Travassos, 1929
Family Thelastomatidae Travassos, 1929
Family Travassosinematidae Rao, 1958
Family Hystrignathidae Travassos, 1919
Family Protrelloididae Chitwood, 1932
Superfamily Oxyuroidea Cobbold, 1864
Family Oxyuridae Cobbold, 1864
Family Pharyngodonidae Travassos, 1919
Family Heteroxynematidae Skrjabin and Shikhobalova, 1948

INFRAORDER RHIGONEMATOMORPHA
Superfamily Rhigonematoidea Artigas, 1930
Family Rhigonematidae Artigas, 1930
Family Ichthyocephalidae Travassos and Kloss, 1958
Superfamily Ransomnematoidea Travassos, 1930 Family Ransomnematidae Travassos, 1930 Family Carnoyidae Filipjev, 1934
Family Hethidae Skrjabin and Shikhobalova, 1951

INFRAORDER SPIRUROMORPHA
Superfamily Camallanoidea Railliet and Henry, 1915
Family Camallanidae Railliet and Henry, 1915
Superfamily Physalopteroidea Railliet, 1893
Family Physalopteridae Railliet, 1893
Superfamily Rictularoidea Hall, 1915
Family Rictulariidae Hall, 1915
Superfamily Thelazoidea Skrjabin, 1915
Family Thelaziidae Skrjabin, 1915
Family Rhabdochonidae Travassos, Artigas and Pereira, 1928
Family Pneumospiruridae Wu and Hu, 1938
Superfamily Spiruroidea Örley, 1885
Family Gongylonematidae Hall, 1916
Family Spiruridae Örley, 1885
Family Spirocercidae Chitwood and Wehr, 1932
Family Hartertiidae Quentin, 1970
Superfamily Habronematoidea Chitwood and Wehr, 1932
Family Hedruridae Railliet, 1916
Family Habronematidae Chitwood and Wehr, 1932
Family Tetrameridae Travassos, 1914
Family Cystidicolidae Skrjabin, 1946
Superfamily Acuarioidea Railliet, Henry and Sisoff, 1912
Family Acuariidae Railliet, Henry and Sisoff, 1912
Superfamily Filarioidea Weinland, 1858
Family Filariidae Weinland, 1858
Family Onchocercidae Leiper, 1911
Superfamily Aproctoidea Yorke and Maplestone, 1926
Family Aproctidae Yorke and Maplestone, 1926
Family Desmidocercidae Cram, 1927
Superfamily Diplotriaenoidea Skrjabin, 1916
Family Diplotriaenidae Skrjabin, 1916
Family Oswaldofilariidae Chabaud and Choquet, 1953

INFRAORDER ASCARIDOMORPHA
Superfamily Ascaridoidea Baird, 1853
Family Heterocheilidae Railliet and Henry, 1912
Family Ascarididae Baird, 1853
Family Raphidascarididae Hartwich, 1954
Family Anisakidae Railliet and Henry, 1912
Superfamily Cosmocercoidea Skrjabin and Schikhobalova, 1951
Family Cosmocercidae Railliet, 1916
Family Atractidae Railliet, 1917
Family Kathlaniidae Lane, 1914
Superfamily Heterakoidea Railliet and Henry, 1914
Family Heterakidae Railliet and Henry, 1912
Family Aspidoderidae Skrjabin and Schikhobalova, 1947
Family Ascaridiidae Travassos, 1919
Superfamily Subuluroidea Travassos, 1914
Family Subuluridae Travassos, 1914
Family Maupasinidae Lopez-Neyra, 1945
Superfamily Seuratoidea Hall, 1916
Family Seuratidae Hall, 1916
Family Cucullanidae Cobbold, 1864
Family Quimperiidae Gendre, 1928
Family Chitwoodchabaudiidae Puylaert, 1970
Family Schneidernematidae Freitas, 1956

Suborder Myolaimina Inglis, 1983

Superfamily Myolaimoidea Andrássy, 1958
Family Myolaimidae Andrássy, 1958

Suborder Tylenchina Thorne, 1949

INFRAORDER PANAGROLAIMOMORPHA
Superfamily Panagrolaimoidea Thorne, 1937
Family Panagrolaimidae Thorne, 1937

Table 1.1. Continued.

Superfamily Strongyloidoidea Chitwood and McIntosh, 1934
Family Steinernematidae Filipjev, 1934
Family Strongyloididae Chitwood and McIntosh, 1934
Family Rhabdiasidae Railliet, 1916

INFRAORDER CEPHALOBOMORPHA
Superfamily Cephaloboidea Filipjev, 1934 Family Cephalobidae Filipjev, 1934 Family Elaphonematidae Heyns, 1962 Family Osstellidae Heyns, 1962
Family Alirhabditidae Suryawanshi, 1971
Family Bicirronematidae Andrássy, 1978

INFRAORDER TYLENCHOMORPHA
Superfamily Aphelenchoidea Fuchs, 1937
Family Aphelenchidae Fuchs, 1937
Family Aphelenchoididae Skarbilovich, 1947
Superfamily Criconematoidea Taylor 1936
Family Criconematidae Taylor, 1936
Family Hemicycliophoridae Skarbilovich, 1959
Family Tylenchulidae Skarbilovich, 1947
Superfamily Sphaerularioidea Lubbock, 1861 Family Anguinidae Nicoll, 1935 Family Sphaerulariidae Lubbock, 1861 Family Neotylenchidae Thorne, 1941 Family Iotonchidae Goodey, 1935
Superfamily Tylenchoidea Örley, 1880
Family Hoplolaimidae Filipjev, 1934
Family Meloidogynidae Skarbilovich, 1959
Family Tylenchidae Örley, 1880
Family Belonolaimidae Whitehead, 1959
Family Pratylenchidae Thorne, 1949
Superfamily Myenchoidea Pereira, 1931

INFRAORDER DRILONEMATOMORPHA
Superfamily Drilonematoidea Pierantoni, 1916
Family Drilonematidae Pierantoni, 1916
Family Ungellidae Chitwood, 1950
Family Homungellidae Timm, 1966
Family Pharyngonematidae Chitwood, 1950
Family Creagrocercidae Baylis, 1943

Suborder Rhabditina Chitwood, 1933

INFRAORDER BUNONEMATOMORPHA
Superfamily Bunonematoidea Micoletzky, 1922
Family Bunonematidae Micoletzky, 1922
Family Pterygorhabditidae Goodey, 1963

INFRAORDER DIPLOGASTEROMORPHA
Superfamily Cylindrocorporoidea Goodey, 1939
Family Cylindrocorporidae Goodey, 1939
Superfamily Odontopharyngoidea Micoletzky, 1922
Family Odontopharyngidae Micoletzky, 1922
Superfamily Diplogasteroidea Micoletzky, 1922
Family Pseudodiplogasteroididae Körner, 1954
Family Diplogasteroididae Filipjev and Schuurmans Stekhoven, 1941
Family Diplogasteridae Micoletzky, 1922
Family Neodiplogasteridae Paramonov, 1952
Family Mehdinematidae Farooqui, 1967
Family Cephalobiidae Filipjev, 1934

INFRAORDER RHABDITOMORPHA
***Incertae sedis*:** Family Carabonematidae Stammer and Wachek, 1952
***Incertae sedis*:** Family Agfidae Dougherty, 1955
Superfamily Mesorhabditoidea Andrássy, 1976
Family Mesorhabditidae Andrássy, 1976
Family Peloderidae Andrássy, 1976
Superfamily Rhabditoidea Örley, 1880
Family Diploscapteridae Micoletzky, 1922
Family Rhabditidae Örley, 1880
Superfamily Strongyloidea Baird, 1853
Family Heterorhabditidae Poinar, 1975
Family Strongylidae Baird, 1853
Family Ancylostomatidae Looss, 1905
Family Trichostrongylidae Witenberg, 1925
Family Metastrongylidae Leiper, 1908

Furthermore, a toolset of nematode-specific indices have been developed to measure food web status (Ferris, 2010; Ferris and Bongers, 2009; Neher, 2010; Sieriebriennikov et al., 2014). Based on various ecological studies undertaken during the 1970s and 1980s, Bongers et al. (1989) classified nematode taxa into five categories along an r (colonizer) – K (persister) strategists scale. This subsequently became known as the colonizer-persister (c-p) scale (Table 1.2), which ranges from c-p 1 (extreme r-strategists) to c-p 5 (extreme K-strategists) (Ferris and Bongers, 2009; Ferris et al., 2001).

According to Bongers (1990) r-strategists have short life-cycles, respond rapidly to favourable environmental conditions, and are tolerant to disturbance. K-strategists, in turn, have long life-cycles, present low reproduction rates, and are sensitive to disturbance. This c-p scale was used in the development of the Maturity Index family, which consists of various indices primarily used to assess ecosystem disturbance (Bongers, 1990; Sieriebriennikov et al., 2014; Tsiafouli et al., 2017). Relevant to this study is the original Maturity Index, which ranges from 1 – 5 with lower values indicating disturbance.

Table 1.2. Colonizer-persister (c-p) scale (1-5) assigned to nematodes based on their life history traits. Modified from Ferris et al. (2001).

C-P class	Description
c-p 1	Short generation time, small eggs, high fecundity, mainly bacterivores, feed continuously in enriched media, form dauer larvae as microbial blooms subside.
c-p 2	Longer generation time and lower fecundity than the c-p 1 group, very tolerant of adverse conditions and may become cryptobiotic. Feed more deliberately and continue feeding as resources decline. Mainly bacterivores and fungivores.
c-p 3	Longer generation time, greater sensitivity to adverse conditions. Fungivores, bacterivores, and carnivores.
c-p 4	Longer generation time, lower fecundity, greater sensitivity to disturbance. Besides the other trophic roles, smaller omnivore species.
c-p 5	Longest generation time, largest body sizes, lowest fecundity, greatest sensitivity to disturbance. Predominantly carnivores and omnivores.

Ultimately, research and model validations associated with the Maturity Index family led to the development of the Enrichment, Structure, Basal, and Channel indices, which are used to study and compare ecosystem processes (Ferris and Bongers, 2009). The Enrichment and Structure indices are based on the functional guilds of nematodes, which is defined by Ferris and Bongers (2009) as 'a matrix of nematode feeding habits with the biological, ecological, and life history characteristics embodied in the c-p classification'. These indices can be used to evaluate the food web status on a faunal profile plot (Fig. 1.3), which allows the classification of the food web status (Table 1.3), as well as comparisons between different environments (Ferris and Bongers, 2009; Ferris et al., 2001). The Enrichment and Structure indices are thus weighted measures of tolerant (r-strategists) vs. sensitive (K-strategists) nematodes, respectively (Sánchez-Moreno et al., 2018). The Channel Index, in turn, serves as a measure of organic matter decomposition controlled by fungi, while the Basal Index indicates diminished (basal) soil food web conditions (Ferris et al., 2001; Sánchez-Moreno et al., 2018). The above named indices have been widely used as indicators of ecosystem disturbance induced by different pollutants (Caixeta et al., 2016; Gutiérrez et al., 2016; Šalamún et al., 2014) and agricultural practices (Sánchez-Moreno et al., 2018; Zhong et al., 2017).

The use of metabolic footprints, originally proposed by Ferris (2010), further extend the functionality of nematode based assessments. Metabolic footprints measure the magnitude of ecosystem functions and services provided by nematodes, thus, indicating the main pathway of carbon and energy flow in the soil food web (Zhang et al., 2017). The main metabolic footprints include the Enrichment and Structure footprints, which represent the metabolic activity of enrichment and structure nematodes, respectively (Ferris, 2010; Zhang et al., 2015). All the named nematode-specific indices can be easily calculated and graphically illustrated using the Nematode Indicator Joint Analysis (NINJA) web-based tool (available at http://spark.rstudio.com/bsierieb/ninja) (Sieriebriennikov et al., 2014). For this, nematode abundance data are required with a minimum taxonomic resolution of family level.

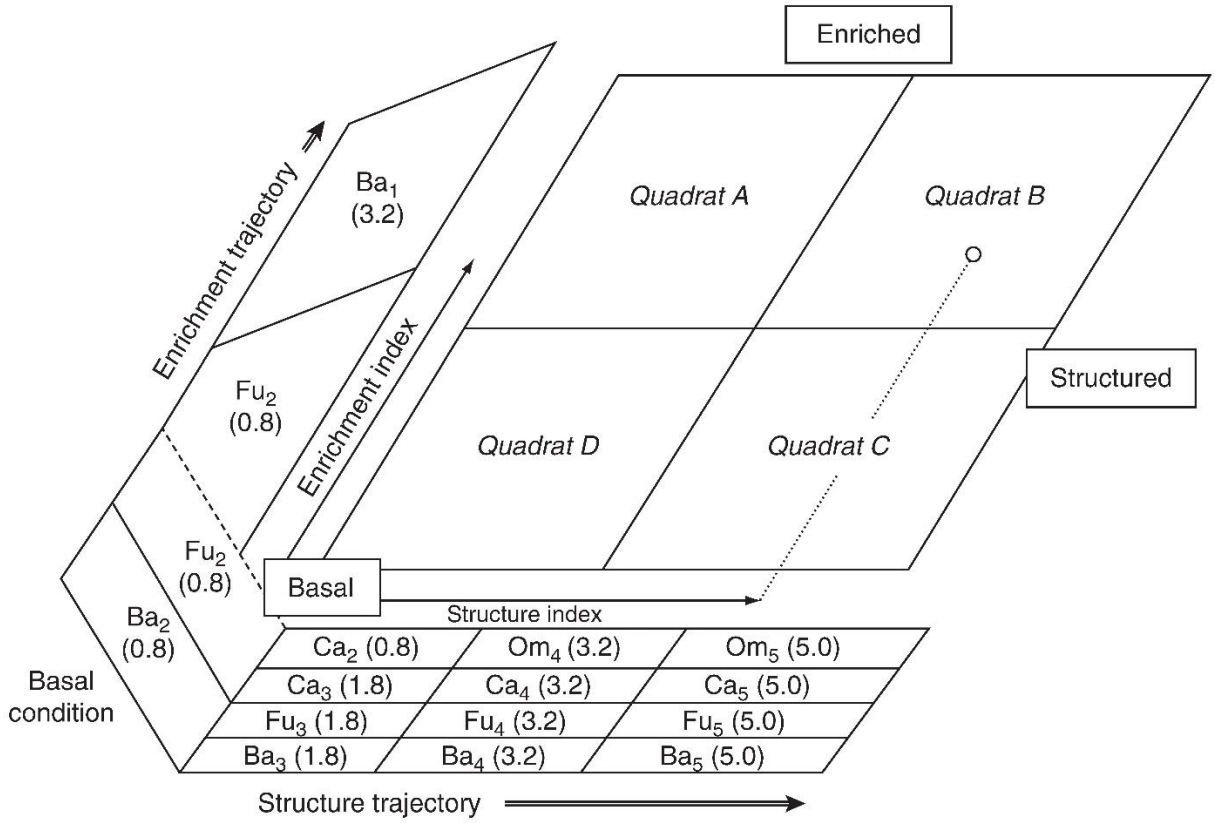

Fig. 1.3. Faunal profile (four quadrats) plotting of nematode assemblage as determined by the Enrichment and Structure indices. These indices are characterised by functional guilds that represent nematode trophic groups (bacterivores, fungivores, omnivores, and carnivores) and life history traits (colonizer-persister scale from 1 - 5). Originally created by Ferris et al. (2001).

Table 1.3. Soil food web status based on nematode faunal profile as suggested by Ferris et al. (2001).

General diagnosis	Quadrat A	Quadrat B	Quadrat C	Quadrat D
Disturbance	High	Low to moderate	Undisturbed	Stressed
Enrichment	N-enriched	N-enriched	Moderate	Depleted
Decomposition channels	Bacterial	Balanced	Fungal	Fungal
C:N ratio	Low	Low	Moderate to high	High
Food web condition	Disturbed	Maturing	Structured	Degraded

1.3.6 Nematodes as bioindicators of toxicity

Nematodes have been widely used to measure the toxicity of, for example, metals, organic pollutants, pesticides, and pharmaceuticals, as well as environmental sample extracts (Hägerbäumer et al., 2015; Höss and Williams, 2009). Although exposure can occur as a result of pollutant accumulation through the cuticle, the primary pathway is likely to be via the gut following the ingestion of pollutants and/or contaminated food (Ferris and Bongers, 2009). Nematodes are regarded as useful indicators of toxicity largely as a result of their ecological relevance (Höss and Williams, 2009). Other benefits, such as those presented by *C. elegans* (Fig. 1.4) (most commonly used nematode species for toxicity testing), include being easy to culture, small in size (adult = 1110-1150 µm), and the ability to grow in axenic liquid media (Hunt, 2017). According to Hunt (2017), these characteristics also render *C. elegans*, under stable laboratory conditions, a good candidate for high-throughput assessment tests.

While interspecific sensitivity is highly variable, a recent study by Hägerbäumer et al. (2018) confirmed *C. elegans* as a representative model of metal and polycyclic aromatic hydrocarbon toxicity for freshwater nematodes species. *Caenorhabditis elegans* is also a useful indicator of toxicity in soils (Höss et al., 2012; Höss et al., 2009; ISO, 2010). Furthermore, the use of nematodes in ecotoxicological studies is constantly evolving with, for example, recent advances in nanoparticle toxicity testing (Jung et al., 2015; Luo et al., 2017; Yang et al., 2017). Model ecosystems can also be used to study the response of natural assemblages to toxicant exposure under controlled conditions (Hägerbäumer et al., 2015).

Various endpoints of toxicity have been used and include nematode feeding, fertility, growth, moulting, movement, reproduction, respiration, and survival (Hägerbäumer et al., 2015; Höss and Williams, 2009; Jiang et al., 2016; Kohra et al., 2002). However, for the assessment of environmental samples (e.g. sediment, soil, or soil water), the ISO 10872 international standard uses sublethal parameters including growth, fertility, and reproduction (ISO, 2010), which is also likely to present varying sensitivity (Schertzinger et al., 2017). Although this protocol has been successfully applied before (Höss et al., 2012; Meyer et al., 2013;

Schertzinger et al., 2017), it is time consuming as the adult nematodes in each sample (and replicate) need to be measured and the number of offspring counted. This justifies the development of high-throughput assessment methods as valuable additions to the available toxicity toolset. An example of an approach showing promise is the measurement of *C. elegans* OCR using the Seahorse respirometer (Koopman et al., 2016; Luz et al., 2015b). However, current protocols make use of the instrument's capability of injecting compounds while the assay is being performed (Koopman et al., 2016).

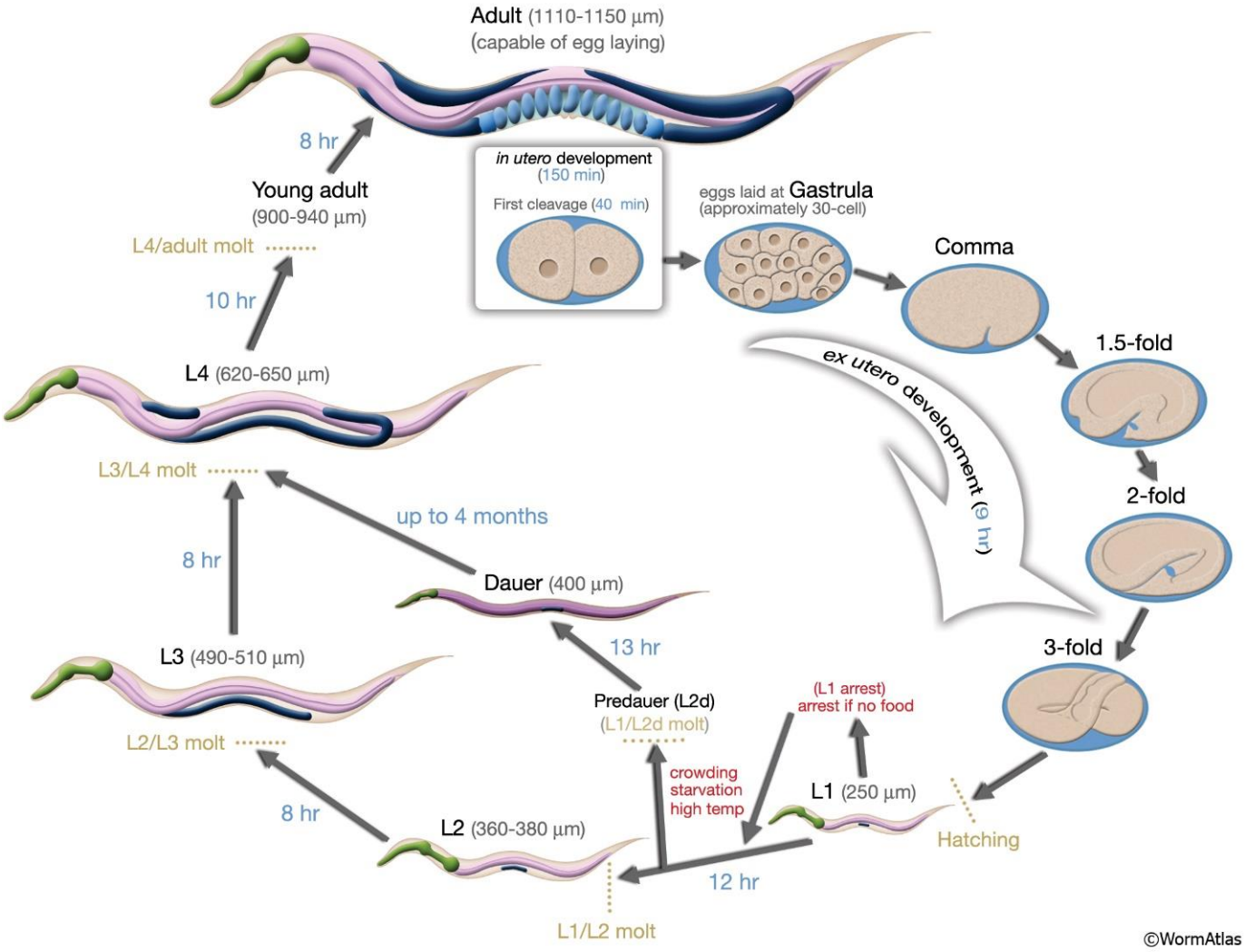

Fig. 1.4. The life cycle of *Caenorhabditis elegans* under normal and unfavourable (dauer larva) conditions. Blue numbers indicate the time spent (at 22 °C) in a specific life stage. Image provided by Hall and Altun (2008).

A literature survey revealed that no Seahorse bioassay protocol currently exists for evaluating the effect of *C. elegans* exposure to toxicants or environmental samples. Furthermore, published works focused on *C. elegans* fourth larval stage and older nematode stadia. However, it is likely that exposing first stage *C. elegans* larvae will present greater benefits such as 1) simplifying preparations for the high-throughput assessment approach and 2) greater sensitivity as larvae have been shown to be less tolerant than adults (Avila et al., 2011; Hunt, 2017).

1.3.7 Ecological risk assessment and the TRIAD approach

The ERA framework was published in 1992 by the United States Environmental Protection Agency (USEPA) as a means of evaluating the adverse ecological effects resulting from exposure to one or more stressors (Gutiérrez et al., 2015; Norton et al., 1992; Suter, 2007; USEPA, 1992). It is thus a method used to consider the impact of anthropogenic activities on the environment and if well executed, an ERA can limit or even prevent ecosystem damage (Rohr et al., 2016). Within the context of an ERA, the word 'stressor' refers to a chemical, physical, and/or biological factor that can adversely affect the ecological components (individuals, populations, communities, and/or ecosystems) of an environment (Norton et al., 1992; Suter, 2007). The ERA framework is regarded as a capable tool for assessing contaminated sites and is often used to inform environmental policy makers (Gutiérrez et al., 2015; Rohr et al., 2016; Swartjes, 2011).

Ecological risk assessments can be executed using different approaches, however, a useful and integrative method is the weight-of-evidence approach known as TRIAD (Dagnino et al., 2008; Gutiérrez et al., 2015). Although this methodology was first used in the assessment of sediment quality (Long and Chapman, 1985), it was later adapted for the assessment of contaminated soils and is now standardised as an International Organization for Standardization (ISO) protocol (ISO, 2017). The site-specific ERA model, as outlined by ISO (2017), constitutes five steps (Fig. 1.5). Briefly, during the first step, a desk-based study and

often the development of a conceptual site model is undertaken. This considers information on soil management, sources of contamination, exposure pathways of toxicants, as well as ecological receptors of concern (ISO, 2017). If a pollutant linkage is established, the ERA continues to the second step, which entails basic considerations of the TRIAD assessment that is to follow. This largely includes the design (sampling, analyses, data management, timeframe, etc.) of the practical investigation plan, which is formulated taking into account the ecological receptors, conditions, and ecosystem services at risk as a result of contamination. This step also considers the assessment criteria (e.g. reference values and/or reference sites), as well as interpretation methodology (e.g. scaling and weighting) of the results (Ashton et al., 2008; ISO, 2017). The next (third) step is represented by the practical execution of the soil quality TRIAD, which refers to the three components of soil health assessments, namely, the chemistry, ecology, and ecotoxicology LOEs.

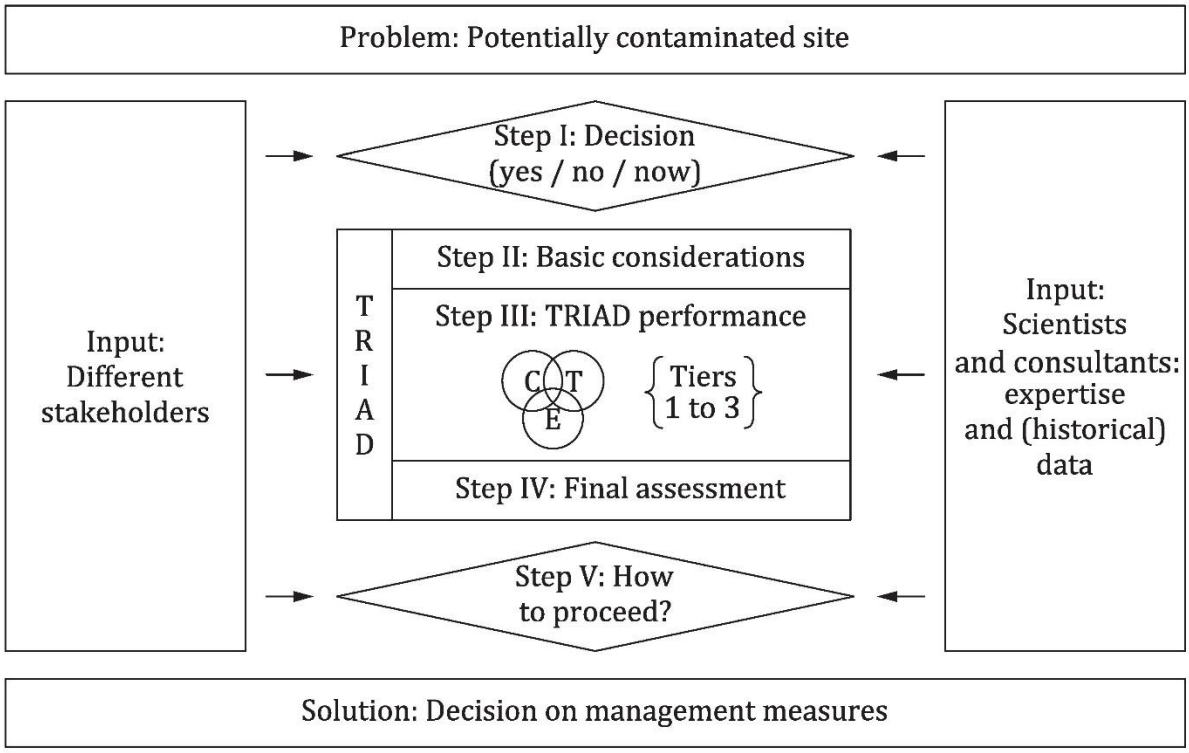

Fig. 1.5. Progression of a site-specific ecological risk assessment (ERA) of soil contamination. The TRIAD approach is represented by the chemistry (C), ecotoxicology (T), and ecology (E) lines-of-evidence, which are scaled and weighted to provide a final assessment on the risk. Originally created by ISO (2017).

The chemistry LOE is represented by the presence and concentration of the constituent(s) (toxicant) of concern and can include the total and/or bioavailable fractions (Jensen et al., 2006). In soil environments, this LOE is focused on, for example, metals and/or salts that may pose a threat to soil fauna and subsequently soil ecosystem health. The concentrations of such toxicants are usually compared to screening values or reference site concentrations (Ribé et al., 2012). The ecology LOE, in turn, considers the ecosystem health of soils by, for example, studying the diversity and abundance of soil invertebrates, or more specifically, the associated nematode assemblage (ISO, 2017; Jensen et al., 2006). The nematode-specific Maturity Index, for example, is often used in ERAs (Jensen et al., 2006; Semenzin et al., 2009). Lastly, the ecotoxicology LOE is represented by tests (bioassays) that measure the toxicity of an environmental sample. Various groups (e.g. microfauna or macrofauna) and/or species (e.g. *C. elegans*) tests can be used. It is important, however, to select tests that display sufficient sensitivity (e.g. with sublethal endpoints such as growth or reproduction) in order to accurately measure the toxic effect of a contaminated environmental sample. ISO (2010), for example, provides a protocol for assessing the toxicity of soil or extracted soil water (aqueous media) by considering the growth, fertility, and reproduction of *C. elegans* individuals exposed to such samples.

These three LOEs are often executed within tiers (e.g. tiers 1, 2, and 3), with higher tiers being more complex and specific with regard to the tests used and inferences made (ISO, 2017; Jensen et al., 2006). The main reason for implementing this approach is cost-effectiveness as the TRIAD assessment can be exited at any tier that provides sufficient information for the final assessment (ISO, 2017). It is important to note, however, that the TRIAD approach provided by ISO (2017) is not always executed in the proposed manner or sequence. For example, some tiers may be excluded, while the order in which the different lines-of-evidence are executed may be deviated from. Although there are several scientific and/or logistical reasons why this may be necessary, alterations should be justified. The fourth step involves the final assessment during which results are scaled, weighted, integrated, and interpreted. All tests conducted as part of the soil TRIAD should be scalable between 0 and 1, which makes

it possible to compare, combine, and utilize the results of the different LOE in the ERA. When considering the weighting of results, the different LOEs are usually equally weighted. However, when multiple tests are executed per LOE, weighting can be considered 1) if functional groups, as well as key and/or endangered species are relevant, 2) to minimize uncertainty or variability of test results, and 3) to correct bias in measured and calculated results if, for example, budget constraints did not allow for sufficient replication in dynamic ecosystems. Following, scaled results are integrated within each LOE into a risk number after which information from all three LOEs are integrated into one number of risks. An important consideration is the deviation in risk number between the LOEs; a high deviation might suggest that further investigations are necessary (ISO, 2017; Jensen et al., 2006). The final step entails deciding on whether there is an ecological risk at the studied site, which also represents the actual findings of the ERA. If a risk is evidenced, action will likely be required by stakeholders and policy makers (ISO, 2017).

1.3.8 Final considerations

From literature the water quality status of freshwater systems associated with the Crocodile (West) Catchment is of major concern. South Africa is already experiencing severe water shortages and with a growing human population, ensuring food security will become increasingly challenging. Furthermore, low quality irrigation water poses a direct threat to crop production (e.g. by causing leaf burn or lodging) and also threatens soil health as a result of exposure to salts, metals, and nutrients. Unfortunately, current irrigation water quality guidelines were not formulated following a holistic soil health framework, i.e. incorporating soil physico-chemical properties, as well as ecosystem health assessments. The soil quality TRIAD approach, however, does provide such a framework that can be used to study the effect of anthropogenic activities on farmland soils. By integrating the chemistry, ecology, and ecotoxicology LOEs into an ERA, the health status of irrigated farmlands can be evaluated, which represented the primary focus of this study.

1.4 Structure of thesis

This thesis is subdivided into the following chapters:

1. **Introduction, literature review, and thesis structure** formulates the rational of the study and presents the aims, objectives, and hypotheses. Emphasis is placed on major shortcomings in current irrigation water quality guidelines with regard to soil health from a biotic perspective.

2. **Article 1** investigates the historical irrigation water quality of the Hartbeespoort, Crocodile (West), and Marico-Bosveld (reference system) irrigation schemes over a period of 10 years (January 2005 – December 2015). Further emphasis is placed on temporal (seasonal and long term) and spatial variation in water quality, as well as potential factors contributing to the observed trends. This published article evidences the potential threat posed by deteriorating irrigation water quality to crop production and provides the basis (and justification) for this PhD study. This chapter, together with the introduction and literature review, represents step one in the ERA and establishes a pollution pathway.

3. **Article 2** considers the effects of irrigation water and soil water quality on soil ecosystem health as inferred by nematode assemblage structures. Also, the main factors contributing to the enrichment of soils are investigated. This chapter provides information on the assessment criteria (ERA: step two) of the ERA. Findings reported in this chapter are representative of the chemistry and ecology LOEs of the soil quality TRIAD (ERA: step three) and are integrated into Chapter 5 (Article 4).

4. **Article 3** describes a new protocol developed as a high-throughput assessment method for testing the toxicity of pollutants or drugs by considering *C. elegans* OCR inhibition as a sublethal endpoint of toxicity. This approach is used in Chapter 5 (Article 4) to evaluate the toxicity of environmental (aqueous) samples.

5. **Article 4** reports on the ecological risk (ERA: step four) posed to irrigated soils associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes. Findings from the chemistry and ecology LOEs (Chapter 3: Article 2) are integrated, while the results of the ecotoxicology LOE are reported, discussed, and also integrated. These LOEs are representative of a higher tier assessment as a lack of information, i.e. irrigation water quality guidelines that take into account soil ecosystem health, prevented lower tier investigations. A final risk number per farmland/irrigation scheme is calculated, followed by a discussion on the significance of the ecological risk posed to the health of irrigated soils.

6. **Conclusion and recommendations** considers the key findings of the PhD study. This final chapter also further discusses the outcome of the ERA and provides recommendations (ERA: step five) for future studies.

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CHAPTER 2: ARTICLE 1

Irrigation water quality and the threat it poses to crop production: evaluating the status of the Crocodile (West) and Marico catchments, South Africa.

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Irrigation water quality and the threat it poses to crop production: evaluating the status of the Crocodile (West) and Marico catchments, South Africa

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Abstract Ensuring food security is becoming increasingly difficult due to limited freshwater resources. Low-quality irrigation water also poses a severe threat to crop yield and quality. The aim of this study was to evaluate the water quality associated with the Crocodile (West) and Marico catchments, which represent one of South Africa's most developed regions. Sources of irrigation water include the hypertrophic Hartbeespoort Dam, as well as the heavily impacted Crocodile (West) River. Analysis of historical irrigation water quality data (from January 2005 to December 2015) revealed that the Hartbeespoort and Crocodile (West) irrigation schemes were exposed to calcium sulfate enrichment, likely as a result of extensive mining activities in the Bushveld Igneous Complex. Also, significant differences in water quality parameters occurred between these irrigation schemes and the reference system (Marico-Bosveld Irrigation Scheme), while important salt (chloride and sodium) and nutrient (inorganic nitrogen and orthophosphate (as phosphorus)) concentrations exceeded threshold values provided by irrigation water quality guidelines. The Hartbeespoort and Crocodile (West) irrigation schemes also presented distinctive temporal (long-term and seasonal) patterns in water quality. Seasonal variation in pH levels at the Hartbeespoort Irrigation Scheme is likely caused by excessive algae growth and cyanobacteria blooms (*Mycrocystis* sp.), which also pose an important threat to human and animal health. Despite mitigation efforts by government and other stakeholders, some of South Africa's major irrigation schemes remain highly impacted as a result of water quality deterioration.

Keywords Food security · Environmental pollution · Crop yield and quality · Hartbeespoort dam

Introduction

Globally, irrigated croplands account for 40% of food production and utilize 70% of freshwater withdrawals (Haddeland et al. 2014; Salmon et al. 2015). However, according to Elliott et al. (2014), water limitations in large irrigated regions (e.g., western USA and West, South, and Central Asia) could result in a 20–60 million ha reversal from irrigated to rain-fed farmland. This will drastically reduce crop yield and with the global population expected to increase by 50% before plateauing (Elliott et al. 2014), ensuring food security will become increasingly difficult. Furthermore, irrigation schemes are negatively impacted by anthropogenic activities that

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result in the deterioration of available water resources (Lu et al. 2015; Zhang et al. 2015).

Freshwater systems, especially in developing countries, are threatened by inadequate water management and treatment (Capps et al. 2016). An appropriate example is the Crocodile (West) River that represents the largest water system in the Crocodile (West) Catchment. This also forms part of the larger Crocodile (West) Marico Water Management Area, which is considered to be one of South Africa's most developed regions (DEAT 2005). The Crocodile (West) River system is subsequently exposed to sewage effluent, industrial wastewater, as well as agricultural and urban runoff (DEAT 2005; DWA 2013; Ballot et al. 2014; Kemp et al. 2016). Pollutants that include bacteria, dissolved and particulate trace metals, nutrients, persistent organic pollutants, pesticides, pharmaceuticals, and salts are prevalent in this river system (DEAT 2005; Ansara-Ross et al. 2012; Amdany et al. 2014; Ballot et al. 2014). As part of the River Health Program, DEAT (2005) reported that many of the rivers (e.g., Apies, Jukskei, and Pienaars) that either directly or indirectly serve as tributaries to the Crocodile (West) River, as well as the Crocodile (West) River itself, presented poor water quality status.

The Hartbeespoort Dam, which also forms part of this river system, is well known to be in a hypertrophic state as a result of being exposed to treated and even untreated sewage effluent (Van Dyk et al. 2012; Ballot et al. 2014). Subsequently, this freshwater body has suffered severe blooms of potentially toxin-producing *Microcystis* species from as early as the 1950s (Ballot et al. 2014). Unfortunately, this threat still prevails today as a recent report by Matthews and Bernard (2015) on the occurrence of cyanobacteria classified the Hartbeespoort Dam as the most impacted water body in South Africa.

It is evident that the 65,000 ha of irrigated lands associated with the Crocodile (West) River system are at threat (DWAF 2004; DEAT 2005). For this reason, a research project was undertaken to investigate the threat that irrigation water quality poses to crop production in the Crocodile (West) Catchment. This paper forms the basis of such a research undertaking and is aimed at studying the historical water quality of the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes. Emphasis will be placed on short- and long-term temporal variations by studying water quality trends, as well as the factors influencing it. Furthermore, irrigation water will be checked for its compliance with the South Africa Water Quality Guidelines for Agricultural Use: Irrigation.

Methods

Study area

The study areas included the Hartbeespoort and Crocodile (West) irrigation schemes that form part of the Crocodile (West) Catchment, as well as the Marico-Bosveld Irrigation Scheme, which is associated with the Marico Catchment (Fig. 1). Water utilized by the Hartbeespoort Irrigation Scheme is sourced from the Hartbeespoort Dam, a major reservoir of the Crocodile (West) River system, via the Eastern and Western canals that extend 78 and 58 km, respectively (Meyer and McKay 2012). Farmers irrigating in the Crocodile (West) Irrigation Scheme, however, abstract water directly from a 117 km stretch of the Crocodile (West) River starting approximately 56 km downstream of the Hartbeespoort Dam. The Marico-Bosveld Dam, in turn, forms part of the Groot-Marico River system and also provides farms with irrigation water via a canal system. The latter served as the minimally impacted reference system, since this system is not subjected to substantial anthropogenic pollution (Kemp et al. 2016; Wolmarans et al. 2017). The Crocodile (West) and Groot-Marico River systems, including the tributaries and reservoirs, represent the main freshwater systems associated with the Crocodile (West) and Marico catchments, respectively. These river systems ultimately join at the confluence of the Limpopo River (DWA 2013).

The Crocodile (West) and Marico catchments are represented by a very diverse geological setting with the mineral-rich Bushveld Igneous Complex dominating the region north of the Magaliesberg mountains (DWAF 2004; DEAT 2005). The predominant soil types are moderate to deep sandy loam in the south-eastern regions, while deep, clayey loam soils can be found in the remainder of the region. The mean annual temperatures range from 18 to 20 °C and the mean annual rainfall from 400 to 800 mm with the eastern sections enjoying the highest annual rainfall (DEAT 2005). The wet and high-flow season runs from December to May, while the dry and low-flow season extends from June to November.

Data extraction and validation

Historical water quality data (combined total of 1496 records) for a 10-year period (January 2005–December 2015) of four monitoring sites (Fig. 1) were sourced from the Department of Water and Sanitation: Resource Quality Information Services (RQIS 2016). Two of these monitoring sites represent canal discharge points at the Hartbeespoort (RQIS: 90240; −25.726540°, 27.848839°) and Marico-Bosveld (RQIS: 90323; −25.470440°, 26.392580°) Dams. The third monitoring site (RQIS: 90233; −24.695140°, 27.409060°) is located on the Crocodile (West) River, approximately 170 km downstream of the Hartbeespoort Dam. These first three monitoring sites were used to study the water quality of the Hartbeespoort, Marico-Bosveld, and Crocodile (West) irrigation schemes, respectively. An additional Crocodile (West) River monitoring site (RQIS: 90214; −25.723767°, 27.847873°), located directly downstream of the Hartbeespoort Dam wall, was also included to study the change in river water quality over a spatial scale. For the sake of clarity, this site will be referred to as the upstream river site, while the Crocodile (West) Irrigation Scheme is represented by the downstream river site (Fig. 1). Verified flow data (total cubic meters per month) at the downstream river site, as well as for the major inflow (upper Crocodile (West) River at Kalkheuwel; DWS: A2H012; −25.81056, 27.90983) to the Hartbeespoot Dam, were obtained from the Department of Water and Sanitation: Hydrological Services (DWS 2016). This was

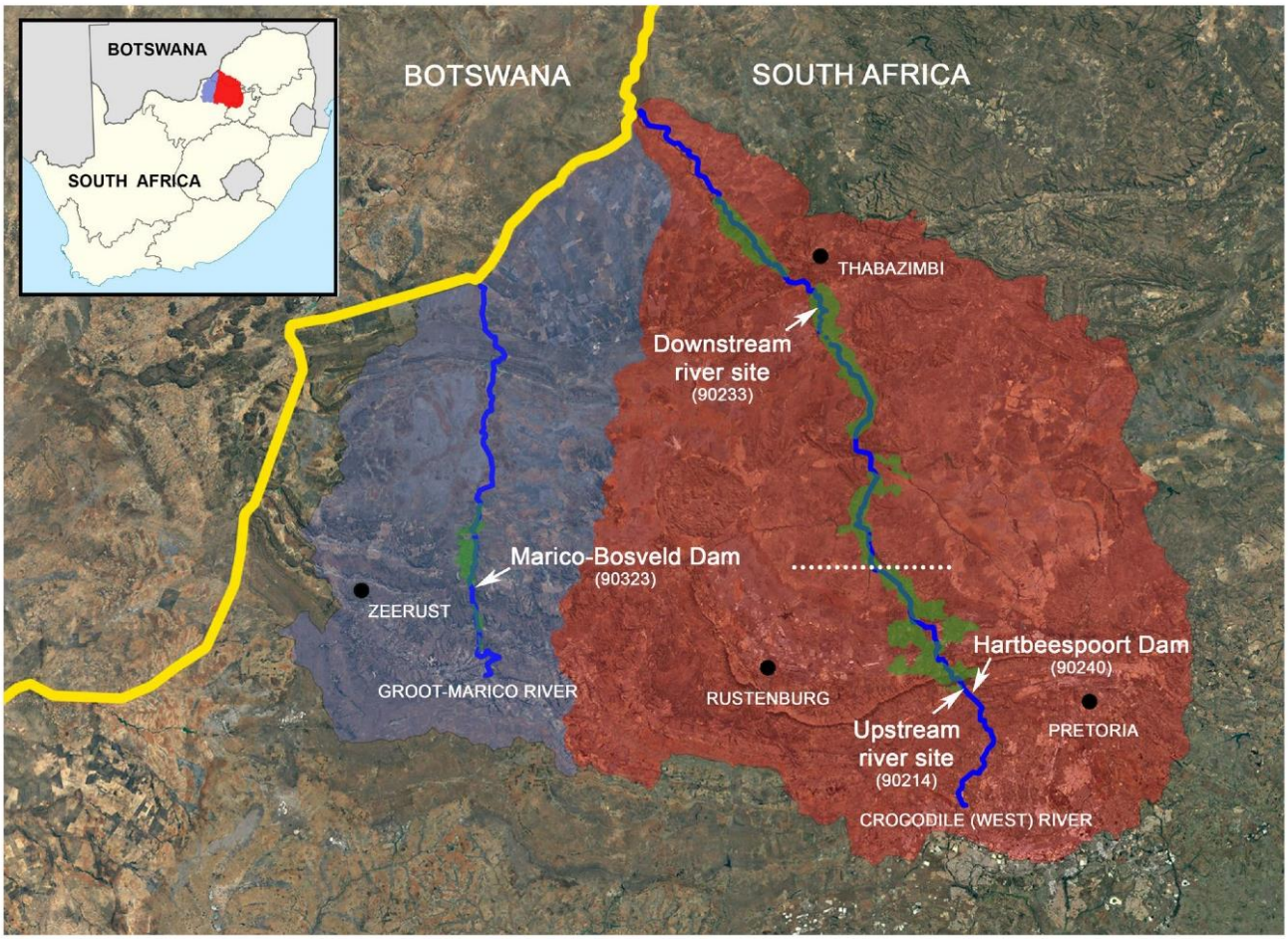

Fig. 1 Satellite imagery of the Crocodile (West) (red polygon) and Marico (blue polygon) catchments and their respective major water systems (Crocodile (West) and Marico Rivers). The selected Department of Water and Sanitation: Resource Quality Information Services monitoring sites are also indicated (e.g., RQIS no. 90214). Extensive farmlands are represented by green polygons, which are associated with the Hartbeespoort Dam (below white dotted line), Crocodile (West) (above white dotted line), and Marico-Bosveld irrigation schemes

used to study seasonal patterns and the effect that dilution might have on water quality parameters.

Records that listed the concentrations of major ions were validated and quality checked by calculating charge balance errors; records with errors greater than 5% were discarded (Freeze and Cherry 1979; Clark 2015). Specific water quality parameters were selected for further investigations: electrical conductivity (EC), pH, sodium adsorption ratio (SAR), total alkalinity (TAL), and major ion (calcium (Ca), chloride (Cl), fluoride (F), magnesium (Mg), potassium (K), silicon (Si), sodium (Na), and sulfate (SO_4)) and nutrient (orthophosphate (as phosphorous) (PO_4-P) and inorganic nitrogen (N)) concentrations.

Statistical analysis

Relationships between measured water quality parameters were analyzed graphically and numerically across spatial and temporal regimes. While spatial variation was considered between the different irrigation schemes and along the Crocodile (West) River, short- (seasonal) and long-term temporal variations were investigated at each of the monitoring points.

A Durov diagram was created using the Aquachem 2014.2 software package (Schlumberger Water Services) to compare the water character (chemical composition) and degree of enrichment (natural or anthropogenic) between the different irrigation schemes. Following, irrigation-relevant water quality parameters (EC, Cl, SO_4, PO_4-P, inorganic N, and Na) were studied over the selected time period using line graphs. These parameters were then subjected to D'Agostino and Pearson omnibus normality test in order to determine the appropriate statistical tests to be used. Following, the unpaired *t* test with Welch's correction (parametric data) or Mann-Whitney test (non-parametric data) was used to test for significant differences between the means. Long-term variation in water quality concentrations was studied using Pearson's correlation coefficient and Spearman's rank correlation coefficient tests for parametric and non-parametric data, respectively. In order to study the potential influence that flow had on EC, pH, and nutrients (inorganic N and PO_4-P), linear regression models were used. The above mentioned statistical tests were performed and graphs created using GraphPad Prism 7 software package.

Furthermore, correlations between the water quality parameters (monthly averaged data) of the three irrigation schemes were illustrated on principal component analysis (PCA) biplots using Canoco 5 software package. Lastly, the same statistical approach (unpaired *t* test with Welch's correction or Mann-Whitney test) as before was used to determine the significance for the remaining water quality parameters between the respective irrigation schemes.

Results

Water quality comparison between irrigation schemes

Monitoring site records of the selected 10-year time period (January 2005–December 2015) was used to evaluate the irrigation water quality of the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes, as well as the upstream river site (Fig. 1). Averaged high (December to May) and low (June to November) flow data of all available parameters are provided in Table 1.

The Durov diagram (Fig. 2) showed that both the Hartbeespoort and Crocodile (West) irrigation schemes presented similar cation (Na and K) and anion (Cl and SO_4) enrichment with respect to the reference scheme. And while the highest EC readings, as well as the biggest range, were recorded at the Crocodile (West) Irrigation Scheme, the Hartbeespoort Irrigation Scheme presented the highest pH range. The reference system (Marico-Bosveld Irrigation Scheme), in turn, presented no enrichment, as well as lower EC readings, and lower EC and pH variations.

Temporal and spatial variations in selected irrigation water quality guideline parameters (EC, Cl, SO_4, PO_4, inorganic N, and Na) were assessed on line graphs. The first line graph (Fig. 3) illustrates the significant ($p < 0.001$) differences between the reference system and Hartbeespoort Irrigation Scheme with the latter consistently exceeded the EC target value of 40 mS/m as listed in the South Africa Water Quality Guidelines for Agricultural Use: Irrigation (DWAF 1996). Orthophosphate (as phosphorous), in turn, exceeded the long-term trigger value (0.05 mg/L) of the Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC 2000). The reference system mostly conformed to these irrigation water quality guidelines. Long-term variation (water quality trends) was studied using the appropriate correlation tests (Table 2). The Hartbeespoort Irrigation Scheme presented a significant ($p < 0.05$) increase in inorganic N and SO_4, while increases (with weaker correlations) in EC, Na, and PO_4-P

Table 1 Averaged high (December May) and low (June November) flow water quality data (January 2005–December 2015) with standard deviations at four monitoring sites associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes, as well as the Crocodile (West) River

Monitoring site	Flow period	EC (mS/m)	pH	SAR	TAL (as $CaCO_3$) (mg/L)	Ca (mg/L)	Cl (mg/L)	F (mg/L)
Hartbeespoort Irrigation Scheme	High	51.87 ± 4.77	8.46 ± 0.42	1.58 ± 0.16	121.73 ± 15.28	34.56 ± 5.22	51.26 ± 8.07	0.31 ± 0.09
	Low	56.89 ± 4.20	8.28 ± 0.29	1.59 ± 0.16	133.34 ± 13.88	39.07 ± 4.85	54.41 ± 7.53	0.31 ± 0.11
Crocodile (West) Irrigation Scheme (downstream river site)	High	72.60 ± 13.33	8.21 ± 0.33	2.00 ± 0.33	171.14 ± 29.97	42.28 ± 8.00	83.61 ± 22.27	0.46 ± 0.10
	Low	83.44 ± 8.07	8.31 ± 0.28	2.17 ± 0.27	197.44 ± 18.23	49.91 ± 6.73	101.98 ± 15.46	0.46 ± 0.09
Marico-Bosveld Irrigation Scheme	High	28.51 ± 5.26	8.33 ± 0.27	0.22 ± 0.07	143.11 ± 36.72	24.31 ± 6.57	4.92 ± 1.69	0.27 ± 0.14
	Low	30.71 ± 4.54	8.38 ± 0.20	0.23 ± 0.05	152.23 ± 25.31	26.08 ± 4.43	5.00 ± 1.24	0.26 ± 0.11
Upstream river site	High	52.17 ± 4.60	8.20 ± 0.40	1.54 ± 0.17	125.90 ± 16.81	35.32 ± 6.11	50.35 ± 8.88	0.31 ± 0.10
	Low	57.18 ± 4.34	8.22 ± 0.32	1.61 ± 0.16	136.36 ± 15.17	38.42 ± 5.29	54.49 ± 7.99	0.29 ± 0.10

Monitoring site	Mg (mg/L)	N (inorg.) (mg/L)	PO_4-P (mg/L)	K (mg/L)	Si (mg/L)	Na (mg/L)	SO_4 (mg/L)
Hartbeespoort Irrigation Scheme	14.50 ± 1.65	1.65 ± 0.93	0.22 ± 0.11	7.89 ± 1.02	4.39 ± 1.32	43.52 ± 4.85	48.68 ± 6.71
	17.07 ± 3.08	2.61 ± 1.28	0.20 ± 0.12	8.24 ± 0.96	2.61 ± 1.32	46.57 ± 4.69	52.89 ± 7.77
Crocodile (West) Irrigation Scheme (downstream river site)	25.30 ± 6.57	0.74 ± 0.54	0.16 ± 0.11	8.37 ± 1.04	5.89 ± 1.15	66.26 ± 15.32	66.81 ± 13.63
	29.56 ± 3.63	1.14 ± 0.85	0.15 ± 0.12	8.55 ± 1.31	3.90 ± 1.79	77.95 ± 10.75	82.49 ± 10.39
Marico-Bosveld Irrigation Scheme	17.80 ± 4.30	0.14 ± 0.11	0.02 ± 0.02	1.73 ± 0.40	4.68 ± 1.13	5.96 ± 2.06	7.01 ± 2.54
	20.22 ± 4.39	0.17 ± 0.11	0.02 ± 0.07	1.63 ± 0.63	4.61 ± 0.97	6.47 ± 1.53	6.50 ± 1.95
Upstream river site	14.73 ± 2.32	1.90 ± 0.91	0.26 ± 0.15	7.91 ± 1.08	4.27 ± 1.26	42.6 ± 4.96	48.31 ± 9.56
	16.72 ± 2.99	2.77 ± 0.98	0.20 ± 0.14	8.41 ± 1.33	2.46 ± 1.08	46.41 ± 4.72	53.34 ± 11.43

were also recorded. The Marico-Bosveld Irrigation Scheme, however, presented significant ($p < 0.05$) decreases in EC, Cl, and PO_4-P. Supplementary data (Online Resource 1) illustrate the most substantial ($r > 0.5$) increases over the selected 10-year time period.

Using the same approach, water quality data were also compared between the upstream and downstream (Crocodile (West) Irrigation Scheme) river sites. Line graphs (Fig. 4) clearly illustrate the significant ($p < 0.001$) differences in water quality parameters with the Crocodile (West) Irrigation Scheme generally presenting the highest EC readings and Cl, SO_4, and Na concentrations. However, nutrient levels were significantly ($p < 0.001$) higher at the upstream river site. Electrical conductivity readings at both monitoring sites exceeded the DWAF target value of value 40 mS/m. Furthermore, the Crocodile (West) Irrigation Scheme exceeded the same guideline's target values for Cl (100 mg/L) and Na (70 mg/L), while both monitoring sites exceeded ANZECCs long-term trigger value for PO_4-P (0.05 mg/L). Correlation tests evidenced that over the selected time period EC, inorganic N, PO_4-P, and SO_4 presented significant ($p < 0.05$) increases at the upstream and downstream river sites. Inorganic N presented the most substantial increase at both river sites.

Seasonal variation and effect of flow

The line graphs illustrated that not only long-term temporal variation but also substantial seasonal variation occurred. Temporal trends at the three irrigation schemes were further visualized on a series of PCA biplots using monthly averaged data of selected water quality parameters. The first PCA biplot (Fig. 5a) was used to illustrate differences between the respective irrigation schemes. With 97.7% of the variation explained on F1 (93.6%) and F2 (4.1%), Fig. 5a clearly shows that the reference system was negatively

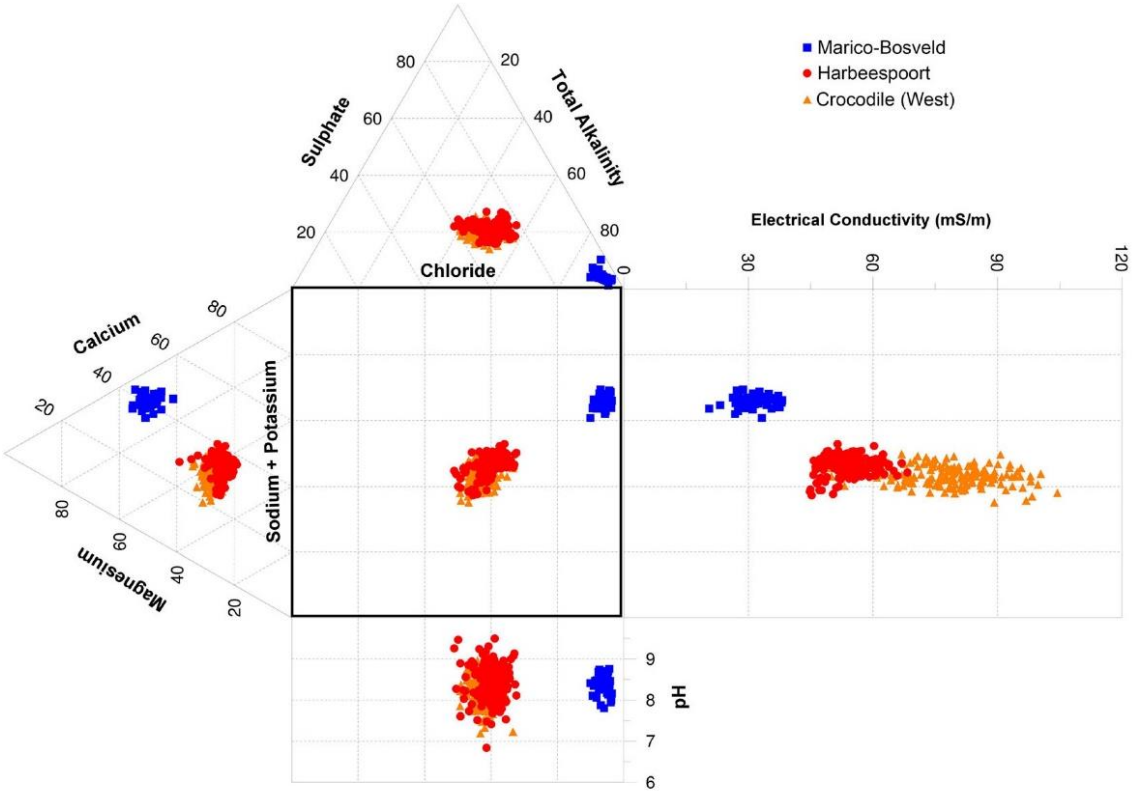

Fig. 2 Durov diagram illustrating the water characteristics of the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes

correlated with the Hartbeespoort and Crocodile (West) irrigation schemes, as well as to most of the water quality parameters. These parameters, apart from pH, differed significantly ($p < 0.05$) between the Hartbeespoort Irrigation Scheme and the reference system. Similarly, significant ($p < 0.05$) differences, with the exception of Si, were recorded between the Crocodile (West) Irrigation Scheme and the reference system. However, since minimal variation was explained on F2 (Fig. 5a), a second PCA biplot (Fig. 5b) with 97% of the variation explained on F1 (73.5%) and F2 (23.5%) was used to illustrate differences between the Hartbeespoort and Crocodile (West) irrigation schemes. This clearly showed that the Hartbeespoort Irrigation Scheme is positively correlated with nutrients (inorganic N and PO_4-P), while the Crocodile (West) Irrigation Scheme is positively correlated with the remainder of the water quality parameters.

The final two PCA biplots (Fig. 6) were used to study monthly patterns (with temporal progression indicated as dotted lines) at the Hartbeespoort and Crocodile (West) irrigation schemes. For both these analyses, total monthly flow was also considered. The Hartbeespoort Irrigation Scheme PCA biplot (Fig. 6a), with 94.9% of the variation explained on F1 (77.9%) and F2 (17%), revealed a strong, positive correlation between most of the water quality parameters and the July (H07) to December (H12) time period. Furthermore, pH and flow were positively correlated with the December (H12) to March (H03) time period, while Si presented the strongest correlation to the February (H02) to May (H05) time period. However, April (H04) and May (H05) were negatively correlated to most of the parameters. With the Crocodile (West) Irrigation Scheme PCA biplot (Fig. 6b), 97.23% of the variation was explained on F1 (90.16%) and F2 (7.07%). It clearly illustrated that flow, Si, and PO_4-P were positively correlated to the December (C12) to May (C05) time period, while the June (C06) to August (C08) time period was positively correlated with most of the remaining water quality parameters. Some parameters (PO_4-P, K, TAL, Na, and Cl) were also positively correlated with the September (C09) to November (C11) time period.

Evident from the line diagrams and PCA biplots was the influence that flow had on some of the water quality parameters. Subsequently, monthly averaged pH and EC (a proxy for the dissolved salts) readings, as well as

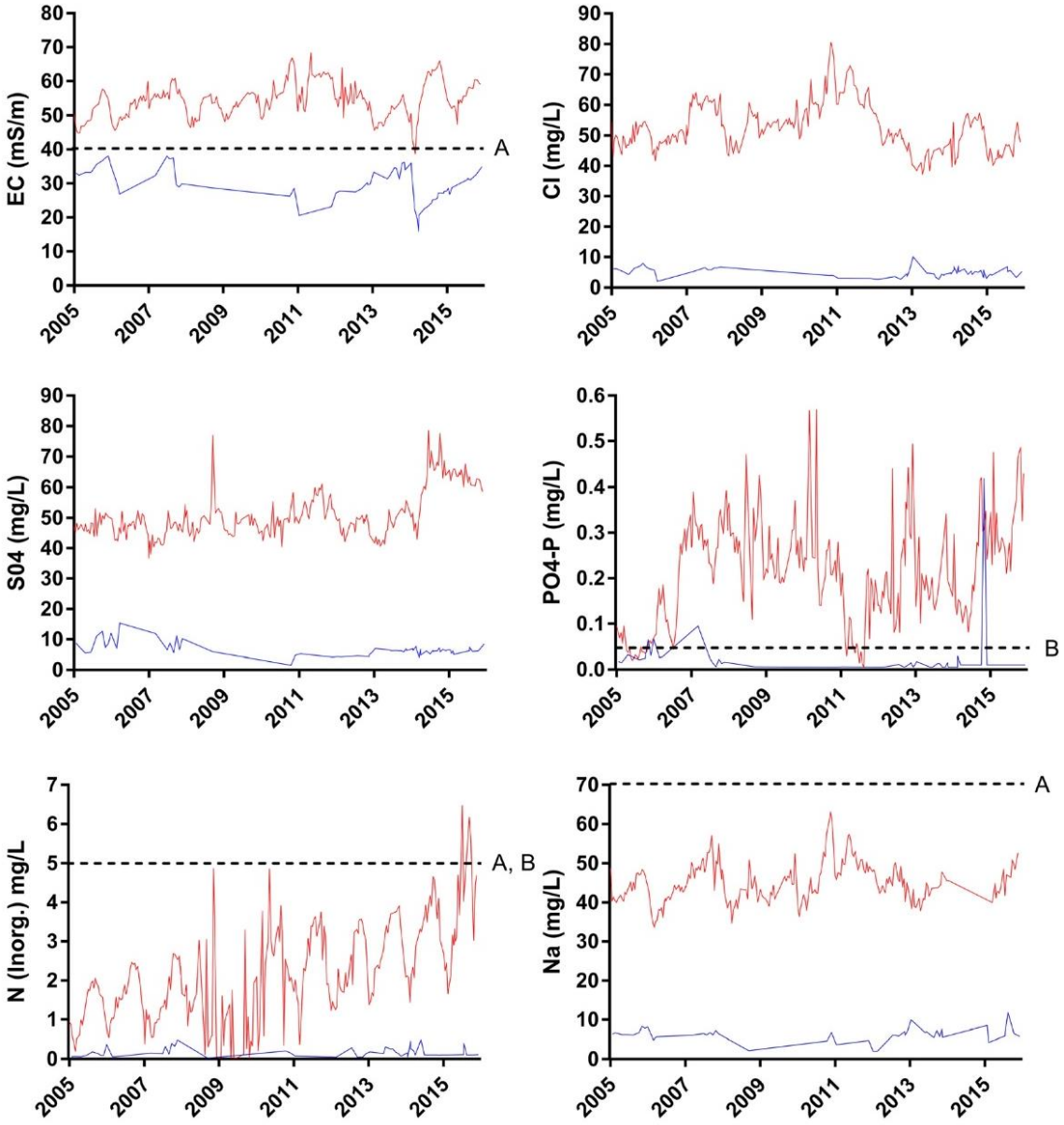

Fig. 3 Time series data (January 2005–December 2015) of selected irrigation water quality parameters at the Hartbeespoort (red line) and Marico-Bosveld (blue line) irrigation schemes. Dotted lines represent the South Africa Water Quality Guidelines for Agricultural Use: Irrigation target value (**A**) and Australian and New Zealand Guidelines for Fresh and Marine Water Quality long-term trigger value (**B**)

Table 2 Correlation coefficient (*r* value) between irrigation water quality parameters and the selected time period (January 2005–December 2015) indicate water quality trends at the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes, as well as the Crocodile (West) River

Monitoring site	Cl	EC	N (inorg.)	Na	PO_4-P	SO_4
Hartbeespoort Irrigation Scheme	− 0.15*	+ 0.29*	+ 0.62*	+ 0.20*	+ 0.25*	+ 0.51*
Crocodile (West) Irrigation Scheme (downstream river site)	+ 0.08	+ 0.26*	+ 0.58*	+ 0.17*	− 0.19*	+ 0.22*
Marico-Bosveld Irrigation Scheme	− 0.31*	− 0.44*	+ 0.09	− 0.01	− 0.38*	− 0.20
Upstream river site	− 0.13	+ 0.34*	+ 0.6*	+ 0.17*	+ 0.24*	+ 0.43*

*$p < 0.05$, significant results

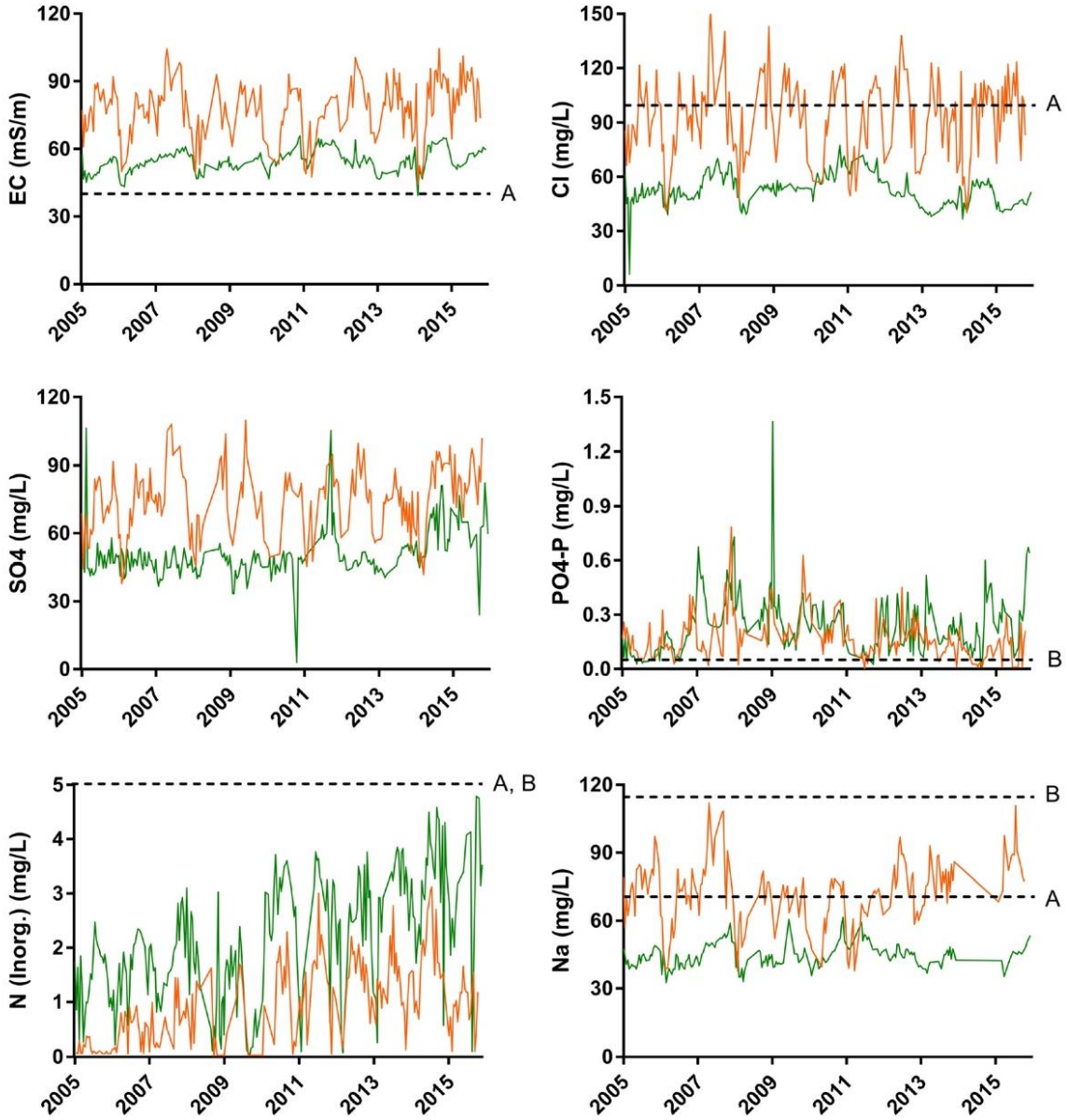

Fig. 4 Time series data (January 2005–December 2015) of selected irrigation water quality parameters at the upstream river site (green line) and the Crocodile (West) Irrigation Scheme (downstream river site) (orange line) along the Crocodile (West) River. Dotted lines represent the South Africa Water Quality Guidelines for Agricultural Use: Irrigation target value (**A**) and Australian and New Zealand Guidelines for Fresh and Marine Water Quality long-term trigger value (**B**)

nutrient (inorganic N and PO_4-P) concentrations, were linearly regressed against total monthly flow. For the Hartbeespoort Irrigation Scheme (Fig. 7), a significant and positive linear relationship was recorded between flow and pH ($R^2 = 0.84$), while negative relationships were recorded between flow and EC ($R^2 = 0.46$), as well as flow and inorganic N ($R^2 = 0.72$). The Crocodile (West) Irrigation Scheme (Fig. 8), in turn, presented significant ($p < 0.05$) and negative linear relationships between flow and pH ($R^2 = 0.57$) and flow and EC ($R^2 = 0.7$).

Discussion

Anthropogenic enrichment

The importance of minimalizing anthropogenic impact on freshwater resources utilized for irrigation has been well documented (Srinivasan and Reddy 2009; Khan et al. 2013; Rahman et al. 2014; Lu et al. 2015; Zhang et al. 2015). Nevertheless, the Durov diagram criteria provided by Hodgson (2004) suggest that the

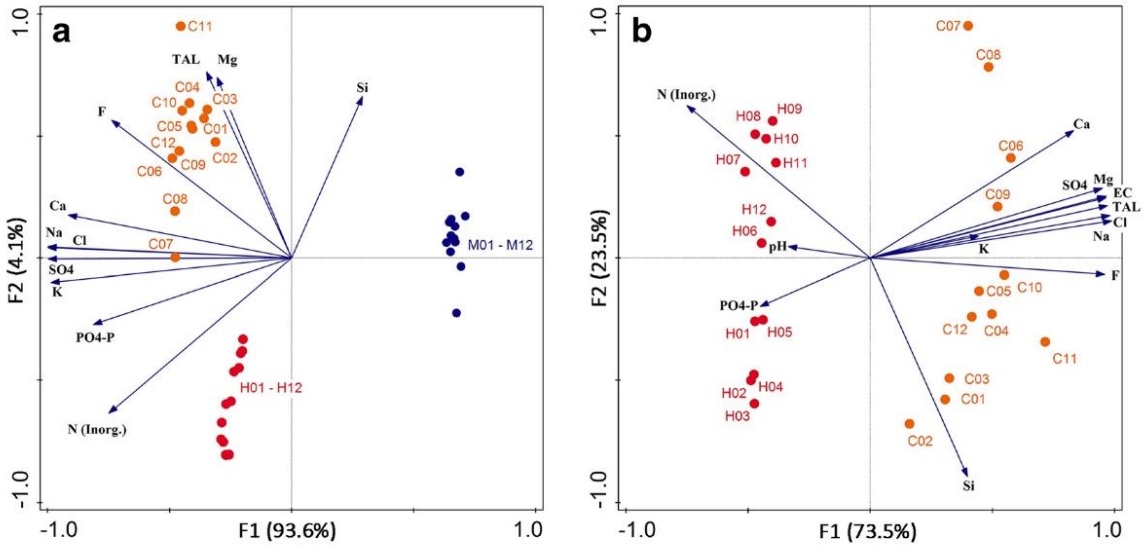

Fig. 5 PCA biplots illustrate spatial variation in major ion concentrations (monthly averaged) between the **a** Hartbeespoort (H01–H12), Crocodile (West) (C01–C12), and Marico-Bosveld (M01–M12) irrigation schemes, as well as between the **b** Hartbeespoort and Crocodile (West) irrigation schemes

enrichment observed at the Hartbeespoort and Crocodile (West) irrigation schemes may be linked to calcium sulfate pollution as a result of mining activity. This is not surprising as various mines, especially platinum-group metal mines, are associated with the Bushveld Igneous Complex in Brits and Rustenburg (North-West Province, South Africa), as well as the surrounding areas (Walsh and Wepener 2009; Van der Walt et al. 2012; Ololade and Annegarn 2013; Rauch and Fatoki 2013). Mining activity in especially the Rustenburg area, also with the two largest platinum mines in the world (Impala and Anglo Platinum), has substantially increased in the twenty-first century largely as a result of a rise in demand (Van der Walt et al. 2012; Ololade and Annegarn 2013). These activities have resulted in elevated Ni, Cr, Pt, Pd, Rh, and Ir concentrations in water and sediments of the Hex River, which is a tributary of the Crocodile (West) River system (Almécija et al. 2017). This may pose a severe threat to food safety and security (Zhang et al. 2013, 2015; Lu et al. 2015).

Anthropogenic pollution in the Crocodile (West) River system was further evidenced as significant water quality differences between the Hartbeespoort Irrigation Scheme and the reference system (Marico-Bosveld Irrigation Scheme), as well as significant differences between higher (upstream river site) and lower (downstream river site; Crocodile (West) Irrigation Scheme) reaches of this river system. Although water characteristics are naturally influenced by geological, hydrological, and climatic factors (Meybeck et al. 1996; Wolmarans et al. 2017), studies have shown that the Crocodile (West) River system is subjected to and severely impacted by anthropogenic pollution (e.g., salts, nutrients, pesticides, and pharmaceuticals) (DEAT 2005; Walsh and Wepener 2009; Amdany et al. 2014; Ballot et al. 2014).

Consequences of nutrient loading

Although increased crop yield have been recorded as a result of irrigating with nutrient contaminated wastewater (Singh et al. 2012), the latter can also contain toxicants and have unwanted effects on freshwater systems. For example, the consequence of nutrient loading is sometimes visible as increased algal and water hyacinth growth and even cyanobacteria (e.g., *Microcystis*) blooms in the Hartbeespoort Dam. This poses a severe threat to animal and human health, as well as the food production industry (Ballot et al. 2014). According to Ballot et al. (2014) severe blooms of potentially toxin-producing *Microcystis* species have occurred from as early as the 1950s in the Hartbeespoort Dam. Some *Microcystis* species can even produce hepatotoxic and neurotoxic secondary metabolites (Ballot et al. 2014; Zhang et al. 2015). Studies in Southeast China have shown that a positive correlation existed between hepatocellular carcinoma risk and human populations that utilized polluted water from eutrophic freshwater systems (Zhang et al.

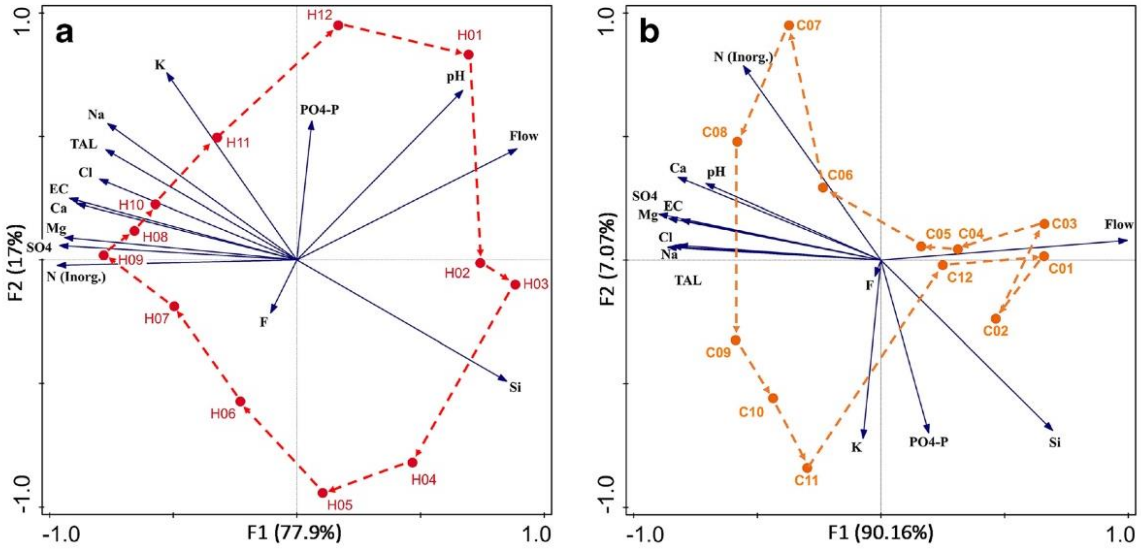

Fig. 6 PCA biplots illustrate temporal variation in major ion concentrations (monthly averaged) at the **a** Hartbeespoort and **b** Crocodile (West) irrigation schemes

2015). Also, during the 1970s, a case of cattle mortalities was linked to toxins produced by *Microcystis aeruginosa* in the Hartbeespoort Dam (Ballot et al. 2014). Cyanobacteria also present additional threats to water quality. Following senescence, the decomposition of cyanobacteria can result in oxygen

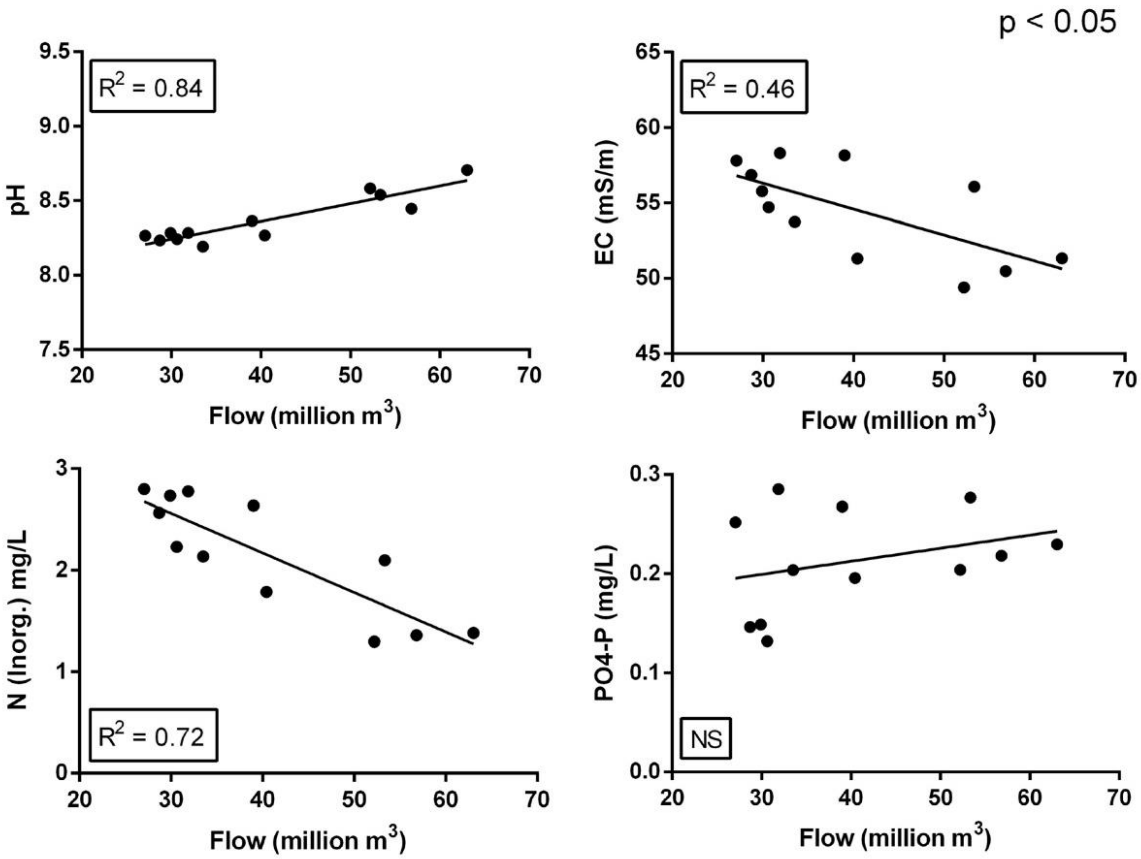

Fig. 7 Linear regression models illustrating the relationship between total monthly flow (as million cubic meter (m^3)) against pH, electrical conductivity, inorganic nitrogen, and orthophosphate (as phoshorus) at the Hartbeespoort Irrigation Scheme. *R*-square values are provided and non-significant (NS) models indicated. Statistical test was regarded significant when $p < 0.05$

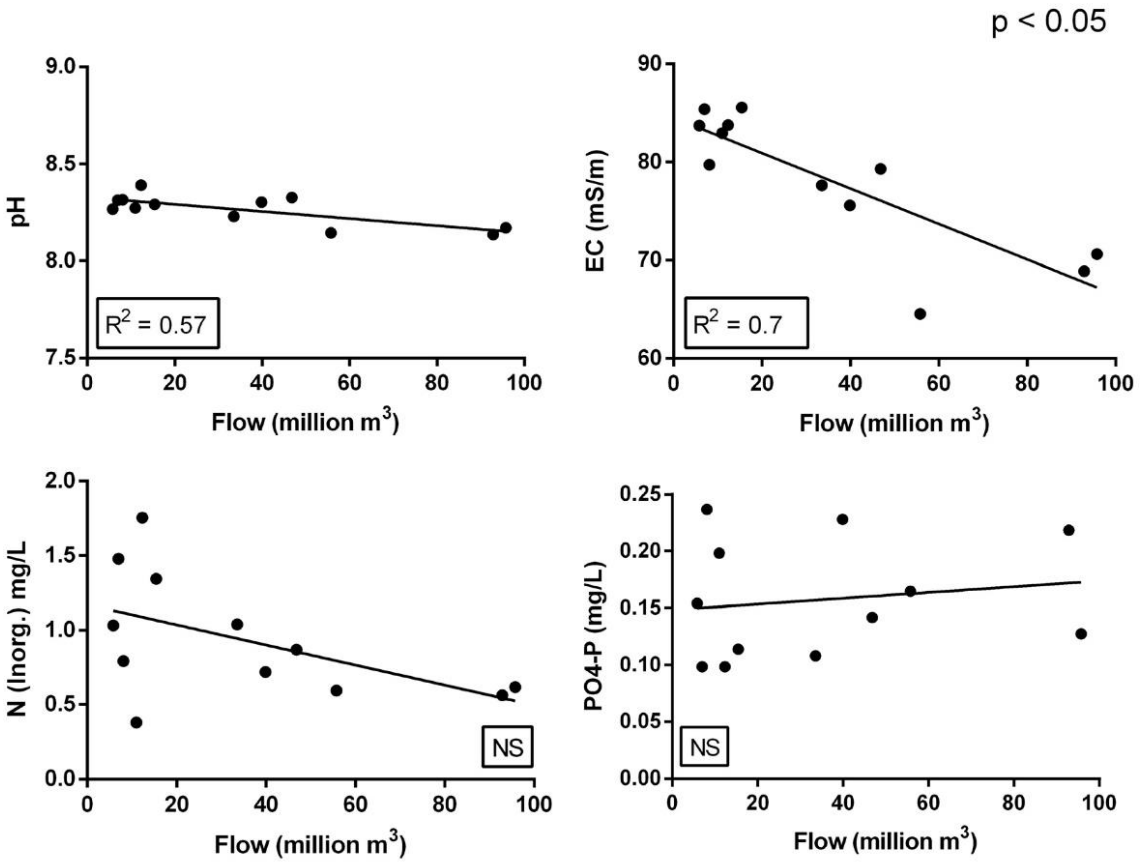

Fig. 8 Linear regression models illustrating the relationship between total monthly flow (as million cubic meter (m^3)) against pH, electrical conductivity, inorganic nitrogen, and orthophosphate (as phoshorus) at the Crocodile (West) Irrigation Scheme. *R*-square values are provided and non-significant (NS) models indicated. Statistical test was regarded significant when $p < 0.05$

depletion and the production of ammonia, which can easily kill other aquatic organisms (e.g., fish and invertebrates) (Havens 2008).

Spatial variation in the Crocodile (West) river system

The significant higher levels of EC, Cl, SO_4, and Na associated with the Crocodile (West) Irrigation Scheme (downstream river site), in comparison with the upstream river site, can be the result of a number of natural and anthropogenic influences (Meybeck et al. 1996). However, it is likely that runoff from the extensive agricultural areas, as well as the influx of polluted water from tributaries, substantially contributed to the observed differences (Walsh and Wepener 2009). The significantly higher nutrient levels directly downstream of the Hartbeespoort Dam (upstream river site), in comparison to the Crocodile (West) Irrigation Scheme (downstream river site), can be expected as it is well known that the Hartbeespoort Dam is hypertrophic as a result of anthropogenic nutrient loading (Ballot et al. 2014). Although the water that reaches the Crocodile (West) Irrigation Scheme is partially sourced from the Hartbeespoort Dam, inflow from other tributaries (e.g., Sterkstroom, Elands, and Pienaars Rivers) possibly carry less nutrient loads and thus dilutes inorganic N and PO_4-P concentrations (DEAT 2005).

Threats to crop production

High levels of inorganic N can cause excessive crop growth, pollute groundwater, and stimulate the growth of algae and aquatic plants in irrigation infrastructure, which can limit the effective distribution of water (DWAF 1996; ANZECC 2000). Also, the recorded elevated salt levels (measured as EC) especially at the Crocodile (West) Irrigation Scheme may cause salinity-induced water stress, which can reduce plant growth and ultimately crop yield (DWAF 1996). When the EC target value of 40 mS/m is exceeded, especially with high frequency irrigation and foliar wetting, yield of moderately salt-sensitive crops (e.g., maize, sunflower,

and various vegetables) is reduced up to 5%. However, peak EC levels exceeding 100 mS/m were recorded in Crocodile (West) Irrigation Scheme, which can lead to a further 5% reduction in the yield of moderately salt-sensitive crops (DWAF 1996). A specific anion of concern is Cl, which is regarded as the most toxicity relevant irrigation water quality parameter (Ayers and Westcot 1985). Since Cl is not adsorbed by the soil, it is available for plant uptake that can result in foliar damage (e.g., leaf burn) (Ayers and Westcot 1985; DWAF 1996; ANZECC 2000). According to ANZECC (2000) Cl concentrations exceeding 40 mg/L are unsuitable for tobacco, with some loss in quality occurring between 25 and 40 mg/L. Although tobacco was once extensively cultivated in the Hartbeespoort and Crocodile (West) irrigation schemes, poor water quality has contributed to farmers abandoning its production. While Na levels associated with the Crocodile (West) Irrigation Scheme also exceeded the DWAF (1996) target value, most crops (e.g., soybean and wheat) cultivated in this area, with the exception of citrus, are not classified as being sensitive (DWAF 1996; ANZECC 2000).

Factors causing seasonal variation

The final section of this discussion is focused on the distinct seasonal patterns in water quality parameters at the Hartbeespoort and Crocodile (West) irrigation schemes. The strong negative correlations evidenced between flow and the majority of the water quality parameters at both irrigation schemes are indicative of a dilution effect occurring during the high-flow periods (Walsh and Wepener 2009). Further evidence of this dilution effect was provided by the negative linear relationships that existed between flow and EC. Although this was most evident at the Crocodile (West) Irrigation Scheme, the Hartbeespoort Irrigation Scheme's weaker relationship between flow and EC, as well as lower variation in EC readings, can likely be attributed to the concentration buffer capacity of the Hartbeespoort Dam, which experienced more gradual changes over a temporal scale.

Yet, a strong negative and linear relationship was evidenced between flow and inorganic N at the Hartbeespoort Irrigation Scheme. This may, however, not only be as a result of dilution, but also of nutrient uptake by algae growing during the hot summer months (Van Ginkel 2011). This was further evidenced by another phenomenon of interest; the observed positive correlation between pH and what is considered the hottest summer months at the Hartbeespoort Irrigation Scheme. As a result of the hypertrophic state of the Hartbeespoort Dam, it is likely that pH is being influenced by excessive algae and cyanobacteria growth (Havens 2008). According to Havens (2008), favorable conditions for cyanobacteria growth in eutrophic systems include increased light intensity and higher water temperature. Thus, during the hot summer months, photosynthetic activity diminishes CO_2 from the dam water, which results in an increase in pH (Havens 2008). Although a positive linear relationship existed between flow and pH, it is likely an indirect effect of algae growth (Van Ginkel 2011). A rise in pH values can have a negative impact on water quality and its suitability for irrigation (DWAF 1996; ANZECC 2000). According to DWAF (1996) pH values greater than 8.4, as was occasionally recorded at the Hartbeespoort Irrigation Scheme, can result in (1) problems with foliar damage, (2) reduced nutrient availability, (3) negative impact on soil fauna, and (4) encrustation in irrigation equipment.

Orthophosphate (as phosphorus) also presented a positive correlation to the hottest summer months. This may, however, be twofold as (1) runoff from agricultural and industrial areas during the wet-period can result in an increased influx of nutrients and (2) PO_4-P is released through internal loading (Owuor et al. 2007). According to the latter authors, PO4-P is released from sediments in the Hartbeespoort Dam as a result of anoxic conditions when thermal water layers are formed.

Lastly, Si is considered as it did not conform to the typical temporal trends observed for most of the water quality parameters at the Hartbeespoort and Crocodile (West) irrigation schemes. This is likely because Si, mostly in the form of silica (SiO_2), originates from the weathering and erosion of rocks in the surrounding environment after which surface runoff carries it into water systems (Zhen-Gang 2017). In fact, Schouwstra et al. (2000) noted that the majority of common minerals associated with the Bushveld Complex are silica based. It is thus likely that Si will occur in meaningful concentrations in most of the Crocodile (West) Catchment's freshwater systems.

Conclusions

It is evident that major irrigation schemes associated with the Crocodile (West) Catchment remains subjected

to low-quality irrigation water. Considering especially the temporal variation evidenced in this paper, it is important to continue monitoring and assessing the water quality associated with this catchment.

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CHAPTER 3: ARTICLE 2

Beneficial nematodes as bioindicators of ecosystem health in irrigated soils

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3.1 Abstract

Irrigated crop production, which accounts for 40% of global produce, is threatened by the deterioration of freshwater resources as a result of pollution originating from anthropogenic activities. While the effects of irrigating with low quality water on crop yield and quality is well documented, little remains known about the threat posed to soil ecosystems. The present study represents, with nematodes as bioindicators, the ecological line of evidence (of the soil quality TRIAD approach) and is aimed at evaluating the soil health status of farmlands associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld (reference system) irrigation schemes (South Africa). Irrigation water and soil samples were collected during the winter and summer growing seasons of 2016 and analysed for physico-chemical properties (pH, electrical conductivity, organic content, particle size distribution, and metal, nutrient, and salt concentrations). Results indicated that the Hartbeespoort and Crocodile (West) irrigation schemes utilized water characterized by elevated salt (as indicated by the electrical conductivity) and nutrient (inorganic nitrogen and phosphorus) concentrations. The associated soil ecosystems were classified, using nematode-specific indices, as either degraded or disrupted. However, the same was evidenced at the reference system, which suggests that irrigation water quality was not the main factor influencing soil ecosystem health. Instead, it is likely that conventional farming practices (e.g. tillage) were the main drivers behind the observed disruptive effects. A redundancy analysis triplot evidenced a strong correlation between inorganic nitrogen, crop production, and r-strategists, indicating that the nematode assemblages responded rapidly to agricultural activities such as the addition of fertilizers. No significant influence of irrigation water quality on soil ecosystem health was thus evidenced during the study period, suggesting that this effect is more difficult to elucidate.

Keywords: Conventional agriculture; Hartbeespoort Dam; Nutrient enrichment; Soil nematode assemblages

3.2 Highlights

- Irrigation water presented elevated salt and nutrient concentrations.
- Soil ecosystem health was classified as degraded/disturbed at all the studied farmlands.
- Nematode assemblages responded to inorganic nitrogen (N) availability.
- Agricultural activities likely present the greatest threat to soil health.

3.3 Introduction

Irrigation plays an important role in food production and accounts for approximately 40% of global yields (Salmon et al., 2015). However, pollution resulting from anthropogenic activities pose a severe threat to freshwater systems, potentially rendering it unfit for irrigation purposes (Lu et al., 2015). The primary concerns for utilizing low quality irrigation water include its impact on crop yield and quality, as well as animal and human health (ANZECC, 2000; DWAF, 1996; Zhang et al., 2015b). For this reason, region specific irrigation water quality guidelines have been developed, and are updated when necessary, in order to assess the suitability of water for irrigation (ANZECC, 2000; DWAF, 1996). Such guidelines also take into account the impact on the health (or quality) of soils, however, primarily from a physico-chemical perspective. Consequently, important biological components are not considered even though it has been well documented that soil inhabiting fauna play an important role in maintaining healthy soils (Kibblewhite et al., 2008; Lehman et al., 2015; Neher, 2001; Tsiafouli et al., 2015), which in turn influences crop yield and quality.

Soil inhabiting fauna are linked to vital ecosystem functions such as carbon transformation, nutrient cycling, soil structure maintenance, and the biological control of pests (Kibblewhite et al., 2008). These services are delivered by different functional groups, which include microflora (e.g. fungi and bacteria), microfauna (e.g. nematodes and protozoa), mesofauna (e.g. potworms), and macrofauna (e.g. earthworms), only to name a few (Brussaard, 2012; Kibblewhite et al., 2008). According to Kibblewhite et al. (2008) soil health is a direct expression of the integrity of these functional groups/assemblages, which in turn is influenced by the physico-chemical condition of the associated habitat. As a result of the role that biota play in delivering soil ecosystems functions, they can serve as bioindicators of ecosystem disturbance (Pulleman et al., 2012).

Nematodes can be found in freshwater, marine, and terrestrial environments (Hodda et al., 2009) and when occupying the latter, are associated with the soil water layer (Neher, 2010).

They represent several trophic groups, fulfil important roles in ecosystem processes, and respond rapidly to environmental disturbance (Gutiérrez et al., 2016; Neher, 2001; Sánchez-Moreno et al., 2018). By applying general community and nematode-specific indices, nematodes are frequently utilized as indicators of ecosystem disturbance induced by pollutants that include, for example, salts (Šalamún et al., 2014), metals (Caixeta et al., 2016; Park et al., 2011; Rodríguez Martín et al., 2014), and pharmaceuticals (Gutiérrez et al., 2016). Nematodes are also used as indicators of disturbance induced by different agricultural practices (Ito et al., 2015; Sánchez-Moreno et al., 2018; Zhong et al., 2017).

The aim of this research was to determine whether irrigation water quality influenced the soil ecosystem health of farmlands associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes with nematodes serving as bioindicators of ecosystem disturbance. The potential impact of agricultural practices was also considered.

3.4 Material and methods

3.4.1 Site description

The study areas included selected farmlands (Table 3.1) associated with the Hartbeespoort and Crocodile (West) irrigation schemes (Crocodile [West] River system), as well as the Marico-Bosveld Irrigation Scheme (Marico River system). While the Hartbeespoort Irrigation Scheme is supplied with water directly from the Hartbeespoort Dam via a series of canals, the Crocodile (West) Irrigation Scheme is located further downstream and water is abstracted directly from the

Table 3.1. The location and sampled size of farmlands associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes. Additional information on cultivated crops, applied fertilizers, and agricultural practices are also provided.

Irrigation Scheme	Farm	Land coordinates	Farmland area	Predominant soil texture (USDA)	Crop and cultivar (First sampling interval)			Crop and cultivar (Second sampling interval)		
					Crop (cultivar)	Fertilizer application	Agricultural practice	Crop (cultivar)	Fertilizer application	Agricultural practice
Hartbeespoort	HB 1	-25.672080°, 27.802742°	1.17 ha	Clay	*Glycine max* L. Merrill, soybean (PHB 95Y20R)	None	Tillage	*Zea mays* L., maize (DKC 78-45 BRGEN)	Nitrogen (220 kg/ha), Phosphorous (30 kg/ha), Potassium (40 kg/ha)	Tillage
Hartbeespoort	HB 2	-25.651883°, 27.742367°	27.27	Clay	*Glycine max*, soybean	Ammonium sulfate (32.4 kg/ha), Calcium nitrate (150 kg/ha), Ferrous sulfate (2.4 kg/ha), Potassium humate (2.6 kg/ha), Potassium sulfate (410 L/ha), Urea (129.5 kg/ha), Zinc sulfate (2.4 kg/ha)	Tillage	*Daucus carota* L., carrot (Bangor)	Vermicast (8000 kg/ha)	Tillage
Hartbeespoort	HB 3	-25.567079°, 27.647483°	30.2	Sandy Loam	*Glycine max*, soybean (Pioneer B53)	None	Tillage	*Beta vulgaris* L., beetroot (Falcon) Sakata	Monoammonium phosphate (500 kg/ha), Lime (500 kg/ha)	Tillage
Hartbeespoort	HB 4	-25.421221°, 27.619690°	3.32	Sandy Loam	*Glycine max*, soybean	None	Tillage	*Triticum aestivum* L., wheat	Monoammonium phosphate (200 kg/ha), Urea (400 kg/ha)	Tillage
Crocodile (West)	CW 5	-24.983933°, 27.549934°	13.8	Loam	*Glycine max*, soybean	None	Tillage	*Triticum aestivum* L., wheat	Nitrogen (214 kg/ha), Phosphorous (50 kg/ha), K (73 kg/ha)	Tillage
Crocodile (West)	CW 6	-24.794570°, 27.439316°	52.7	Sandy Loam / Loamy Sand	*Glycine max*, soybean (PHB94Y80R)	Phosphorous (20 kg/ha), Potassium (30 kg/ha)	Tillage	*Triticum aestivum* L., wheat (Duzi)	Nitrogen (182 kg/ha), Phosphorous (42 kg/ha), K (60 kg/ha)	Tillage
Marico-Bosveld	MB 7	-25.434568°, 26.380073°	25.7 ha	Sandy Loam	*Glycine max*, soybean (Pan 1583)	Potassium chloride (100 kg/ha)	Strip tillage	*Glycine max*, soybean (Pan 1583 R)	Potassium chloride (100 kg/ha)	No tillage
Marico-Bosveld	MB 8	-25.369658°, 26.389365°	23 ha	Sandy Loam	*Glycine max*, soybean	None	No tillage	*Zea mays*, maize (DKC73-72) Monsanto	Cattle manure (7 ton/ha), Monoammonium phosphate (200 kg/ha), Urea (150 kg/ha)	Tillage

Crocodile (West) River (Du Preez et al., 2018). This river system, however, is known to be subjected to anthropogenic pollution sourced from agricultural and urban runoff, industrial wastewater, as well as treated and untreated sewage effluent (Ballot et al., 2014; DEAT, 2005; Du Preez et al., 2018). Subsequently, various pollutants are present within this system and include metals, nutrients, persistent organic pollutants, pesticides, pharmaceuticals, and salts (Amdany et al., 2014; Ansara-Ross et al., 2012; DEAT, 2005). Since the Marico River system is subjected to minimal anthropogenic impact (Kemp et al., 2016; Wolmarans et al., 2017), the Marico-Bosveld Irrigation Scheme served as the reference system.

3.4.2 Field sampling

Sampling was undertaken during March/April (first sampling interval) and September/October (second sampling interval) 2016 at eight farmlands associated with the Hartbeespoort (four), Crocodile (West) (two), and Marico-Bosveld (two) irrigation schemes. During sampling, each farmland (Table 3.1) was divided into quadrants, which were randomly sampled. From each farmland, two sets of soil samples were collected for abiotic and biotic analyses, respectively. For both sets, rhizosphere soils were collected up to a depth of 20 cm using a clean hand shovel. The first set of soil samples consisted of 12 composite samples (each with 5 sub-samples) and was used for physico-chemical (abiotic) analyses. The second set consisted of 20 composite samples (each with 5 sub-samples) and was used to characterise the associated nematode community (biotic) assemblages.

Water samples were collected from the Crocodile (West) River, as well as from on-site irrigation dams, for abiotic (water quality) assessments. While the analysis of river water samples served the purpose of further investigating findings by Du Preez et al. (2018), irrigation dam water samples were collected to investigate the quality of water being applied on the studied farmlands, as well as its relation to soil water (capillary water that occupies soil pores) quality. The first river sampling point (river HB; -25.672806°, 27.790278°) was located

downstream of the Hartbeespoort Dam and the second (river CW; -24.668278°, 27.376444°) downstream of the Crocodile (West) Irrigation Scheme. Fresh water samples could not be collected from the Marico River as sluice gates at the Marico-Bosveld Dam remained closed for the duration of the study. From each river site, three replicates were collected. From irrigation dams, three replicates were collected from outlet valves during pump operation, which ensured that the samples were representative of water being applied on the farmlands.

Abiotic (soil and water) samples were transported at -20 °C and stored at the same temperature until further processing. Soil samples for biotic assessments were transported in cool bags and stored at 6-8 °C (for a maximum period of 10 days) until nematode extraction was undertaken.

3.4.3 Abiotic sample preparation and analyses

It should be noted that since nematodes are directly exposed to pollutants in solution, extracted soil water represented the main focus of this study. Each composite soil sample was thawed, homogenised, oven dried at 40 °C for 48 h, and sieved (< 2 mm). The organic carbon (C) content was calculated using the loss-on-ignition method (Donkin, 1991) and the particle size distribution following Laker and Dupreez (1982). Soil water was extracted using the saturated paste extraction method (FSSA, 2002).

Extracted soil water, as well as water samples collected from the Crocodile (West) River and irrigation dams, were vacuum filtered through a 0.45 µm Sartorius CN sterile membrane in order to remove all suspended particles. The pH was measured using a Mettler Toledo FE20 meter and electrical conductivity using a WTW Cond 3210 meter. Major anions (nitrite [NO_2], nitrate [NO_3], sulfate [SO_4], and chloride [Cl]) concentrations were measured using a Metrohm 930 Compact IC Flex, while major cations (calcium [Ca], fluoride [F], magnesium [Mg], phosphorus [P], sodium [Na] and potassium [K]) and trace element (metal) concentrations

were measured using an Agilent 7500 CE series ICP-MS. Ammonium (NH_4) concentrations were determined using a Pharo 300 Spectroquant. Total alkalinity (TAL) (pH < 8.2) was quantified by means of titration and the sodium adsorption ratio (SAR) calculated following the DWAF (1996) procedure. For all soil and water samples, the average and standard deviation were calculated per river site, irrigation dam, and farmland per sampling season.

3.4.4 Nematode extraction, counting, and identification

Nematodes were extracted from a 200 cm^3 representative portion of the biotic soil samples using the decanting and sieving followed by sugar centrifugal flotation method (Marais et al., 2017). Following, the nematodes were transferred to filtered tap water, brought up to a final volume of 10 ml, and stored at 6-8 °C until further processing. Counting and identification of two representative (agitated) 1 ml aliquots were undertaken using a Nikon Eclipse 50i light microscope (100x magnification). Nematodes were identified up to family level and the average and standard deviation for each nematode family calculated per farmland per sampling season.

3.4.5 Statistical analyses

River and irrigation dam water quality was assessed by plotting relevant parameters (EC readings and Na, SO_4, Cl, Inorganic N, and P concentrations) and their respective guideline threshold values on bar charts with whiskers indicating the standard deviation. The relationships between soil physico-chemical parameters at the studied farmlands were illustrated on principal component analysis (PCA) biplots using Canoco 5 software package. Following, the D'Agostino and Pearson omnibus test was used to determine if the data presented a normal distribution. It should be noted that this test was used for all statistical analyses of which normality is a prerequisite. Significant differences in physico-chemical

parameter means were tested using one-way Analysis of Variance (ANOVA) (parametric data) or Krukal-Wallis (non-parametric data) test. Multiple comparisons between the studied farmlands were executed using Tukey's and Dunn's tests for parametric and non-parametric data, respectively.

Next, the relationship between major irrigation and soil water chemical parameters (combined for both sampling intervals) were studied using a correlation matrix and linear regression models. Subsequently, Pearson's correlation coefficient and Spearman's rank correlation coefficient tests were used for parametric and non-parametric data, respectively. Linear regression models were created for parameters that presented a significant correlation between soil and irrigation water. With respect to the linear regression models, normality was achieved by means of $\log_{10}$ transformation. All univariate analyses were performed using GraphPad Prism 7 software package.

In order to study the soil ecosystem structure and disturbance, nematode data were analysed using the Nematode Indicator Joint Analysis (NINJA) web-based tool (available at http://spark.rstudio.com/bsierieb/ninja) (Sieriebriennikov et al., 2014). Various nematode-specific community indices can be calculated this way, however, most relevant to this study is the colonizer-persister (c-p) series percentage distribution, Maturity Index (MI), Enrichment and Structure indices (EI and SI, respectively), as well as the Enrichment and Structure footprints (EF and SF, respectively). Briefly, the c-p series is a scale from 1 (extreme r-strategists) to 5 (extreme K-strategists) ranging from tolerant to sensitive species (Ferris and Bongers, 2009). The MI, in turn, is calculated as the weighted mean frequency of the c-p series classes and is used to classify soil ecosystems from 1 (disturbed/enriched) to 5 (mature/structured) (Ferris and Bongers, 2009; Ferris et al., 2001). Furthermore, the EI and SI indices are used to create a faunal profile, which serves as a graphic representation of the condition of the soil food web (Ferris et al., 2001). Lastly, the metabolic footprint analysis measures the magnitude of ecosystem functions and services (i.e. facilitating carbon and energy flow in soil food webs) provided by enrichment and structure nematodes, or lower and

higher trophic levels, respectively (Ferris, 2010). These metabolic footprints are plotted on the faunal profiles. Coloniser-persister series and MI results were plotted on bar charts and box-and-whisker graphs (whiskers indicating minimum and maximum values), respectively, using Graphpad Prism 7 software package. Using the same software package, faunal profiles and the associated metabolic footprints were plotted on xy plots.

Finally, the effect that selected soil physical parameters (particle size distribution and organic C), soil water chemical parameters (pH, EC, inorganic N, P, and K) parameters, and crop cultivation presented on the associated ecosystems were studied using a constrained redundancy analysis (RDA). Data from the two sampling intervals were combined and the results illustrated on a triplot using Canoco 5 software package. For the calculation of *P* values related to this RDA triplot, the Bonferroni correction was applied in order to account for type 1 errors resulting from multiple comparisons. It should be noted that significance for all relevant univariate and multivariate analyses presented is this study was regarded at $P < 0.05$.

3.5 Results

3.5.1 River and irrigation water quality

Selected parameters of river (Fig. 3.1) and irrigation dam (Fig. 3.2) water quality were plotted on bar charts to study its compliance with irrigation water quality guidelines at the time of sampling. This included the a) *South African Water Quality Guidelines for Agricultural Use: Irrigation* (DWAF, 1996) and b) *Australian and New Zealand Guidelines for Fresh and Marine Water Quality* (ANZECC, 2000).

It is evident that EC measurements downstream of the Hartbeespoort Dam (river HB) and Crocodile (West) Irrigation Scheme (river CW) exceeded the DWAF (1996) target value for EC of 400 µs/cm during both sampling intervals. Also, the DWAF (1996) target value for Cl (100 mg/L) was exceeded at river CW, while inorganic N concentrations at river HB exceeded

the DWAF (1996) target value of 5 mg/L during both sampling intervals. Total P concentrations, in turn, exceeded the ANZECC (2000) long-term trigger value (0.05 mg/L) at both river sites also during the first and second sampling intervals. Furthermore, the selected parameters, with the exception of total P at the Crocodile (West) Irrigation Scheme, presented higher readings/concentrations during the second sampling interval.

Selected irrigation water quality parameters at the Hartbeespoort and Crocodile (West) irrigation schemes (Fig. 3.2) presented similar trends to the river sites. Electrical conductivity readings from on-site irrigation dams exceeded the target value during both sampling intervals. Likewise, the long-term trigger value for total P was exceeded at both irrigation schemes, while inorganic N measurements at the Hartbeespoort Irrigation Scheme, especially during the second sampling interval, did not comply with the set target value. In fact, concentrations of more than 50 mg/L were recorded during the second sampling interval at HB 3. In some cases, the DWAF (1996) target values for Na (70 mg/L) and Cl were also exceeded. The Marico-Bosveld Irrigation Scheme (reference system) conformed to guideline threshold values, as did metal concentrations (Supplementary material: Table 3A) in all of the collected irrigation water samples.

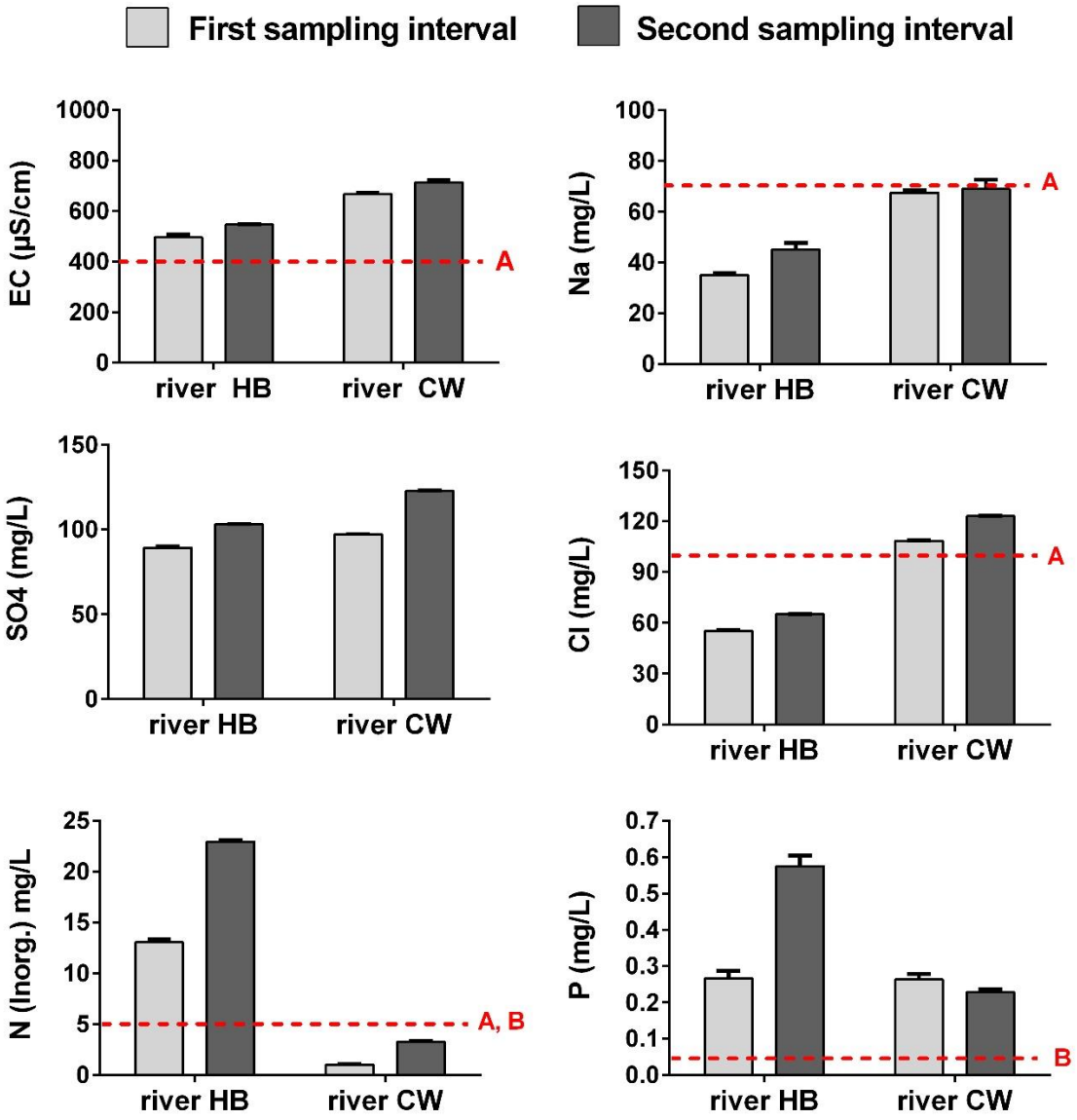

Fig. 3.1. Selected irrigation water quality parameters recorded at river HB (downstream of the Hartbeespoort Dam) and river CW (downstream of the Crocodile [West] River Irrigation Scheme) during the first (March/April 2016) and second (September/October 2016) sampling intervals. Threshold values as provided by the (A) *South African Water Quality Guidelines for Agricultural Use: Irrigation* (DWAF, 1996) and (B) *Australian and New Zealand Guidelines for Fresh and Marine Water Quality* (ANZECC, 2000) are indicated with dotted lines. Whiskers denote standard deviations.

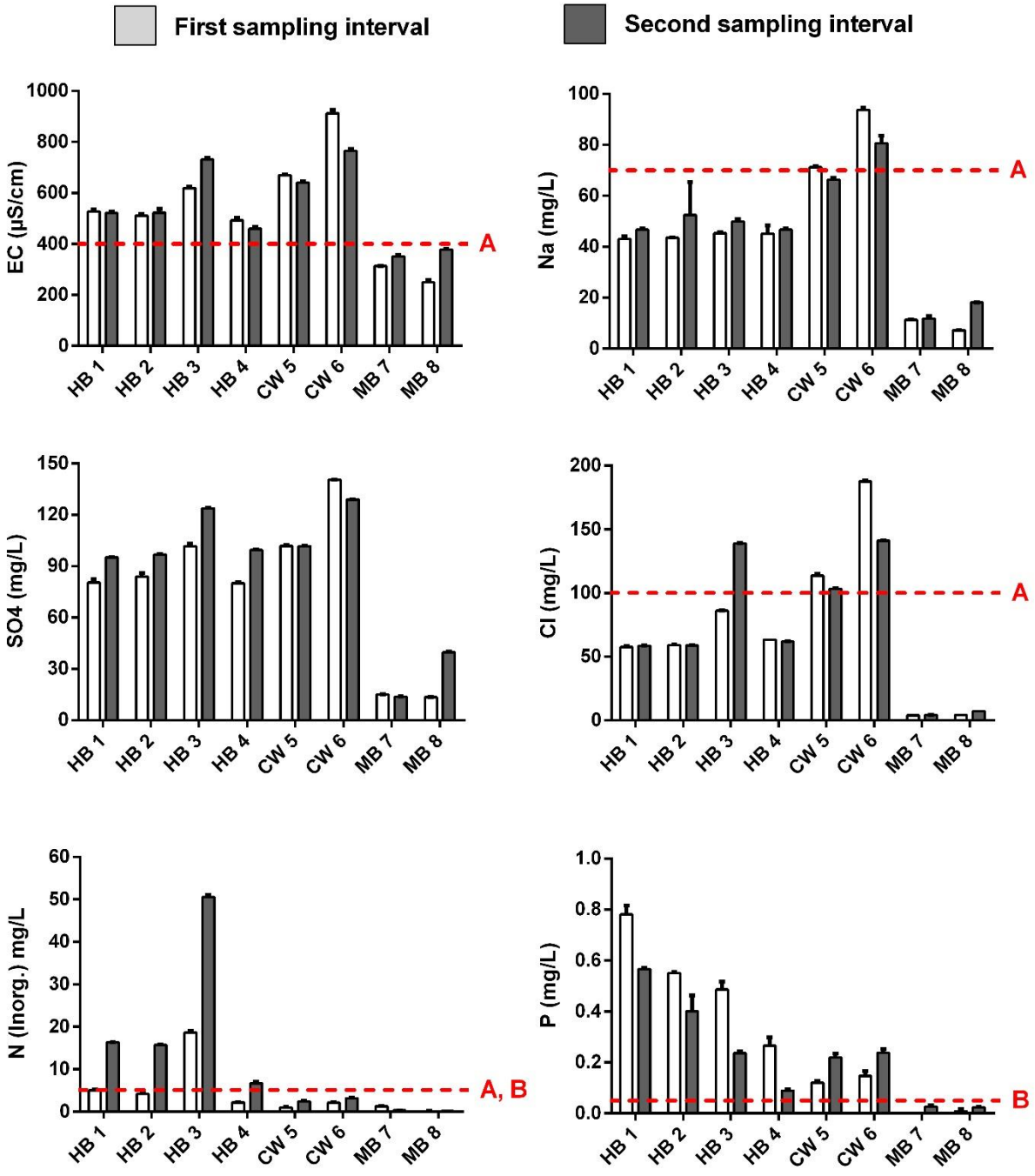

Fig. 3.2. Selected irrigation water quality parameters recorded at farmlands associated with the Hartbeespoort (HB 1 – HB 4), Crocodile (West) (CW 5 and CW 6), and Marico-Bosveld (MB 7 and MB 8) irrigation schemes during the first (March/April 2016) and second (September/October 2016) sampling intervals. Threshold values as provided by the (A) *South African Water Quality Guidelines for Agricultural Use: Irrigation* (DWAF, 1996) and (B) *Australian and New Zealand Guidelines for Fresh and Marine Water Quality* (ANZECC, 2000) are indicated with dotted lines. Whiskers denote standard deviations.

3.5.2 Soil physico-chemical properties

The relationships between soil physical (Table 3.2) and soil water chemical (Table 3.3) parameters and the respective irrigation schemes were studied using PCA biplots (Fig. 3.3). It should be noted that metal concentrations in soil water did not contribute meaningfully to ecological and abiotic variations and were thus excluded from analyses.

The first PCA biplot (Fig. 3.3a), with 71.9% of the variation explained on F1 (40.59%) and F2 (31.31%), illustrated a negative relationship between the Hartbeespoort/Crocodile (West) irrigation schemes and the Marico-Bosveld Irrigation Scheme during the first sampling interval. Furthermore, farmlands associated with the Hartbeespoort and Crocodile (West) irrigation schemes were grouped into two negatively related clusters (HB 1, HB 2, and CW 5, vs. HB 3, HB 4, and CW 6).

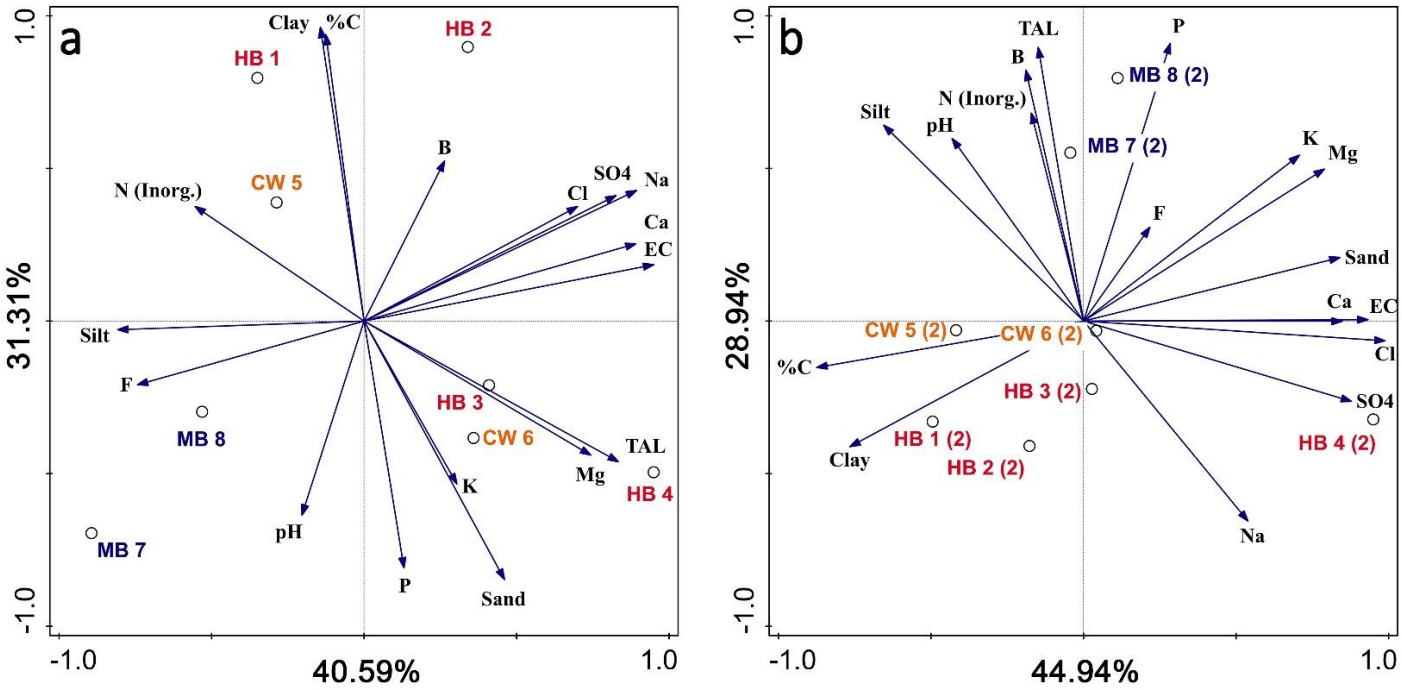

Fig. 3.3a and b. Principal component analysis biplots illustrate the spatial variation in soil physical and soil water (capillary water that occupies soil pores) chemical parameters at farmlands associated with the Hartbeespoort (HB 1 – HB 4), Crocodile (West) (CW 5 and CW 6), and Marico-Bosveld (MB 7 and MB 8) irrigation schemes during the (a) first (March/April 2016) and (b) second (September/October 2016) sampling intervals.

Table 3.2. Mean ± standard deviation of soil physical parameters (particle size distribution and organic C) of farmlands associated with the Hartbeespoort (HB 1 – HB 4), Crocodile (West) (CW 5 and CW 6), and Marico-Bosveld (MB 7 and MB 8) irrigation schemes during the first (March/April 2016) and second (September/October 2016) sampling intervals. Within columns and sampling intervals superscript indicate farmland(s) (as numbered from 1-8) between which significant ($P < 0.05$) differences were recorded.

Farmland	> 2mm	Sand	Silt	Clay	LOI
			First sampling interval		
HB 1	1.40 ± 0.70 [2,4]	23.76 ± 2.06 [3,4,6,7,8]	23.42 ± 1.68 [5]	52.81 ± 2.22 [3,4,5,6,7,8]	2.55 ± 0.42 [3,4,5,6,7,8]
HB 2	9.86 ± 5.95 [1,6,7]	38.62 ± 6.65 [3,4,6]	11.78 ± 1.70 [5,7,8]	49.60 ± 6.56 [3,4,5,6,7,8]	2.48 ± 0.33 [6,7,8]
HB 3	5.17 ± 4.16 [7]	56.60 ± 6.39 [1,2,5]	25.23 ± 4.94	18.16 ± 2.45 [1,2,5]	1.64 ± 0.20 [1,4,7]
HB 4	14.68 ± 8.66 [1,6,7]	78.41 ± 8.20 [1,2,5]	7.24 ± 4.16 [5,7,8]	14.35 ± 9.25 [1,2,5]	1.05 ± 0.35 [1,3,5,6,8]
CW 5	2.51 ± 1.29 [7]	38.15 ± 3.39 [3,4,6]	35.72 ± 3.89 [1,2,4,6]	26.13 ± 1.50 [1,2,3,4,6,7,8]	2.12 ± 0.22 [1,4,6,7,8]
CW 6	1.25 ± 0.41 [2,4]	65.27 ± 7.70 [1,2,5]	19.77 ± 5.58 [5]	14.97 ± 3.24 [1,2,5]	1.62 ± 0.49 [1,2,4,5,7]
MB 7	0.08 ± 0.07 [2,3,4,5,8]	53.90 ± 3.66 [1]	30.43 ± 2.33 [2,4]	15.67 ± 1.57 [1,2,5]	1.05 ± 0.13 [1,2,3,5,6,8]
MB 8	3.72 ± 2.01 [7]	52.90 ± 2.52 [1]	28.88 ± 3.33 [2,4]	18.22 ± 2.76 [1,2,5]	1.60 ± 0.23 [1,2,4,5,7]
			Second sampling interval		
HB 1	1.85 ± 1.44 [2,4]	24.58 ± 2.70 [2,3,4,5,6,7,8]	22.51 ± 2.65 [2,4,5,7,8]	52.92 ± 1.32 [3,4,6,7,8]	2.55 ± 0.15 [3,4,6,7]
HB 2	15.73 ± 6.55 [1,3,5,6,7,8]	39.12 ± 3.85 [2,3,4,6,7,8]	12.38 ± 1.22 [1,3,4,5,6,7,8]	48.49 ± 3.56 [3,4,6,7,8]	2.37 ± 0.26 [3,4,6,7]
HB 3	4.38 ± 3.11 [2,4]	61.02 ± 2.73 [1,2,4,5,7,8]	21.87 ± 2.17 [2,4,5,7,8]	17.11 ± 1.36 [1,2]	1.41 ± 0.27 [1,2,5]
HB 4	13.72 ± 6.09 [1,5,6,7,8]	81.03 ± 7.43 [1,2,3,5,6,7,8]	8.67 ± 3.69 [1,2,3,5,6,7,8]	10.29 ± 4.29 [1,2,5]	0.81 ± 0.38 [1,2,5]
CW 5	1.89 ± 0.65 [2,4]	40.46 ± 2.94 [1,3,4,6,7,8]	33.59 ± 1.49 [1,2,3,4,6,8]	25.95 ± 2.59 [4,7]	2.40 ± 0.16 [3,4,6,7]
CW 6	0.86 ± 0.35 [2,4]	65.62 ± 7.58 [1,2,4,5,7,8]	19.07 ± 4.46 [2,4,5,7,8]	15.31 ± 3.37 [1,2]	1.16 ± 0.34 [1,2,5]
MB 7	0.08 ± 0.06 [2,4]	54.18 ± 2.59 [1.2.3.4.5.6]	31.14 ± 2.36 [1,2,3,4,6]	14.68 ± 1.08 [1,2,5]	1.26 ± 0.11 [1,2,5]
MB 8	3.41 ± 2.94 [2,4]	54.22 ± 4.27 [1,2,3,4,5,6]	28.84 ± 3.22 [1,2,3,4,5,6]	16.94 ± 1.92 [1,2]	1.66 ± 0.22

Table 3.3. Mean ± standard deviation of soil water (capillary water that occupies soil pores) chemical parameters (pH, electrical conductivity, as well as major nutrients and ions) of farmlands associated with the Hartbeespoort (HB 1 – HB 4), Crocodile (West) (CW 5 and CW 6), and Marico-Bosveld (MB 7 and MB 8) irrigation schemes during the first (March/April 2016) and second (September/October 2016) sampling intervals. Within columns and sampling intervals superscript indicate farmland(s) (as numbered from 1-8) between which significant ($P < 0.05$) differences were recorded.

Farmland	pH	EC	TAL (as $CaCO_3$)	N (Inorg.)	P	K
		µS/cm	mg $CaCO_3$/L	mg/L	mg/L	mg/L
First sampling interval						
HB 1	8.08 ± 0.30 [7]	405.90 ± 178.47	95.45 ± 41.74 [3,4,6]	9.45 ± 8.11	0.26 ± 0.09 [4,7]	4.26 ± 2.19 [3,4,6,8]
HB 2	8.20 ± 0.52 [7]	707.27 ± 159.08 [7,8]	98.30 ± 12.44 [3,4,6]	37.55 ± 43.16	0.13 ± 0.06 [3,4,6,7]	7.36 ± 2.81 [3,4,6]
HB 3	8.52 ± 0.32 [5,6]	696.73 ± 149.58	152.55 ± 34.20 [1,2,5,7,8]	14.46 ± 15.87	0.52 ± 0.17 [2]	29.90 ± 6.92 [1,2,7]
HB 4	8.46 ± 0.21 [5,6]	791.27 ± 297.49 [7,8]	178.36 ± 42.04 [1,2,5,7,8]	3.46 ± 1.27 [5,7]	0.78 ± 0.27 [1,2,5]	20.63 ± 10.79 [1,2,7]
CW 5	7.98 ± 0.15 [3,4,7,8]	528.00 ± 170.31	107.23 ± 22.51 [3,4]	59.55 ± 45.48 [4,6]	0.25 ± 0.06 [4,6,7]	10.65 ± 4.42
CW 6	8.01 ± 0.23 [3,4,7,8]	591.27 ± 200.37 [7]	143.41 ± 41.05 [1,2,7]	4.90 ± 5.02 [5,7]	1.45 ± 1.78 [2,5]	21.98 ± 11.63 [1,2,7]
MB 7	8.85 ± 0.25 [1,2,5,6]	275.67 ± 65.61 [2,4,7]	96.58 ± 19.02 [3,4,6]	32.33 ± 19.01 [4,6]	0.97 ± 0.17 [1,2,5]	6.17 ± 1.82 [3,4,6]
MB 8	8.41 ± 0.51 [5,6]	402.27 ± 531.14 [2,4]	94.64 ± 41.93 [3,4]	14.78 ± 12.69	0.41 ± 0.16	51.23 ± 124.95 [1]
Second sampling interval						
HB 1	8.02 ± 0.33 [8]	427.75 ± 198.68 [4,8]	64.08 ± 19.20 [8]	86.38 ± 97.11	0.20 ± 0.17 [7,8]	3.71 ± 1.68 [3,4,6,7,8]
HB 2	8.36 ± 0.36 [4]	539.08 ± 168.63	65.29 ± 14.72 [8]	40.47 ± 22.64	0.10 ± 0.06 [7,8]	5.50 ± 1.66 [3,8]
HB 3	7.94 ± 0.22 [8]	453.08 ± 174.78 [4]	55.50 ± 11.33 [7,8]	38.61 ± 22.15 [8]	0.14 ± 0.06 [7,8]	15.31 ± 4.86 [1,2]
HB 4	7.72 ± 0.24 [2,7,8]	1688.00 ± 1062.52 [1,3,5]	54.50 ± 16.16 [7,8]	43.31 ± 37.8 [8]	0.48 ± 0.51 [7,8]	19.93 ± 19.58 [1]
CW 5	8.13 ± 0.36	339.00 ± 121.71 [4,8]	70.95 ± 16.63 [8]	38.43 ± 42.32 [7,8]	0.15 ± 0.07 [7,8]	8.06 ± 3.08 [8]
CW 6	8.03 ± 0.36	475.45 ± 220.94	65.09 ± 21.91 [8]	44.08 ± 39.57 [7,8]	0.25 ± 0.48 [7,8]	11.50 ± 5.37 [1]
MB 7	8.15 ± 0.18 [4]	537.42 ± 109.10	82.63 ± 16.23 [3,4]	92.48 ± 38.32 [5,6]	1.06 ± 0.18 [1,2,3,4,5,6]	10.35 ± 2.13 [1]
MB 8	8.58 ± 0.29 [1,3,4]	732.58 ± 152.98 [1,5]	132.79 ± 22.35 [1,2,3,4,5,6]	97.51 ± 37.82 [3,4,5,6]	1.92 ± 3.76 [1,2,3,4,6]	29.19 ± 5.23 [1,2,5]

Table 3.3. Continued.

Farmland	Cl	SO4	Ca	Mg	Na	B	F
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
				First sampling interval			
HB 1	29.12 ± 15.74	88.60 ± 52.85 [2]	36.26 ± 9.84	7.27 ± 4.07 [2,3,4,6]	39.60 ± 17.68 [2,4,5,6,7]	0 [2,7]	0.27 ± 0.17 [8]
HB 2	74.09 ± 22.65 [7,8]	319.06 ± 129.92 [1,5,7,8]	55.48 ± 14.96 [5,7,8]	16.74 ± 4.83 [1]	69.10 ± 12.99 [1,7,8]	0.40 ± 0.01 [1,3,4,5,6]	0.35 ± 0.17
HB 3	57.89 ± 24.58 [7,8]	153.14 ± 63.69 [7,8]	49.08 ± 13.91 [7,8]	19.88 ± 4.89 [1,8]	53.47 ± 10.24 [7,8]	0 [2,7]	0.30 ± 0.12 [8]
HB 4	65.56 ± 71.86 [7,8]	224.27 ± 176.85 [7,8]	63.01 ± 33.95 [7,8]	21.04 ± 9.77 [1]	73.64 ± 28.48 [1,7,8]	0.02 ± 0 [2,7]	0.23 ± 0.14 [7,8]
CW 5	38.90 ± 23.96 [7]	67.62 ± 28.58 [2]	29.33 ± 9.34 [2]	12.37 ± 4.52 [7,8]	64.16 ± 20.80 [1]	0 [2,7]	0.42 ± 0.13
CW 6	30.01 ± 17.62	155.77 ± 108.23 [7,8]	35.32 ± 15.53	17.34 ± 8.39 [1,7,8]	67.02 ± 19.65 [1]	0.05 ± 0 [2]	0.34 ± 0.24
MB 7	6.40 ± 2.73 [2,3,4,5]	9.76 ± 3.81 [2,3,4,6]	23.72 ± 6.89 [2,3,4]	13.19 ± 3.86 [5,6]	10.18 ± 2.69 [1,2,4]	0.02 ± 0.01 [1,3,4,5]	0.46 ± 0.06 [4]
MB 8	52.56 ± 143.15 [2,3,4]	29.73 ± 19.72 [2,3,4,6]	20.48 ± 10.10 [2,3,4]	11.27 ± 5.89 [3,5,6]	15.83 ± 6.77 [2,3,4]	0.01 ± 0.02	0.53 ± 0.11 [1,3,4]
				Second sampling interval			
HB 1	17.48 ± 7.90 [4]	29.30 ± 13.23 [4]	34.70 ± 19.27 [4]	8.26 ± 4.59 [4,7,8]	37.76 ± 12.02 [7]	0.04 ± 0.01 [5,6,7,8]	0.28 ± 0.06 [4,5,7,8]
HB 2	27.14 ± 11.98	97.39 ± 45.00 [4]	42.58 ± 18.05	13.55 ± 5.12 [8]	50.67 ± 13.29 [7]	0.05 ± 0.03 [6,7,8]	0.28 ± 0.07 [4,5,7,8]
HB 3	38.95 ± 26.05	51.49 ± 26.54 [4]	30.59 ± 11.20 [4]	11.91 ± 5.39 [8]	36.36 ± 15.05 [7]	0.06 ± 0.01	0.38 ± 0.09 [5]
HB 4	128.55 ± 209.19 [1,5]	642.98 ± 381.15 [1,2,3,5,6,7,8]	175.31 ± 104.70 [1,3,5,6]	39.93 ± 35.89 [1,5]	91.52 ± 76.11 [7]	0.03 ± 0.01 [5,6,7,8]	0.54 ± 0.30 [1,2]
CW 5	18.08 ± 9.76 [4]	20.32 ± 6.74 [4]	21.85 ± 9.00 [4,7,8]	6.99 ± 3.37 [4,7,8]	36.00 ± 10.87 [7]	0.13 ± 0.07 [1.4]	0.73 ± 0.13 [1,2,3]
CW 6	42.43 ± 27.25	42.39 ± 25.19 [4]	24.20 ± 11.46 [4,7,8]	12.22 ± 6.34 [8]	49.82 ± 21.63 [7]	0.16 ± 0.01 [1,2,4]	0.46 ± 0.17
MB 7	34.99 ± 8.53	22.86 ± 5.58 [4]	47.52 ± 10.96 [5]	27.68 ± 6.50 [1,5]	14.87 ± 3.94 [1,2,3,4,5,6]	0.17 ± 0.03 [1,2,4]	0.49 ± 0.10 [1,2]
MB 8	34.11 ± 12.15	84.38 ± 26.63 [4]	56.14 ± 13.36 [5]	35.06 ± 9.01 [1,2,3,5,6]	32.48 ± 7.09	0.16 ± 0.01 [1,2,4]	0.45 ± 0.09 [1,2]

The second PCA biplot (Fig. 3.3b), with 73.88% of the variation explained on F1 (44.94%) and F2 (28.94%), presented, unlike the first sampling interval, a single cluster of farmlands (not including HB 4 [2]) associated with the Hartbeespoort and Crocodile (West) irrigation schemes. This cluster presented a negative relationship to the reference system, as well as to the majority of the studied parameters.

The significance of these relationships was tested as differences in mean values of soil physical (Table 3.2) and soil water chemical (Table 3.3) parameters. Most parameters presented multiple significant ($P < 0.05$) differences within and between the respective irrigation schemes, thus, emphasis was placed on clear spatial and/or temporal trends. When considering the soil physical parameters, Clay content was significantly ($P < 0.05$) higher at HB 1 and HB 2 during both sampling intervals. Furthermore, nearly all the studied farmlands presented significant ($P < 0.05$) differences in sand and silt content during the second sampling interval.

For soil water chemical parameters, EC readings at the reference system (MB 7 and MB 8) were significantly ($P < 0.05$) lower than at HB 2 and HB 4 during the first sampling interval. Furthermore, during the second sampling interval, inorganic N concentrations at the Crocodile (West) Irrigation Scheme (CW 5 and CW 6) were significantly ($P < 0.05$) lower than at the reference system. Total P, in turn, presented substantial spatial variation during the second sampling interval as concentrations recorded at the reference system were significantly ($P < 0.05$) higher than at the majority of the farmlands associated with the Hartbeespoort and Crocodile (West) irrigation schemes. Also, during the first sampling interval, significantly ($P < 0.05$) lower concentrations of Cl, SO_4, and Ca were recorded at the reference system than at HB 2, HB 3, and HB 4. Sulfate concentrations at HB 4, as indicated by the second PCA biplot, were significantly ($P < 0.05$) higher than all the remaining farmlands during the second sampling interval. During the same sampling interval, significantly ($P < 0.05$) lower concentrations of Mg were recorded at the majority of the farmlands when compared to MB 8, while Na concentrations were significantly ($P < 0.05$)

lower at MB 7 than at farmlands associated with the Hartbeespoort and Crocodile (West) irrigation schemes.

3.5.3 Correlating irrigation water and soil water characteristics

The correlation matrix evidenced that some irrigation water parameters, i.e. Na (R^2 = 0.82), P (R^2 = -0.56), and SO_4 (R^2 = 0.53), were correlated with their soil water counterparts. These relationships were further illustrated using linear regression models (Fig. 3.4). Sodium adsorption ratio values presented the strongest positive (R^2=0.84) relationship, while weaker positive relationships were also evidenced for Na and SO_4 concentrations. Phosphorus, however, did not present a significant (P > 0.05) linear relationship.

3.5.4 Ecological classification of soils

Nematode assemblages were represented by 34 families that included herbivores, bacterivores, fungivores, omnivores, and predators (Table 3.4). Absolute abundance values are provided as supplementary material (Table 3B). Although herbivores presented the greatest diversity (at family level), only beneficial (also known as terrestrial, non-parasitic or free-living) nematodes are considered in the analysis of the nematode-specific indices (Table 3.5) applied in this study

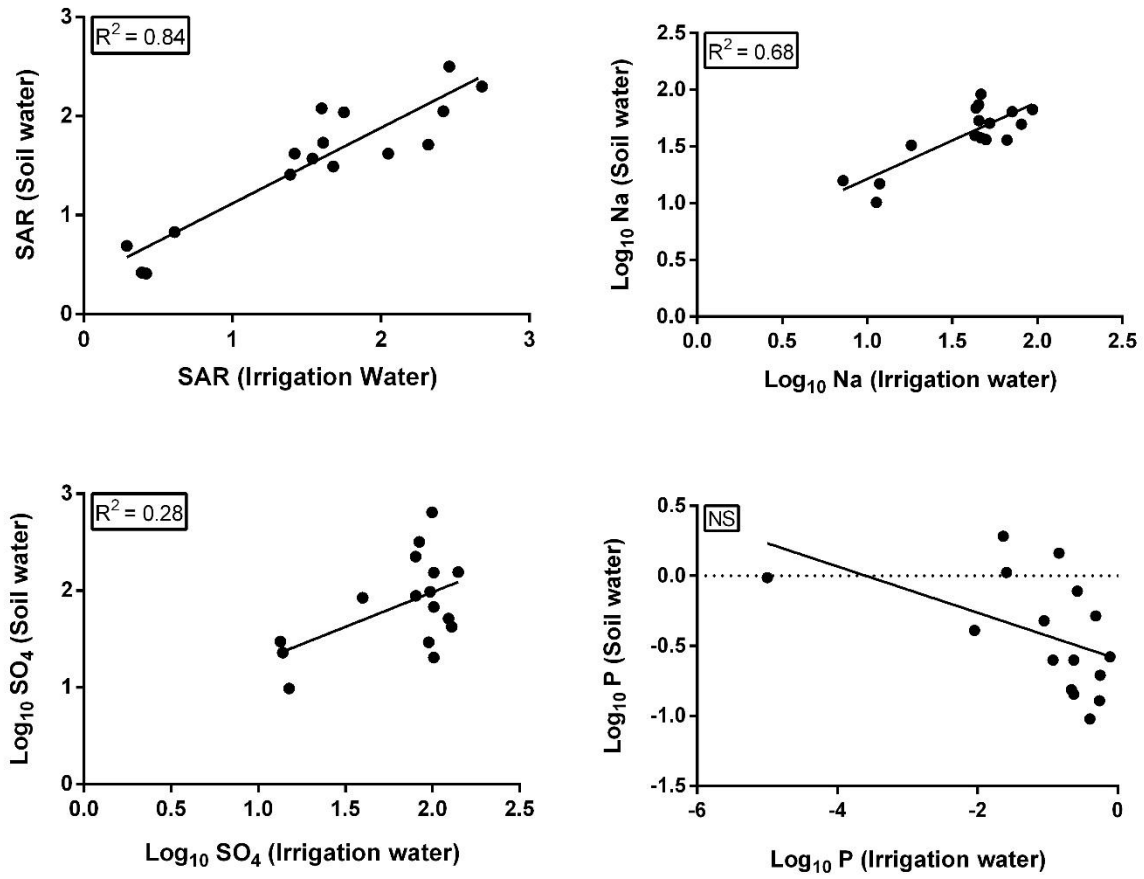

Fig. 3.4. Linear regression models illustrate the relationship between selected irrigation and soil water (capillary water that occupies soil pores) chemical parameters at the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes. Significance was regarded at $P < 0.05$. Not significant models were indicated as NS.

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Table 3.4. Nematode families listed per trophic group as recorded from farmlands associated with the Hartbeespoort (HB 1 – HB 4), Crocodile (West) (CW 5 and CW 6), and Marico-Bosveld (MB 7 and MB 8) irrigation schemes during the first (March/April 2016) and second (September/October 2016) sampling intervals. The colonizer-persister (c-p) classification, as well as corresponding generation time (gen. time), fecundity (fec.), and sensitivity (s.) characteristics, are provided for each family.

	Characteristics				First sampling interval								Second sampling interval							
	c-p class	**Gen. time**	**Fec.**	**Sensitivity**	**HB 1**	**HB 2**	**HB 3**	**HB 4**	**CW 5**	**CW 6**	**MB 7**	**MB 8**	**HB 1**	**HB 2**	**HB 3**	**HB 4**	**CW 5**	**CW 6**	**MB 7**	**MB 8**
Herbivores																				
Anguinidae					x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Belondiridae					x															
Dolichodoridae					x	x	x	x	x	x	x	x	x		x	x	x	x		x
Hoplolaimidae					x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Longidoridae					x	x							x	x		x				
Pratylenchidae					x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Rotylenchulidae						x		x		x	x	x	x			x				x
Trichodoridae								x	x											
Tylenchidae					x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Tylenchulidae													x		x		x			
Bacterivores																				
Alaimidae	**4**	Longer	Lower	Greater S.	x	x	x	x	x	x	x	x		x	x	x	x	x		
Alloionematidae	**1**	Short	High	Tolerant								x								
Cephalobidae	**2**	Longer	Lower	Tolerant	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Diploscapteridae	**1**	Short	High	Tolerant	x						x					x				x
Monhysteridae	**1**	Short	High	Tolerant		x	x	x	x	x	x	x		x		x	x	x		
Panagrolaimidae	**1**	Short	High	Tolerant	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Plectidae	**2**	Longer	Lower	Tolerant	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Prismatolaimidae	**3**	Longer	Lower	More S.	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x
Rhabditidae	**1**	Short	High	Tolerant	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Fungivores																				
Aphelenchidae	**2**	Longer	Lower	Tolerant	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Aphelenchoididae	**2**	Longer	Lower	Tolerant	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Diphtherophoridae	**3**	Longer	Lower	More S.			x		x		x				x				x	
Leptonchidae	**4**	Longer	Lower	Greater S.	x	x		x												

Table 3.4. Continued.

	Characteristics				First sampling interval								Second sampling interval							
	c-p class	**Gen. time**	**Fec.**	**Sensitivity**	**HB 1**	**HB 2**	**HB 3**	**HB 4**	**CW 5**	**CW 6**	**MB 7**	**MB 8**	**HB 1**	**HB 2**	**HB 3**	**HB 4**	**CW 5**	**CW 6**	**MB 7**	**MB 8**
Omnivores																				
Aporcelaimidae	**5**	Longest	Lowest	Greatest S.	x	x	x	x	x	x	x	x								
Dorylaimidae	**4**	Longer	Lower	Greater S.	x							x	x	x	x	x	x	x	x	x
Neodiplogasteridae	**1**	Short	High	Tolerant	x				x	x	x									
Nordiidae	**4**	Longer	Lower	Greater S.												x				
Qudsianematidae	**4**	Longer	Lower	Greater S.		x														
Predators																				
Discolaimidae	**5**	Longest	Lowest	Greatest S.															x	
Ironidae	**4**	Longer	Lower	Greater S.						x										
Mononchidae	**4**	Longer	Lower	Greater S.	x			x					x	x	x	x	x	x	x	
Mylonchulidae	**4**	Longer	Lower	Greater S.			x		x											
Tobrilidae	**3**	Longer	Lower	More S.					x		x									x
Tripylidae	**3**	Longer	Lower	More S.		x	x	x	x	x	x			x	x	x	x		x	x

Table 3.5. Mean ± standard deviation of nematode-specific indices indicate the soil health status of farmlands associated with the Hartbeespoort (HB 1 – HB 4), Crocodile (West) (CW 5 and CW 6), and Marico-Bosveld (MB 7 and MB 8) irrigation schemes during the first (March/April 2016) and second (September/October 2016) sampling intervals. These include the Maturity (MI), Enrichment (EI), Structure (SI), Basal (BI), and Channel (CI) indices, as well as the Enrichment (EF), Structure (SF), and Composite (CF) metabolic footprints. The Shannon Diversity (SDiv) Index is also listed.

Farm	MI	EI	SI	BI	CI	EF	SF	CF	SDiv
First sampling interval									
HB 1	2.15 ± 0.12	9.03 ± 8.71	25.56 ± 15.91	69.74 ± 16.56	58.70 ± 25.32	25.21 ± 29.81	133.99 ± 125.85	690.49 ± 315.36	1.41 ± 0.25
HB 2	2.35 ± 0.19	18.92 ± 7.82	45.48 ± 17.09	48.11 ± 14.41	61.28 ± 26.42	66.31 ± 47.71	681.27 ± 572.93	1630.14 ± 872.19	1.86 ± 0.20
HB 3	2.29 ± 0.32	31.39 ± 11.40	42.54 ± 22.40	43.39 ± 15.47	65.49 ± 33.39	67.01 ± 79.70	178.70 ± 243.72	808.52 ± 273.13	1.81 ± 0.15
HB 4	2.33 ± 0.24	16.25 ± 6.66	40.92 ± 16.52	52.39 ± 13.70	91.96 ± 24.11	27.93 ± 14.95	557.98 ± 412.22	1397.67 ± 644.40	1.40 ± 0.30
CW 5	2.11 ± 0.11	27.67 ± 8.20	25.46 ± 11.73	57.54 ± 8.98	49.21 ± 16.84	199.32 ± 131.67	452.82 ± 263.10	1814.79 ± 479.51	1.67 ± 0.13
CW 6	2.16 ± 0.17	20.13 ± 10.59	29.03 ± 19.00	58.69 ± 13.70	67.38 ± 34.23	68.55 ± 87.19	170.08 ± 185.18	868.52 ± 416.29	1.36 ± 0.23
MB 7	1.83 ± 0.15	54.96 ± 17.20	15.41 ± 12.55	41.74 ± 16.39	13.74 ± 11.03	520.56 ± 437.14	59.85 ± 60.12	1225.32 ± 659.70	1.89 ± 0.19
MB 8	1.89 ± 0.15	48.13 ± 16.05	9.67 ± 14.30	49.32 ± 16.49	34.62 ± 23.25	195.02 ± 167.42	65.76 ± 164.02	1182.92 ± 492.72	1.69 ± 0.39
Second sampling interval									
HB 1	1.96 ± 0.08	28.19 ± 18.38	6.92 ± 9.16	68.42 ± 18.25	44.15 ± 35.54	106.51 ± 160.05	26.10 ± 32.94	390.34 ± 275.79	1.28 ± 0.33
HB 2	1.87 ± 0.20	73.74 ± 8.70	45.28 ± 16.54	21.10 ± 7.29	4.32 ± 2.88	483.09 ± 391.08	161.65 ± 128.07	1452.92 ± 782.50	1.52 ± 0.34
HB 3	1.83 ± 0.26	66.15 ± 14.62	28.44 ± 16.38	28.21 ± 10.53	18.07 ± 12.77	432.34 ± 444.59	52.33 ± 32.05	984.97 ± 644.49	1.89 ± 0.19
HB 4	1.90 ± 0.13	50.55 ± 15.56	16.48 ± 13.19	44.58 ± 13.17	23.12 ± 19.70	376.87 ± 258.92	56.69 ± 42.77	832.59 ± 460.05	1.50 ± 0.36
CW 5	1.97 ± 0.19	57.33 ± 14.05	34.24 ± 15.30	33.97 ± 9.85	18.96 ± 17.05	538.61 ± 405.82	182.10 ± 124.85	1197.85 ± 581.70	2.03 ± 0.17
CW 6	1.93 ± 0.27	59.69 ± 22.05	37.08 ± 14.33	31.10 ± 15.78	21.02 ± 17.97	450.55 ± 742.01	104.71 ± 79.49	754.33 ± 818.91	1.80 ± 0.22
MB 7	1.80 ± 0.14	64.01 ± 9.22	13.67 ± 13.66	33.40 ± 7.38	21.71 ± 11.38	327.12 ± 269.09	33.26 ± 33.75	558.90 ± 370.12	1.82 ± 0.13
MB 8	1.73 ± 0.16	68.51 ± 10.92	15.58 ± 12.36	29.14 ± 9.31	9.03 ± 5.59	586.05 ± 458.28	51.21 ± 42.58	1072.55 ± 636.10	1.94 ± 0.22

The percentage distribution of the c-p series were plotted (Fig. 3.5) per site per sampling interval. It is evident that soil ecosystems associated with the three irrigation schemes, during both sampling intervals, were dominated by c-p 1 and c-p 2 nematodes, which are classified as tolerant (Table 3.4).

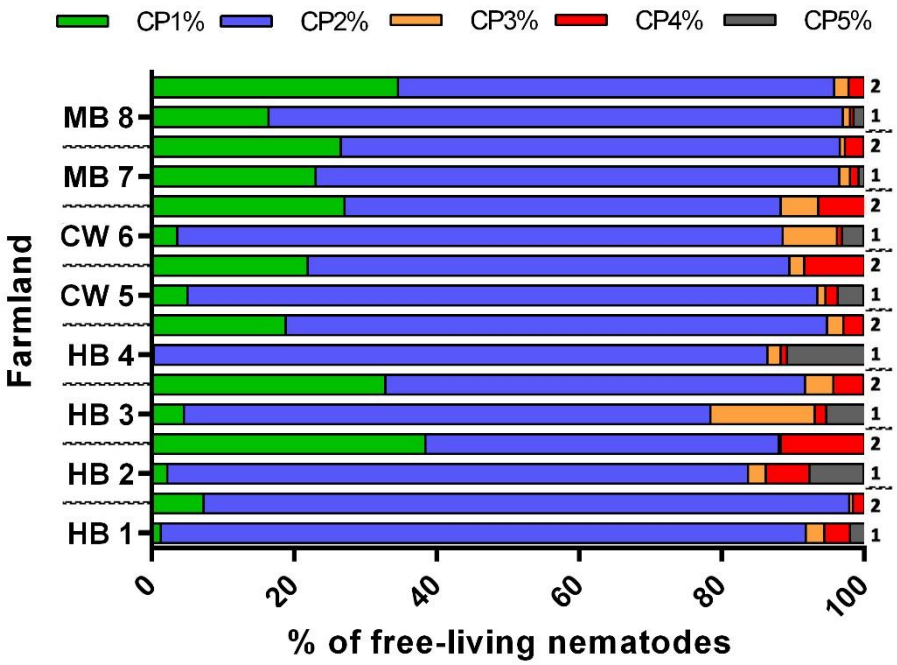

Fig. 3.5. Colonizer-persister series percentage distribution of nematodes associated with the Hartbeespoort (HB 1 – HB 4), Crocodile (West) (CW 1 and CW 2), and Marico-Bosveld (MB 1 and MB 2) irrigation schemes during the first (March/April 2016) and second (September/October 2016) sampling intervals.

Sensitive groups (c-p 3, c-p 4, and c-p 5) that are indicative of healthier soils constituted at most 21.7% (HB 3) of the extracted nematodes and were least prominent at the Marico-Bosveld Irrigation Scheme (< 5%). It should be noted that for the Hartbeespoort Irrigation Scheme, the greatest proportion of sensitive nematodes were recorded during the first sampling interval, while the opposite was true for the Crocodile (West) and Marico-Bosveld irrigation schemes. Box-and-whisker plots (Fig. 3.6) further evidenced the maturity levels of the nematode communities. The Hartbeespoort and Crocodile (West) irrigation schemes presented the highest median values during the first and second sampling intervals, respectively. The Marico-Bosveld Irrigation Scheme, in turn, scored the lowest median values

during both sampling intervals. Also, lower median values were recorded at all three systems during the second sampling interval. High variability, illustrated as minimum and maximum whiskers, were especially evident at the Hartbeespoort Irrigation Scheme.

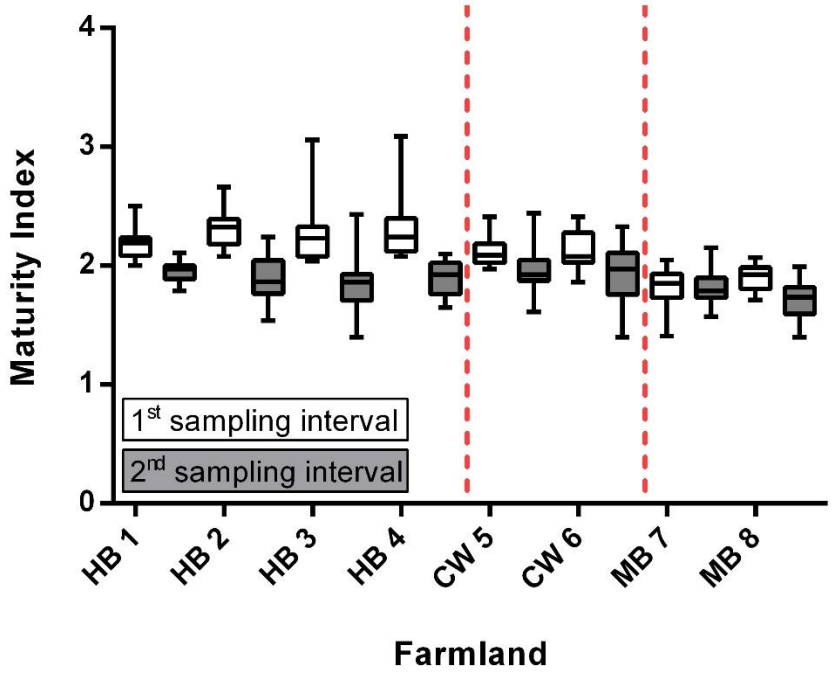

Fig. 3.6. Box-and-whisker plots, with whiskers indicating minimum and maximum values, of Maturity Index scores at the Hartbeespoort (HB 1 – HB 4), Crocodile (West) (CW 1 and CW 2), and Marico-Bosveld (MB 1 and MB 2) irrigation schemes during the first (March/April 2016) and second (September/October 2016) sampling intervals.

The faunal and metabolic footprint analyses (Fig. 3.7) illustrate distinct differences especially between the Hartbeespoort/Crocodile (West) irrigation schemes and the Marico-Bosveld Irrigation Scheme. The Hartbeespoort and Crocodile (West) irrigation schemes shifted, as indicted by the centre point of the rhombus, from degraded/depleted (first sampling interval) to disturbed/N-enriched (second sampling interval). The Marico-Bosveld Irrigation Scheme, in turn, presented minimal variation between the two sampling intervals. Furthermore, the relative magnitude of the metabolic footprints can be interpreted by comparing the size of the vertical (Enrichment Footprint) and horizontal (Structure Footprint) axes of each rhombus. This shows that during the second sampling interval a substantial increase in Enrichment footprints,

but with some reduction in Structure footprints, were recorded at both the Hartbeespoort and Crocodile (West) irrigation schemes. The nematode assemblage associated with the Marico-Bosveld Irrigation Scheme presented comparable Enrichment footprints, but minimal Structure footprints.

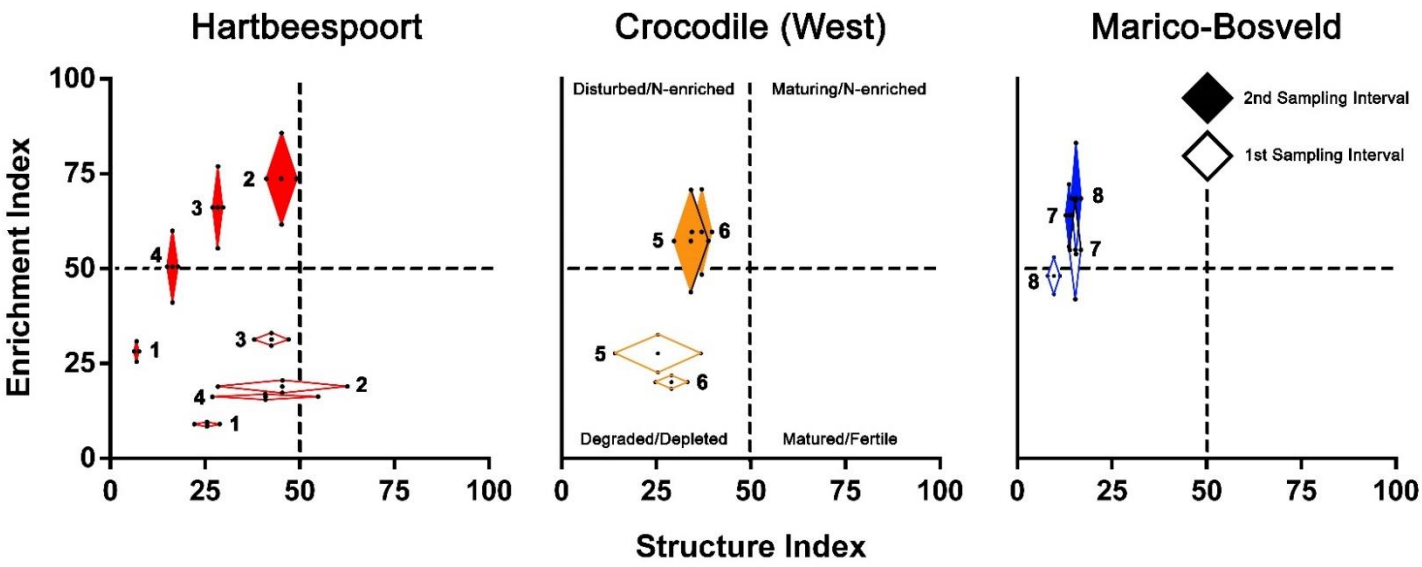

Fig. 3.7. Faunal profile illustrating the food web status (center point of rhombus), as well as Enrichment (vertical axis of rhombus) and Structure footprints (horizontal axis of rhombus) at the (a) Hartbeespoort (HB 1 – HB 4), (b) Crocodile (West) (CW 5 and CW 6), and (c) Marico-Bosveld (MB 6 and MB 7) irrigation schemes during the first (March/April 2016) and second (September/October 2016) sampling intervals.

3.5.5 Integrating biotic and abiotic components of soil health

An RDA triplot (Fig. 3.8), with 46.31% variation explained on F1 (33.31%, $P < 0.05$) and F2 (13%, $P < 0.05$), illustrates how selected biotic indices (Table 3.5) are constrained by selected soil physical (Table 3.2) and soil water chemical (Table 3.3) parameters. The type of cultivated crop was also considered as a factor that contributed to the observed variation. A positive correlation was evidenced between the Enrichment and Shannon Diversity indices, pH, inorganic N, sand and silt content, as well as beetroot, carrot, and wheat production. The MI was positively correlated to EC, clay and organic C content, as well as soybean production. The SI presented a weak positive correlation to both of these clusters, while maize production was negatively correlated to the selected biotic indices. The conditional effect, i.e. the effect

that an explanatory variable contributes by taking into account the influence effected by the other variables, was also considered. Crop production, i.e. the combined effect of the different crop factors as illustrated on the RDA triplot, contributed 17.7% ($P < 0.05$) of the total explained variation. Particle size distribution (clay, sand and silt content) presented a combined effect of 26.4% ($P < 0.05$). Inorganic N contributed 29.5% ($P < 0.05$), while the remaining parameters (EC, pH, total P and K) presented individual effects of less than 10%. Organic C content did not present a significant ($P > 0.05$) effect.

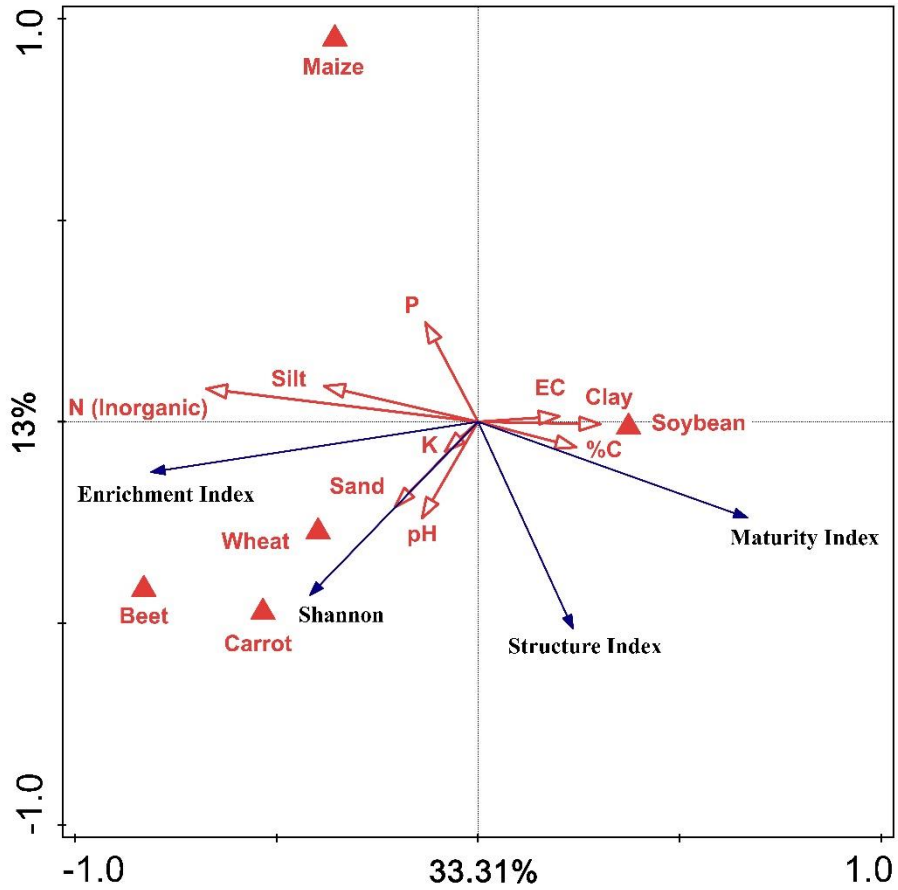

Fig. 3.8. Redundancy analysis triplot illustrates correlations between nematode-specific indices (Maturity, Enrichment, and Structure indices), Shannon Diversity Index (Shannon), and selected soil physical (particle size distribution [sand, silt, and clay] and percentage organic C) and soil water (capillary water that occupies soil pores) chemical (pH, electrical conductivity [EC], inorganic nitrogen [N], phosphorous [P], and potassium [K]) parameters. A permutation test revealed that all axes are significant ($P < 0.05$).

3.6 Discussion

3.6.1 River and irrigation water quality

Irrigation water quality at the Crocodile (West) River system was evidenced as EC readings and Cl, total P, and inorganic N concentrations exceeding guideline threshold values. However, Du Preez et al. (2018) identified especially long term increases (temporal variation) in salt and nutrient levels as a cause for concern and this study evidenced inorganic N concentrations at river HB during the first (> 10 mg/L) and second (> 20 mg/L) sampling intervals greatly exceeding previously reported concentrations (< 7 mg/L). This is likely due to operational failures at upstream sewage works and the subsequent spillage of untreated sewage, as was recently reported by the media, into the Crocodile (West) River system, (BritsPos, 2017; Nkosi, 2016). Spatial variation was further evidenced as higher EC readings and Na, SO_4, and Cl concentrations at river CW, which can likely be attributed to the inflow of tributaries and runoff from agricultural areas (Du Preez et al., 2018).

Since water for the Hartbeespoort and Marico-Bosveld irrigation schemes is canalized directly from the Hartbeespoort and Marico-Bosveld dams, respectively, the water quality of on-site irrigation dams is expected to be of similar quality. Likewise, water for the Crocodile (West) Irrigation Scheme is abstracted directly from the Crocodile (West) River. This is largely substantiated by the results, with the exception of above average Cl (> 130 mg/L) and inorganic N (> 50 mg/L) concentrations recorded at HB 3 during the second sampling interval. However, since this farmland represents only one of four associated with the Hartbeespoort Irrigation Scheme, these elevated concentrations are likely the result of on-site anthropogenic activities (e.g. application of fertilizer).

The evidenced low quality irrigation water can present various crop production related challenges. Salinity induced water stress can result in reduced growth of moderately salt-sensitive crops (e.g. sunflower, maize, and vegetables), while high Cl levels renders the water unfit for the production of tobacco (DWAF, 1996). According to DWAF (1996) inorganic N

concentrations exceeding 30 mg/L, as was recorded at the Hartbeespoort Irrigation Scheme, can result in excessive plant growth and cause lodging, delayed crop maturity, and poor quality produce. Considering that high P concentrations were also recorded at the Hartbeespoort Irrigation Scheme, it is likely that severe algae and aquatic plant growth will lead to blocked irrigation equipment and increased maintenance costs (ANZECC, 2000; DWAF, 1996).

3.6.2 Factors influencing soil physico-chemical properties

A number of factors (e.g. irrigation water quality, agricultural practices, soil structure, climatic variations, etc.) can contribute to the observed differences in soil physico-chemical properties between the respective irrigation schemes (Bronick and Lal, 2005; Lal, 2015). Soil physical parameters (particle size distribution and organic C content), for example, varied over a spatial scale as HB 1 and HB 2 presented clay dominated soils, while the remaining farmlands presented sandy loam or loamy soils. Also, the strong relation presented between clay content and organic C is a well-known phenomenon (Chapin et al., 2011). According to Chapin et al. (2011) clay soils present greater potential for aggregate formation, and by binding to organic matter, the aggregates partly protect the organic matter from decomposition.

Inorganic N, P, and K, available to crops for uptake when dissolved in soil water, are likely to be primarily influenced by the application of fertilizers (Parker, 2010). This was evidenced by the positive relation observed between the above named nutrients and the reference system, which showed significantly higher inorganic N and total P concentrations in soil water, when compared to the Hartbeespoort and Crocodile (West) irrigation schemes, during the second sampling interval. This even though the reference system was subjected to minimal nutrient loading via irrigation water. Furthermore, only a few salt ions presented significantly lower concentrations at the reference system. Nonetheless, it should be noted that Na concentrations were lower, in some cases significantly, at the reference system when compared to the studied farmlands associated with the Hartbeespoort and Crocodile (West) irrigation schemes.

Furthermore, interactions between irrigation and soil water were evidenced as significant linear relationships for the SAR, as well as Na and SO_4 concentrations. According to Hasheminejhad et al. (2013), a close relationship between the SAR of irrigation water and soils is indicative of steady-state conditions. This means that minimal change in the concentrations of the related salt ions occurred over both the temporal and spatial scales relevant to this study (Schaetzl and Thompson, 2015). As also indicated by the SAR, Na concentrations in soils were likely affected by irrigation water quality. Sulfates in solution, on the other hand, are possibly influenced by irrigation water quality, as well as the addition of fertilizers. This is supported by 1) the linear relationship, although weak, evidenced between irrigation and soil water, and 2) the addition of sulfate containing fertilizers reported for this study.

3.6.3 Ecosystem health status of irrigated soils and causal factors of disturbance

The soil food web condition, as inferred by nematode assemblage structure, of farmlands associated with the Hartbeespoort and Crocodile (West) irrigation schemes, as well as the reference system, were either classified as degraded or disturbed. According to Ferris et al. (2001) this is not unexpected in conventional farming systems where associated practices (e.g. tillage and pesticide application) can greatly disrupt soil ecosystems, while nutrient inputs can alter the soil food web trophic distribution (Briar et al., 2007; Cardoso et al., 2016; Ferris et al., 2001; Ito et al., 2015; Zhong et al., 2017). This was also evident in the c-p series distribution, which was dominated by tolerant species (r-strategists). According to Ito et al. (2015) larger nematodes (c-p 3-5) are more sensitive to tillage, which results in the disruption of soils, change in soil temperatures and moisture content, and crop residue burial.

The Maturity Index, however, evidenced the Marico-Bosveld Irrigation Scheme as the most disturbed during both sampling intervals. This despite the reference system farmlands being subjected to high quality irrigation water, as well as conservation agriculture practices (strip

tillage and no tillage). But this can partly be explained from personal communications with the respective farmers, who confirmed that disking was applied at the end of the 2015 growing season in order to loosen compacted soils. As mentioned before, tillage can have a substantial impact on larger and more sensitive nematodes, which are indicative of healthy soils (Ito et al., 2015; Zhong et al., 2017). According to Tsiafouli et al. (2015) it can take decades for the soil ecosystem to fully recover after being subjected to conventional farming practices.

At the Hartbeespoort and Crocodile (West) irrigation schemes, the metabolic footprints shifted from C and energy flow mainly through K-strategists (first sampling interval) to C and energy flow mainly through r-strategists (second sampling interval) (Ferris, 2010; Zhang et al., 2015c). Thus, during the first sampling interval, fewer resources were available for enrichment nematodes to utilize, while top-down predation pressure was likely effected. During the second sampling interval, however, Enrichment footprints were indicative of increased resource entry (bottom-up effect) (Ferris, 2010; Zhang et al., 2015a). Soil ecosystems associated with the Marico-Bosveld Irrigation Scheme, in turn, were also subjected to resource entry, but with minimal activity presented by a likely disturbed community of structure nematodes.

3.6.4 Factors influencing soil enrichment

We can conclude that conventional farming practices likely resulted in the studied farmlands presenting disturbed soil ecosystems. However, the specific causal factors responsible for temporal and spatial variation in biotic (community and nematode-specific) indices were further investigated. The RDA results indicated that the soil food webs, in especially r-strategists, mainly responded to the availability of inorganic N, as well as crop production and the associated agricultural activities. Similar findings were reported by Gruzdeva et al. (2007) who studied, for a period of nine years, the impact of annual mineral fertilizer (N:P:K) application on the nematode community structure of a sown meadow. The authors concluded that bacterivores (largely representative of enrichment nematodes) increased with the addition

of mineral and N-containing fertilizers. This is effected by available nutrients being exploited by the bacterial community, which in turn serves as a food source to bacterivore nematodes (Gruzdeva et al., 2007). Also, as a result of nematode grazing, N (as ammonium) is released and available for uptake by plants and utilization by the soil ecosystem (Gebremikael et al., 2016; Gruzdeva et al., 2007; Neher, 2001).

Finally, the RDA suggests that particle size distribution also contributed meaningful to the studied biotic indices. According to Yeates and Bongers (1999) soil texture influences nematode migration, feeding, and reproduction and therefore contributes to variation in nematode assemblage structure.

3.7 Conclusion

This study showed a link between irrigation and soil water quality, however, the subsequent impact on soil ecosystem health was masked by the likely greater effect of agricultural practices associated with conventional farming. It is therefore necessary to consider an alternative approach, e.g. studying the health of conservation agriculture farmlands subjected to low quality irrigation water, in order to evaluate the irrigation induced pollution threat posed to soils. Although not evidenced in this study, the farmlands associated with the Crocodile (West) Catchment remains threatened by the deteriorating quality of irrigation water.

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3.11 Supplementary material

Table 3A. Metal concentrations in irrigation water associated with farmlands of the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes. Sampling occurred during March/April (first sampling interval) and September/October (second sampling interval) 2016.

First sampling interval	Farmer	Al	Ti	V	Cr	Mn	Fe	Co	Ni	Cu
		ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L
Hartbeespoort	HB 1	51.09 ± 21.57	4.57 ± 0.15	10.90 ± 0.10	2.22 ± 1.45	3.48 ± 0.28	61.90 ± 5.60	1.16 ± 0.05	4.74 ± 0.40	6.11 ± 0.94
Hartbeespoort	HB 2	37.53 ± 4.70	4.31 ± 0.48	11.79 ± 0.44	0.69 ± 0.16	3.20 ± 0.14	47.10 ± 1.32	1.06 ± 0.02	5.02 ± 0.02	5.07 ± 0.67
Hartbeespoort	HB 3	32.39 ± 2.81	4.56 ± 0.24	17.17 ± 0.23	0.00 ± 0.00	4.14 ± 1.53	73.01 ± 7.75	1.03 ± 0.01	6.05 ± 0.39	5.99 ± 0.55
Hartbeespoort	HB 4	52.27 ± 27.44	4.53 ± 0.52	14.79 ± 0.59	1.26 ± 0.81	20.18 ± 27.23	47.26 ± 15.62	1.20 ± 0.59	10.21 ± 10.04	12.02 ± 11.25
Crocodile (West)	CW 5	37.41 ± 6.70	3.56 ± 0.06	18.65 ± 0.46	1.81 ± 1.61	4.24 ± 2.21	35.64 ± 4.83	0.78 ± 0.05	6.45 ± 0.78	5.86 ± 0.63
Crocodile (West)	CW 6	44.37 ± 14.67	3.67 ± 0.12	13.31 ± 0.12	1.10 ± 0.07	3.18 ± 0.89	55.63 ± 5.08	0.67 ± 0.02	5.64 ± 0.45	10.72 ± 7.52
Marico-Bosveld	MB 7	35.04 ± 3.32	3.61 ± 0.06	12.45 ± 0.22	1.97 ± 1.54	5.75 ± 1.10	19.46 ± 1.92	0.35 ± 0.11	1.99 ± 0.28	4.82 ± 0.30
Marico-Bosveld	MB 8	36.93 ± 4.85	3.11 ± 0.19	5.78 ± 0.18	2.53 ± 2.04	3.94 ± 1.30	17.79 ± 11.13	0.27 ± 0.02	1.93 ± 0.18	4.18 ± 0.16

Table 3A. Continued.

First sampling interval	Farmer	Zn	As	Se	Rb	Sr	Mo	Pd	Ag	Sb	Ba	U
		ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L
Hartbeespoort	HB 1	13.92 ± 2.08	7.16 ± 0.09	4.93 ± 0.49	9.84 ± 0.21	117.90 ± 6.42	3.05 ± 0.05	1.79 ± 0.30	14.78 ± 8.72	1.95 ± 0.06	46.19 ± 2.58	2.43 ± 0.05
Hartbeespoort	HB 2	12.63 ± 4.66	7.22 ± 0.04	5.37 ± 0.15	10.02 ± 0.16	111.30 ± 3.08	2.98 ± 0.05	1.91 ± 0.25	9.85 ± 0.73	2.05 ± 0.31	35.73 ± 0.22	2.42 ± 0.02
Hartbeespoort	HB 3	15.50 ± 5.71	6.72 ± 0.22	5.36 ± 0.44	7.67 ± 0.05	180.10 ± 7.37	2.55 ± 0.06	1.71 ± 0.04	7.87 ± 3.53	1.76 ± 0.02	63.37 ± 5.72	2.52 ± 0.03
Hartbeespoort	HB 4	102.56 ± 147.17	7.82 ± 0.15	5.36 ± 0.51	9.85 ± 0.44	100.37 ± 5.92	2.91 ± 0.15	1.60 ± 0.01	6.49 ± 3.24	1.94 ± 0.03	39.14 ± 5.30	2.41 ± 0.04
Crocodile (West)	CW 5	19.54 ± 11.64	8.03 ± 0.16	5.46 ± 0.38	7.08 ± 0.03	127.77 ± 0.83	2.83 ± 0.06	1.66 ± 0.04	7.69 ± 4.17	1.84 ± 0.05	58.05 ± 0.58	3.17 ± 0.00
Crocodile (West)	CW 6	17.13 ± 6.54	7.47 ± 0.06	6.24 ± 0.69	7.83 ± 0.09	193.37 ± 10.47	2.80 ± 0.07	1.66 ± 0.01	5.34 ± 3.12	1.78 ± 0.04	89.58 ± 9.91	5.75 ± 0.05
Marico-Bosveld	MB 7	21.88 ± 6.92	6.89 ± 0.22	4.96 ± 0.12	1.68 ± 0.03	50.35 ± 0.76	1.67 ± 0.01	1.98 ± 0.57	21.02 ± 14.55	1.84 ± 0.45	49.57 ± 1.39	3.23 ± 0.03
Marico-Bosveld	MB 8	15.90 ± 5.30	6.72 ± 0.13	4.87 ± 0.23	1.58 ± 0.01	46.70 ± 2.83	1.52 ± 0.02	1.52 ± 0.02	21.93 ± 28.96	1.57 ± 0.02	40.76 ± 2.28	3.28 ± 0.02

Table 3A. Continued.

Second sampling interval	Farmer	Al	Ti	V	Cr	Mn	Fe	Co	Ni	Cu
		ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L	ug/L
Hartbeespoort	HB 1	34.38 ± 3.64	2.84 ± 0.27	3.68 ± 0.59	0.00 ± 0.00	62.71 ± 7.01	49.75 ± 5.59	0.98 ± 0.08	5.45 ± 0.97	2.54 ± 0.86
Hartbeespoort	HB 2	197.04 ± 287.66	2.55 ± 0.14	2.69 ± 0.71	8.04 ± 0.00	179.02 ± 304.13	91.58 ± 85.77	4.30 ± 6.29	61.30 ± 99.16	51.48 ± 87.49
Hartbeespoort	HB 3	45.82 ± 29.25	4.35 ± 1.38	15.69 ± 0.30	0.00 ± 0.00	31.09 ± 31.17	89.68 ± 11.63	0.81 ± 0.45	4.34 ± 1.99	12.42 ± 18.85
Hartbeespoort	HB 4	25.74 ± 3.13	1.88 ± 0.10	8.63 ± 0.07	0.00 ± 0.00	3.73 ± 1.23	21.41 ± 3.68	0.76 ± 0.02	3.06 ± 0.20	1.17 ± 0.50
Crocodile (West)	CW 5	25.81 ± 4.30	2.31 ± 0.13	8.10 ± 0.60	0.00 ± 0.00	6.00 ± 5.48	34.03 ± 4.84	0.48 ± 0.02	4.37 ± 0.13	1.69 ± 1.07
Crocodile (West)	CW 6	37.87 ± 4.35	2.58 ± 0.07	7.85 ± 0.20	0.00 ± 0.00	3.26 ± 0.59	47.99 ± 3.57	0.40 ± 0.04	4.65 ± 0.19	1.00 ± 0.34
Marico-Bosveld	MB 7	34.88 ± 17.53	2.21 ± 0.18	5.64 ± 0.18	0.00 ± 0.00	19.04 ± 23.52	35.61 ± 15.89	0.41 ± 0.58	5.49 ± 8.37	5.48 ± 7.60
Marico-Bosveld	MB 8	34.18 ± 13.75	2.35 ± 0.19	3.19 ± 0.17	0.00 ± 0.00	5.13 ± 0.60	39.09 ± 10.75	0.03 ± 0.03	0.77 ± 0.23	9.72 ± 3.70

Table 3A. Continued.

Second sampling interval	Farmer	Zn ug/L	As ug/L	Se ug/L	Rb ug/L	Sr ug/L	Mo ug/L	Pd ug/L	Ag ug/L	Sb ug/L	Ba ug/L	U ug/L
Hartbeespoort	HB 1	26.82 ± 12.65	3.18 ± 0.42	3.12 ± 1.02	9.50 ± 0.08	112.50 ± 1.65	6.59 ± 2.83	1.26 ± 0.12	6.06 ± 4.36	1.50 ± 0.20	34.89 ± 1.42	2.18 ± 0.05
Hartbeespoort	HB 2	924.22 ± 1593.29	2.60 ± 0.22	2.37 ± 0.24	10.48 ± 2.14	143.43 ± 56.01	3.76 ± 0.31	1.18 ± 0.12	9.99 ± 2.57	1.51 ± 0.16	49.95 ± 25.16	2.20 ± 0.07
Hartbeespoort	HB 3	129.95 ± 199.29	2.29 ± 0.05	2.53 ± 0.25	5.32 ± 0.11	276.63 ± 0.72	2.67 ± 0.02	1.32 ± 0.09	8.96 ± 1.21	1.25 ± 0.07	69.62 ± 1.95	2.03 ± 0.03
Hartbeespoort	HB 4	4.16 ± 2.08	2.53 ± 0.06	1.93 ± 0.06	9.25 ± 0.05	77.69 ± 2.28	3.31 ± 0.03	1.08 ± 0.02	21.83 ± 29.82	1.35 ± 0.06	21.58 ± 0.59	2.07 ± 0.02
Crocodile (West)	CW 5	3.85 ± 1.49	3.12 ± 0.13	1.81 ± 0.17	6.99 ± 0.17	117.87 ± 1.72	2.96 ± 0.05	1.21 ± 0.11	11.57 ± 3.59	1.28 ± 0.05	51.72 ± 2.02	2.57 ± 0.04
Crocodile (West)	CW 6	8.47 ± 1.24	3.08 ± 0.16	2.13 ± 0.07	7.20 ± 0.19	169.00 ± 4.24	3.36 ± 0.16	1.23 ± 0.14	5.27 ± 2.08	1.31 ± 0.06	72.91 ± 3.01	4.18 ± 0.14
Marico-Bosveld	MB 7	77.97 ± 125.43	2.57 ± 0.07	1.61 ± 0.27	0.87 ± 0.17	63.23 ± 3.76	2.25 ± 0.12	1.06 ± 0.04	9.84 ± 2.56	1.56 ± 0.79	45.01 ± 1.73	3.84 ± 0.09
Marico-Bosveld	MB 8	10.40 ± 5.26	2.66 ± 0.20	1.82 ± 0.26	0.83 ± 0.00	79.87 ± 1.26	2.44 ± 0.10	1.23 ± 0.24	19.45 ± 22.45	1.32 ± 0.18	39.19 ± 0.99	4.85 ± 0.05

Table 3B. Abundance of nematode families recorded from farmlands associated with the Hartbeespoort (HB 1 – HB 4), Crocodile (West) (CW 5 and CW 6), and Marico-Bosveld (MB 7 and MB 8) irrigation schemes during the first (March/April 2016) and second (September/October 2016) sampling intervals.

	First sampling interval								Second sampling interval							
	HB 1	**HB 2**	**HB 3**	**HB 4**	**CW 5**	**CW 6**	**MB 7**	**MB 8**	**HB 1**	**HB 2**	**HB 3**	**HB 4**	**CW 5**	**CW 6**	**MB 7**	**MB 8**
Alaimidae	0,50 ± 2,24	7,78 ± 12,63	6,67 ± 9,70	3,16 ± 10,03	13,00 ± 21,79	4,00 ± 8,21	22,00 ± 29,49	2,22 ± 9,43	0	1,00 ± 4,47	0,50 ± 2,24	5,00 ± 12,35	1,50 ± 3,66	0,53 ± 2,29	0	0
Alloionematidae	0	0	0	0	0	0	0	1,11 ± 4,71	0	0	0	0	0	0	0	0
Anguinidae	9,50 ± 22,82	100,56 ± 78,85	67,78 ± 65,13	92,63 ± 71,25	109,00 ± 70,63	34,00 ± 45,93	37,50 ± 25,93	113,33 ± 81,17	2,50 ± 6,39	53,50 ± 47,49	113,50 ± 190,99	31,50 ± 50,19	62,00 ± 56,72	24,21 ± 24,57	33,16 ± 41,91	46,11 ± 37,75
Aphelenchidae	33,00 ± 45,20	132,22 ± 82,72	95,56 ± 74,69	156,84 ± 104,62	304,00 ± 90,52	69,00 ± 54,09	72,00 ± 67,87	172,22 ± 417,45	86,05 ± 123,30	46,00 ± 39,52	107,00 ± 62,08	186,33 ± 165,10	214,00 ± 156,35	134,21 ± 128,34	185,79 ± 157,17	136,11 ± 104,21
Aphelenchoididae	19,50 ± 33,79	25,00 ± 22,29	63,33 ± 40,15	33,68 ± 25,87	44,00 ± 30,85	46,00 ± 47,28	69,50 ± 49,15	86,67 ± 67,56	31,50 ± 77,21	7,50 ± 11,18	57,00 ± 61,14	30,50 ± 31,03	8,50 ± 14,24	20,00 ± 25,60	58,95 ± 55,77	18,33 ± 22,29
Aporcelaimidae	16,50 ± 17,55	127,78 ± 112,33	32,22 ± 50,01	112,63 ± 85,43	88,00 ± 51,67	31,00 ± 37,54	10,00 ± 12,57	12,22 ± 33,70	0	0	0	0	0	0	0	0
Belondiridae	22,50 ± 20,99	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cephalobidae	897,50 ± 816,46	838,89 ± 346,05	406,67 ± 216,28	738,95 ± 285,93	1706,00 ± 609,37	895,00 ± 835,80	820,00 ± 554,45	555,56 ± 493,37	637,33 ± 494,70	343,00 ± 179,15	316,00 ± 181,82	788,65 ± 367,83	636,50 ± 362,18	384,74 ± 310,97	342,63 ± 175,43	588,89 ± 280,31
Diphtherophoridae	0	0	1,11 ± 4,71	0	3,00 ± 7,33	0	2,00 ± 8,94	0	0	0	2,00 ± 5,23	0	0	0	0,53 ± 2,29	0
Diploscapteridae	1,50 ± 6,71	0	0	0	0	0	5,00 ± 11,00	0	0	0	0	3,00 ± 11,29	0	0	0	27,22 ± 42,54
Discolaimidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,53 ± 2,29	0
Dolichodoridae	132,00 ± 112,09	2,22 ± 9,43	16,67 ± 28,49	5,26 ± 18,67	3,00 ± 9,79	51,00 ± 70,33	2,00 ± 6,16	56,67 ± 82,39	27,25 ± 34,77	0	7,50 ± 9,67	2,50 ± 9,10	36,00 ± 36,04	9,47 ± 11,77	0	23,89 ± 44,74
Dorylaimidae	21,50 ± 42,34	0	0	0	0	0	0	2,22 ± 6,47	13,50 ± 17,55	96,00 ± 77,08	26,00 ± 19,30	30,50 ± 25,44	86,50 ± 66,43	60,00 ± 49,89	18,95 ± 19,41	25,56 ± 22,55
Hoplolaimidae	53,00 ± 71,46	119,17 ± 197,17	42,22 ± 56,94	1661 ± 1589,88	1617,00 ± 608,58	1545,00 ± 849,74	503,00 ± 440,92	853,33 ± 1077,73	5,50 ± 8,26	11,00 ± 19,71	18,50 ± 45,68	97,50 ± 162,35	97,50 ± 59,46	191,58 ± 138,97	45,79 ± 38,05	156,67 ± 170,67
Ironidae	0	0	0	0	0	1,00 ± 4,47	0	0	0	0	0	0	0	0	0	0
Leptonchidae	2,50 ± 11,18	51,39 ± 55,73	0	6,32 ± 11,65	0	0	0	0	0	0	0	0	0	0	0	0

Table 3B. Continued.

	First sampling interval								Second sampling interval							
	HB 1	**HB 2**	**HB 3**	**HB 4**	**CW 5**	**CW 6**	**MB 7**	**MB 8**	**HB 1**	**HB 2**	**HB 3**	**HB 4**	**CW 5**	**CW 6**	**MB 7**	**MB 8**
Longidoridae	0,50 ± 2,24	2,22 ± 9,43	0	0	0	0	0	0	1,50 ± 6,71	3,50 ± 9,88	0	0,50 ± 2,24	0	0	0	0
Monhysteridae	0	87,78 ± 140,94	2,22 ± 6,47	16,84 ± 41,24	8,00 ± 19,89	1,00 ± 4,47	33,00 ± 26,97	13,33 ± 20,58	0	2,00 ± 6,16	0	1,50 ± 4,89	8,50 ± 11,37	2,63 ± 4,52	0	0
Mononchidae	14,00 ± 14,65	0	0	1,05 ± 4,59	0	0	0	0	3,00 ± 9,23	4,00 ± 10,46	4,00 ± 8,21	0,50 ± 2,24	30,50 ± 25,85	0,53 ± 2,29	1,05 ± 3,15	0
Mylonchulidae	0	0	4,44 ± 8,56	0	30,00 ± 41,29	0	0	0	0	0	0	0	0	0	0	0
Neodiplogasteridae	0,50 ± 2,24	0	0	0	14,00 ± 41,09	9,00 ± 23,82	25,00 ± 50,21	0	0	0	0	0	0	0	0	0
Nordiidae	0	0	0	0	0	0	0	0	0	0	0	1,00 ± 4,47	0	0	0	0
Panagrolaimidae	3,00 ± 11,29	26,67 ± 27,65	36,67 ± 67,30	1,05 ± 4,59	94,00 ± 84,88	17,00 ± 36,29	135,50 ± 195,16	111,11 ± 75,53	53,25 ± 104,73	139,00 ± 157,08	110,50 ± 129,27	56,70 ± 104,87	13,00 ± 20,80	19,47 ± 32,74	207,89 ± 221,35	329,44 ± 261,86
Plectidae	14,00 ± 16,67	148,89 ± 94,18	34,44 ± 31,29	101,05 ± 108,01	141,00 ± 81,69	11,00 ± 22,92	215,00 ± 168,32	22,22 ± 39,34	4,50 ± 8,26	22,00 ± 26,28	13,00 ± 14,18	23,00 ± 24,52	174,50 ± 145,18	12,63 ± 20,23	11,58 ± 13,85	41,67 ± 42,74
Pratylenchidae	437,00 ± 182,44	628,33 ± 389,21	472,22 ± 208,43	56,84 ± 86,73	276,00 ± 139,41	5,00 ± 8,89	307,50 ± 507,66	446,67 ± 699,68	192,15 ± 152,84	914,50 ± 630,28	269,00 ± 157,88	238,67 ± 648,98	201,50 ± 132,79	37,37 ± 54,14	70,53 ± 69,48	232,78 ± 234,29
Prismatolaimidae	17,50 ± 19,16	3,33 ± 7,67	97,78 ± 49,89	23,16 ± 36,67	19,00 ± 26,34	97,00 ± 95,87	20,50 ± 24,81	13,33 ± 33,61	5,50 ± 12,76	0	28,50 ± 46,37	24,75 ± 42,22	30,00 ± 45,65	38,95 ± 29,98	4,21 ± 8,38	11,11 ± 22,72
Qudsianematidae	0	29,72 ± 57,10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhabditidae	10,00 ± 14,51	10,00 ± 18,47	3,33 ± 7,67	2,11 ± 6,31	24,00 ± 27,22	20,00 ± 34,34	218,50 ± 230,66	21,11 ± 55,93	19,25 ± 21,66	206,50 ± 156,25	187,00 ± 224,88	187,25 ± 122,19	328,50 ± 259,94	271,05 ± 476,51	33,16 ± 53,96	113,33 ± 123,38
Rotylenchulidae	0	1,94 ± 5,72	0	23,16 ± 32,15	0	41,00 ± 55,24	5,50 ± 10,99	314,44 ± 286,46	0,75 ± 3,35	0	0	7,50 ± 19,16	0	0	0	144,44 ± 185,13
Tobrilidae	0	0	0	0	1,00 ± 4,47	0	1,00 ± 4,47	0	0	0	0	0	0	0	0	1,67 ± 5,14
Trichodoridae	0	0	0	3,16 ± 7,49	1,00 ± 4,47	0	0	0	0	0	0	0	0	0	0	0
Tripylidae	0	41,67 ± 55,97	2,22 ± 6,47	6,32 ± 11,65	6,00 ± 11,42	1,00 ± 4,47	1,00 ± 4,47	0	0	2,50 ± 6,39	0,50 ± 2,24	1,00 ± 4,47	5,00 ± 8,27	0	0,53 ± 2,29	8,89 ± 19,97
Tylenchidae	91,00 ± 113,97	76,94 ± 55,71	60,00 ± 43,93	71,58 ± 75,52	206,00 ± 96,92	127,00 ± 78,75	357,50 ± 188,51	124,44 ± 104,16	54,40 ± 49,24	28,00 ± 31,22	79,00 ± 59,46	50,50 ± 58,80	208,00 ± 150,04	51,58 ± 25,88	80,53 ± 75,90	48,33 ± 41,76
Tylenchulidae	0	0	0	0	0	0	0	0	2,50 ± 7,86	0	1,00 ± 4,47	0	0,50 ± 2,24	0	0	0

CHAPTER 4: ARTICLE 3

Oxygen consumption rate of *Caenorhabditis elegans* as a high-throughput endpoint of toxicity testing using the Seahorse XF^{e}96 Extracellular Flux Analyzer

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4.1 Abstract

The bacterial-feeding nematode *Caenorhabditis elegans* is frequently used to evaluate the toxicity of pollutants, drugs, and environmental samples. One clear benefit is the exposure of an intact organism with functioning phenotypes that are biologically relevant. Typical sublethal endpoints of toxicity include growth, fertility, and reproduction of the test organism. Respiration (or oxygen consumption rate: OCR) of *C. elegans*, in turn, has infrequently been used, but is set to gain more interest with the development of new technologies such as the Seahorse XF^{e}96 Extracellular Flux Analyzer (respirometer). This apparatus complements high-throughput OCR assessments of microorganisms or living cells of organisms with its 96-well format. Therefore, this study was aimed at developing and validating a protocol for using OCR inhibition as a sublethal endpoint of toxicity using the Seahorse respirometer. Bioassay plates containing toxicants (benzylcetyldimethylammonium chloride [BAC-C16] and cadmium [Cd]) at different exposure concentrations were incubated in the presence of a food (*Escherichia coli* OP50) for *C. elegans*, for 48 h at 20 °C. Following incubation, the OCR was measured, averaged per exposure concentration, and concentration-response curves created with a Probit analysis. From the resulting BAC-C16 (R^2 = 0.93; P < 0.001) and Cd (R^2 = 0.98; P < 0.001) curves, the effective concentrations (EC10, EC20, and EC50) were inferred. Growth inhibition of *C. elegans* was also considered and concentration-response curves for BAC-C16 (R^2 = 0.97, P < 0.001) and Cd (R^2 = 0.95, P < 0.001) created. Furthermore, the Pearson correlation coefficient evidenced a strong, positive correlation between *C. elegans* OCR and growth inhibition for BAC-C16 (R^2 = 0.93; P < 0.001) and Cd (R^2 = 0.91; P < 0.001). The data presented in this study show that OCR inhibition of *C. elegans* can be effectively used as an endpoint of toxicity. Together with the high-throughput capability of the Seahorse respirometers, this protocol can be employed to rapidly measure the toxicity of aqueous media. An extension of this protocol is its use in measuring the toxicity of environmental samples.

Keywords: *Caenorhabditis elegans* oxygen consumption; Sublethal endpoints; Concentration-response curves; Effective concentrations

4.2 Highlights

- Rapid assessment of oxygen consumption rate of *Caenorhabditis elegans*.
- Exponential relationship between *Caenorhabditis elegans* length and oxygen consumption rate.
- Oxygen consumption rate of *Caenorhabditis elegans* used as a sublethal endpoint of toxicity.
- Strong, positive correlation between oxygen consumption rate and growth inhibition of *Caenorhabditis elegans*.

4.3 Introduction

Caenorhabditis elegans Maupas, 1900 has been extensively used to study the toxic effect of pollutants (Khare et al., 2015; Sun et al., 2016), drugs (De Boer et al., 2015), and environmental samples (Höss et al., 2012). One clear benefit is the exposure of an intact animal with functioning digestive, endocrine, neuromuscular, reproductive, and sensory systems (Hunt, 2017), i.e. phenotypes that are biologically relevant (Boyd et al., 2010). Furthermore, this species is small in size, easy to culture, and can even be maintained axenically (Hunt, 2017; Scanlan et al., 2018). *Caenorhabditis elegans* assays also present good correlations with mammalian toxicity assessments, which is used to predict safe human exposure levels, yet at a fraction of the cost (Harlow et al., 2016; Hunt, 2017). These qualities and benefits complement *C. elegans* as a model organism for toxicity testing, as well as its use in high-throughput assessment protocols (Boyd et al., 2010), as has been developed for drugs (O'Reilly et al., 2014) and pollutants of environmental concern (Jung et al., 2015).

Commonly used toxicity parameters include feeding, fertility, growth, movement, reproduction, and survival of *C. elegans* (Hägerbäumer et al., 2015; Höss and Williams, 2009). An International Organization for Standardization protocol (ISO 10872), for example, measures *C. elegans* growth, fertility, and reproduction to assess the toxic effect of either environmental or spiked aqueous, sediment, or soil samples (Höss et al., 2012; ISO, 2010). Respiration (or oxygen consumption rate: OCR) of *C. elegans*, in turn, has infrequently been used as an endpoint to study the toxic effect of, for example, disinfection by-products (Zuo et al., 2017), as well as bisphenol A, ethanol, dimethyl sulfoxide, and nonylphenol (Kohra et al., 2002). However, oxygen consumption measurement as an endpoint of toxicity is likely to gain interest with the development of state-of-the-art respirometers, such as the Seahorse XF^{e}96 Extracellular Flux Analyzer, which complements high-throughput assessments with its 96-well format (Koopman et al., 2016). This novel technology was utilized in the development of protocols that measure the change in OCR of *C. elegans* individuals following injection (by the Seahorse respirometer) of pre-loaded compounds (Koopman et al., 2016; Luz et al., 2015a;

Luz et al., 2015b). Using this capability and electron transport inhibitors, Koopman et al. (2016) designed a protocol which allows either the measurement of *C. elegans* organismal and/or cellular respiration. Nonetheless, this work focuses on oxygen consumption of the intact organism (also referred to as basal respiration) as this represents the best interpretable results (Koopman et al., 2016).

The effects studied in these protocols represent an acute response (following injection of a compound), while exposure for longer time periods would allow the study of a more environmentally relevant, chronic response. This is possible due to the short life cycle (approximately 3 days at 20 °C) of *C. elegans* (Boyd et al., 2010; Porta-de-la-Riva et al., 2012) and will promote the use of OCR inhibition as a high-throughput alternative in assessing the toxicity of drugs, specific toxicants, or environmental samples. Information generated using this approach can ultimately contribute, for example, to the outcome of trials and risk assessments.

The aim of this research undertaking was to develop and validate a protocol for using OCR inhibition of *C. elegans* as a sublethal endpoint in toxicity testing by utilizing the high-throughput capabilities of the Seahorse XF^{e}96 Extracellular Flux Analyzer.

4.4 Material and methods

4.4.1 Cultures and reagents

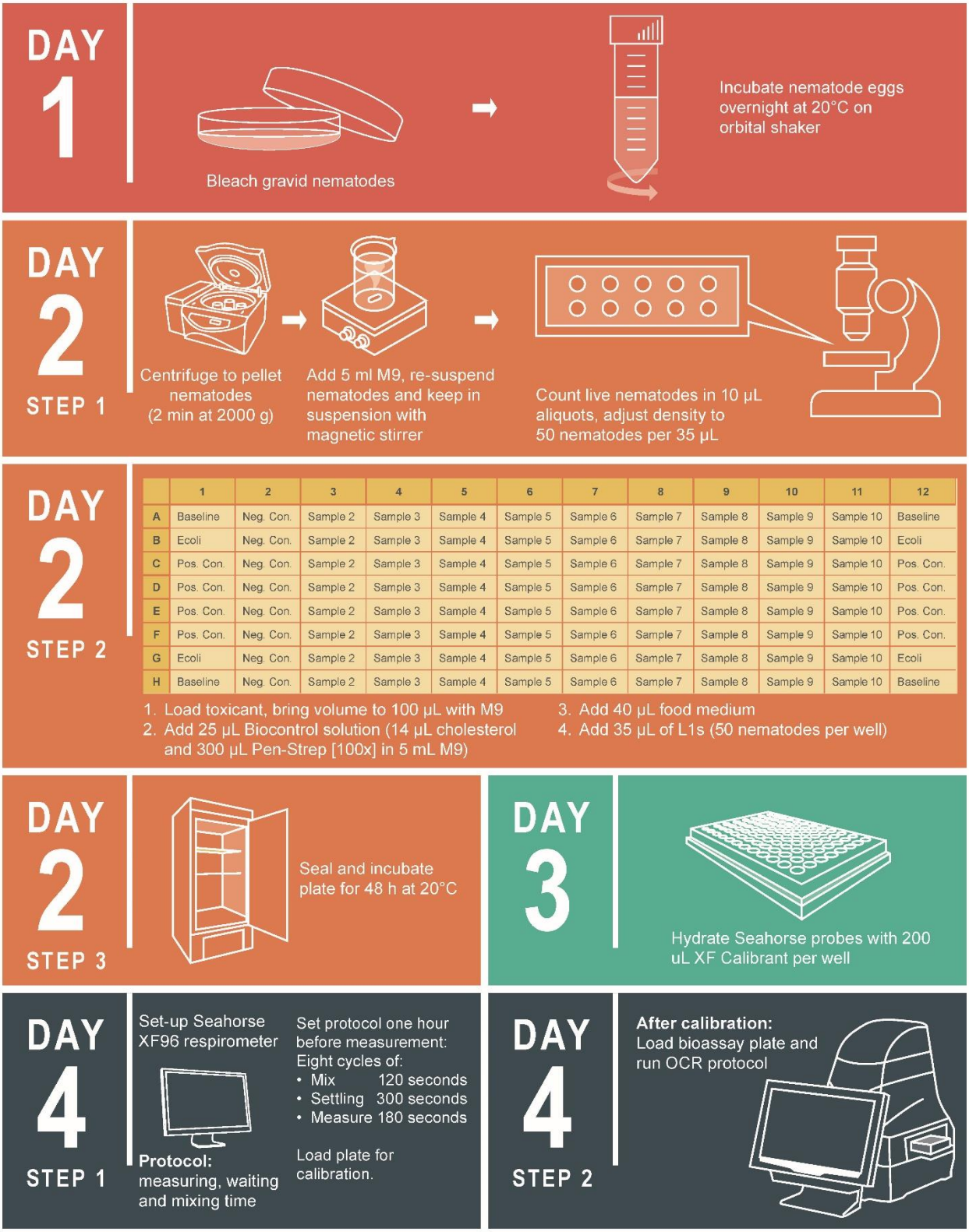
Cultures of *C. elegans* N2 and *Escherichia coli* OP50 (food source) were obtained from the Caenorhabditis Genetics Centre (https://cbs.umn.edu/cgc/home). Stock solutions of sterile M9 medium (buffer) and cholesterol were prepared, as well as a culture of *C. elegans* reared, following ISO (2010). Penicillin-Streptomycin (Pen-Strep) (Gibco 100X) was ordered from ThermoFisher Scientific while the remaining reagents used in this study were obtained from Sigma-Aldrich®.

4.4.2 Stock preparation of the food source (Escherichia coli) of Caenorhabditis elegans

An important step in the preparation of food stocks is the culturing, inactivation, and density adjustment of *E. coli* before the commencement of an exposure. *Escherichia coli* was cultured, washed, and pelleted according to ISO (2010). The pellet was re-suspended in M9 medium after which an aliquot was diluted (1→10) and the optical density (OD) measured at 595 nm (Teixeira-Castro et al., 2015) using a Pharo 300 Spectroquant (spectrophotometer). Finally, the density of the *E. coli* suspension was adjusted to an OD of 3 (595 nm). Thereafter, 5 mL aliquots of food stocks were transferred to 15 mL conical centrifuge tubes and heat inactivated (30 min at 65°C) using a water bath (Gruber et al., 2009). The stocks were stored at -80 °C until further use.

4.4.3 Synchronization of Caenorhabditis elegans

A schematic overview of the experimental procedures, as well as the appropriate timeline, is provided in Fig. 4.1. On day one, *C. elegans* eggs were extracted from culture plates using the sodium hypochlorite (bleaching) method, followed by three wash cycles to remove any residual chemicals (Luz et al., 2015b). Subsequently, synchronized larval stage one (L1) nematodes were obtained after overnight (12-20 h) incubation in 50 mL sterile conical centrifuge tubes at 20 °C on an orbital shaker (100 rpm) (Luz et al., 2015b). This procedure also kills and dissolves *E. coli*, which could otherwise influence the outcome of the bioassay. It should be noted that prior to bleaching, the culture plates were studied using a Nikon SMZ1000 stereo microscope (100x magnification) to confirm the presence of eggs and gravid females. Also, to ensure maximum extraction, enough M9 medium was added to cover the surface of the plates after which a plastic scraper was used to dislodge the eggs.



	1	2	3	4	5	6	7	8	9	10	11	12
A	Baseline	Neg. Con.	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Baseline
B	Ecoli	Neg. Con.	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Ecoli
C	Pos. Con.	Neg. Con.	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Pos. Con.
D	Pos. Con.	Neg. Con.	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Pos. Con.
E	Pos. Con.	Neg. Con.	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Pos. Con.
F	Pos. Con.	Neg. Con.	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Pos. Con.
G	Ecoli	Neg. Con.	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Ecoli
H	Baseline	Neg. Con.	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Baseline

Fig. 4.1. Schematic overview of the experimental procedure and associated timeline for culturing *Caenorhabditis elegans* and its bacterial food source (*Escherichia coli* OP 50). The timeline for preparing and incubating the bioassay plate, as well as for *C. elegans* oxygen consumption rate (OCR) measurement using the Seahorse XF^{e}96 Extracellular Flux Analyzer, is also provided.

4.4.4 Number of nematodes

An important consideration is the number of nematodes per well to be used for OCR measurements. In order to effect a broad OCR range, 50 L1s per well were used. Koopman et al. (2016) reported this to be the largest number of larval stage four (L4) (expected life stage after incubation) nematodes to be used per well without inducing anoxic conditions.

4.4.5 Food density and nematode development bioassay

Since food availability can have a substantial impact on *C. elegans* development (Gruber et al., 2009), a food density (0.1 – 0.7 OD, 595 nm) bioassay (see Section 4.4.7 for layout) was performed (as described in Section 4.4.8), with 50 L1s per well. The OCR of *C. elegans* was measured as detailed in Section 4.4.9.

4.4.6 Toxicant stock solutions

In order to test the viability of OCR inhibition of *C. elegans* as a sublethal endpoint of toxicity, benzylcetyldimethylammonium chloride monohydrate (BAC-C16) and cadmium (Cd) were selected as toxicants. Benzylcetyldimethylammonium chloride monohydrate is routinely used as a positive control for *C. elegans* growth inhibition (Hanna et al., 2016; Höss et al., 2012; ISO, 2010), while Cd is regarded as an environmentally relevant, non-essential metal (Höss et al., 2011; Järup and Åkesson, 2009; Vellinger et al., 2012).

The exposure solutions for BAC-C16 (made up in M9 medium) had the following concentrations: 2, 4, 6, 8, 10, 12, 16, 20, and 24 mg/L. For Cd, the exposure concentrations in M9 medium were 0.25, 0.5, 0.75, 1, 0.25, 1.5, 1.75, and 2 mg/L. For both assays (designed, performed, and measured as described in Sections 4.4.7, 4.4.8, and 4.4.9, respectively) the negative control consisted of M9 medium.

4.4.7 Bioassay plate layout

The bioassay was carried out in 96-well utility plates, which will from hereon be referred to as 'bioassay plates'. The bioassay plate layout (Fig. 4.1) was designed to allow for the maximum number of exposure concentrations. The four corners (A1, H1, A12, and H12) represented the baseline wells, which the Seahorse respirometer uses as a background correction for zero oxygen consumption. Four wells (B1, G1, B12, and G12) labelled "Ecoli" were reserved for *E. coli* food stocks (0.6 OD, 595 nm) containing a biocontrol solution (Pen-Strep and cholesterol; see Section 4.4.8), brought to a final volume of 200 µL with M9 medium. These wells were included to ensure that zero *E. coli* oxygen consumption, which could substantially impact OCR measurements, occurred.

Wells C1 – F1 and C12 – F12 were assigned for future use as a positive control for which BAC-C16 is recommended at a concentration of 8.94 mg/L (EC50 value of OCR inhibition as determined during this study). Oxygen consumption rate inhibition of *C. elegans* for the positive control, compared to the negative control, should range between 20% and 80% (ISO, 2010). Column 2 was represented by the negative control, while columns 3 to 11 were used for the measurement of nine treatments (food density; toxicant concentration) with eight replicates each.

4.4.8 Experimental procedure

Working in a sterile environment, the following preparation steps were executed on day two (Fig. 4.1):

1. A tube of *E. coli* food stock was allowed to thaw and reach room temperature.
2. Synchronized L1 nematodes were pelleted (2 min at 2000 g) and the supernatant discarded in order to remove residual material. Next, the pellet was re-suspended in 5 mL M9 medium using a magnetic stirrer. It should be noted that studying L1 nematodes using a Nikon Eclipse 50i light microscope (1000x magnification) after being subjected

to stirring revealed no physical damage. Similarly, Van Aardt et al. (2016) reported that stirring speed had no effect on the OCR of *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 second-stage juveniles.

3. Suspended nematodes were transferred, in 10 µL aliquots, to a microscope slide and counted using a Nikon SMZ1000 stereo microscope (40-100x magnification). The average number of nematodes in 10 replicates were calculated per 1 µL. Thereafter, the concentration of nematodes in suspension was adjusted to 50 individuals per 35 µL.

4. Stock solutions of the studied toxicants (BAC-C16 or Cadmium [Cd]) were prepared at twice the concentration of the highest exposure concentration in the test as stock solutions were diluted 1:1 with food, biocontrol solution (see below), and nematode suspensions (Fig. 4.1). By taking into account the final volume (200 µL) of each well, the required volume of toxicant stock was calculated for each exposure concentration using the following equation:

$$V_1 = \frac{C_2 V_2}{C_1}$$

where V_1 represents the volume of toxicant stock, C_2 the final exposure concentration, V_2 the final well volume (200 µL), and C_1 the toxicant stock concentration.

5. Biocontrol solution: A solution of cholesterol stock (14 µL), Pen-Strep (200 µL), and M9 medium (5 mL) was prepared. Cholesterol is necessary for the development of *C. elegans* (Kawasaki et al., 2013), while Pen-Strep ensured the inactivation of the *E. coli* food source and prevented bacterial contamination (Teixeira-Castro et al., 2015).

Immediately following the preparation steps, the bioassay plate (Fig. 4.1) was loaded in the following sequence:

6. The required volume of M9 medium (without Pen-Strep and cholesterol) was added to ensure a final volume per well of 200 µL.

7. The calculated volume of toxicant stock per exposure concentration was loaded.
8. *Escherichia coli* food stocks were briefly vortexed at room temperature after which 40 µL was added to all bioassay wells (excluding 'Baseline' wells). This ensured a final OD of 0.6 (595 nm).
9. Finally, 25 µL of the biocontrol solution were added to all bioassay wells (excluding 'Ecoli' wells).
10. The bioassay plate was placed on an orbital shaker (100 rpm) for 15 min in order to ensure sufficient mixture of reagents.
11. Lastly, 35 µL of the nematode solution was added to each control and exposure well. No nematodes were added to the 'baseline' or 'Ecoli' wells. Using a Nikon TS100 inverted microscope (40-100x magnification) each well was checked to ensure correct loading of reagents and nematodes.

The bioassay plate was sealed with parafilm and incubated for 48 h at 20 °C. This incubation period was chosen to effect high OCR readings without risk of oxygen depletion during measurement.

Additional considerations for the execution of this protocol follows:

- A final volume of 200 µL was assayed in order to simplify the calculation of the concentration and volume of solutions. However, according to Agilent (2018) the final well volume can range between 150 µL and 275 µL. Therefore, the protocol can be adjusted accordingly if lower or higher well volumes are required.
- It became evident during the design and validation phases of this protocol that water vapour condensed, especially during incubation, underneath the lid of the bioassay plate. Therefore, the bioassay plate was incubated inside a container lined with polystyrene in order to prevent air flow from resulting in different temperatures between the well content and bioassay plate lid surface.

4.4.9 Seahorse respirometer setup and oxygen consumption rate measurement

4.4.9.1 Cartridge hydration

The Seahorse respirometer makes use of optic fibre bundles, which insert into solid state sensor probes (containing polymer embedded fluorophores) and emit light to excite the fluorophores. These optic fibres then measure the change in fluorophore emission due to oxygen consumption by the test organism. On day three (Fig. 4.1), the Seahorse respirometer cartridge, which houses the probes, was hydrated by adding 200 µL XF calibrant to each well followed by overnight incubation at 37 °C.

4.4.9.2 Temperature requirements

The Seahorse respirometer was designed for cell OCR measurement at 37 °C, contrary to *C. elegans'* typical culture temperature range of between 16 and 25 °C (Porta-de-la-Riva et al., 2012). While the instrument is capable of regulating the temperature in this range, it requires a room temperature of 4 °C, which was logistically not possible. Therefore, the room was cooled to the lowest possible temperature of 16 °C. The respirometer's temperature was set to 24 °C and the internal heater switched off. It should be noted that the Seahorse respirometer generates heat during operation and was therefore only powered on directly before use. On day four (Fig. 4.1), the Seahorse cartridge was removed from the incubator two hours prior to use and left to cool and reach ambient temperature (16 °C). Although these precautions ensured sufficiently low temperatures, fluctuations in room temperature might result in varying bioassay temperatures between runs. However, Koopman et al. (2016) studied the OCR of L4 and adult life stadia of *C. elegans* and found that no significant ($P > 0.05$) difference in OCR was measured between temperature drifts of 20 to 25 °C.

4.4.9.3 Seahorse settings

Prior to OCR measurement, the following Seahorse respirometer protocol was programmed (Fig. 4.1):

1) **Two min mixing**: This involves the raising and lowering of the cartridge in order to replenish the oxygen levels within each well.
2) **Five min waiting**: The cartridge remains stationary in the 'raised' position to allow the nematodes to settle.
3) **Three min measuring**: The cartridge is lowered and a microchamber (of 3 µL) is created at the bottom of each well in which nematode oxygen consumption is measured. The decrease in oxygen is converted to a single OCR value per well.

This represents one measurement cycle, which was repeated eight times. After programming and one h prior to the OCR test, the Seahorse XF cartridge was inserted into the Seahorse respirometer for calibration (approximately 55 min.). Once calibrated, the bioassay plate containing the nematodes was inserted into the Seahorse respirometer after which it underwent an equilibration period (12 min) followed by the above detailed OCR protocol. Oxygen levels during and after OCR measurement were checked to ensure that anoxic conditions were not induced. Agilent Seahorse Wave 2.4 software package was used for exporting OCR data.

Upon completion of OCR measurement, 100 µL Bengal Rose was added to each well and the bioassay plate heat inactivated (10 min at 80 °C) (ISO, 2010). The bioassay plates were stored, for a maximum of 7 days, at 4 °C. Nematode length was measured and growth calculated as described in ISO (2010).

4.5 Statistical analyses

4.5.1 Food density bioassay

The average OCR (of the eight measurement cycles) and length of *C. elegans* were calculated per well and graphically illustrated, at different food densities, using GraphPad Prism 6 software package. Thereafter, the density of food required to allow maximum nematode development was calculated using a segmented regression model. Briefly, the growing phase of the curve was fitted with a quadratic model. The plateau, in turn, was fitted using a constant representing a line running parallel to the density food axis defining the maximum nematode development. The plateau point was determined under a condition of continuity and smoothness as defined in the supplementary material (SAS, 2013). This analysis was performed using SAS/STAT software package 9.4.

The relationship between OCR and nematode length was explained by an exponential growth (non-linear) regression model. In order to further study this relationship, the dependant variable (OCR) was $\log_{10}$ transformed and a linear regression model fitted. The 95% confidence limits were also calculated. These graphs were created and analyses performed using GraphPad Prism 6 software package.

4.5.2 Toxicant concentration-response bioassays

The average nematode OCR and growth of the eight measurement cycles were calculated per exposure concentration for the BAC-C16 and Cd concentration-response bioassays. Using ToxRat Professional 3 software package, the percentage decrease per exposure concentration was calculated against the negative control. Thereafter, the Probit analysis using the linear maximum likelihood regression algorithm was performed. The Chi-squared test was used in order to indicate the goodness-of-fit of the regression line. Furthermore, the effective concentrations (EC10, EC20, and EC50) for OCR and growth inhibition of *C. elegans* were calculated, while 95% confidence limits were based on Fieller's Theorem. Lastly, in order to study the relationship between OCR and growth inhibition of *C. elegans*, the data were

tested for normality using the D'Agostino & Pearson omnibus normality test (Ghasemi and Zahediasl, 2012). The data presented a normal distribution and therefore the Pearson correlation coefficient test was performed.

4.5.3 Oxygen consumption rate response to temperature fluctuations

In order to determine whether temperature had a significant effect on the OCR of *C. elegans*, the negative control data (of eight measurements) of the two toxicant bioassays were used. Temperatures during both assays ranged between 20 °C and 24.5 °C. Firstly, the bioassay data were tested for normality using the D'Agostino & Pearson omnibus normality test (Ghasemi and Zahediasl, 2012). Thereafter, the significance between the measurement means were tested using a one-way analysis of variance (ANOVA) (parametric data) or Kruskal-Wallis test (non-parametric data). These analyses were performed using GraphPad Prism 7 software package.

4.6 Results and discussion

4.6.1 Relationship between food density and Caenorhabditis elegans development and oxygen consumption rate

The relationship between food density and OCR, as well as between food density and nematode length, is illustrated in Fig. 4.2. Nematode length (or rather growth) was clearly inhibited by decreased food availability; a well-studied response often used to investigate the effect of dietary restrictions on *C. elegans* development (Hansen et al., 2008; Uppaluri and Brangwynne, 2015). Therefore, the relationship between *C. elegans* development and OCR was considered as the average OCR at different nematode lengths and visualised as a non-linear exponential growth curve (Fig. 4.3a). In order to infer statistical meaning from this relationship, a linear regression model (Fig. 4.3b) was applied. The slope of the $\log_{10}$ transformed linear model ($Y = 0.003761X - 0.1247$) differed significantly ($P < 0.001$) from zero

with nematode length explaining 98% of the variation in OCR of *C. elegans*. The 95% confidence bands, as illustrated in Fig. 4.3b, indicated a low degree of uncertainty. Previous studies have reported on the change in OCR as a function of *C. elegans* larval development and/or adult aging (Braeckman et al., 2002a; Braeckman et al., 2002b; Braeckman et al., 2002c; De Cuyper and Vanfleteren, 1982; Koopman et al., 2016). However, these observations were mainly made per life stage or for L4 and older nematodes. Nonetheless, findings presented here clearly show that following incubation, *C. elegans* OCR was directly related to its developmental stage as influenced by food availability.

Subsequently, to ensure that no growth (and thus OCR) inhibition occurred as a result of food availability, the minimum density of food that allowed unrestricted nematode development was determined using a segmented regression model. This model indicated that a plateau for nematode growth was reached at a food (*E. coli*) OD of 0.59 (595 nm). Therefore, an OD of 0.6 was used in the remainder of the bioassays.

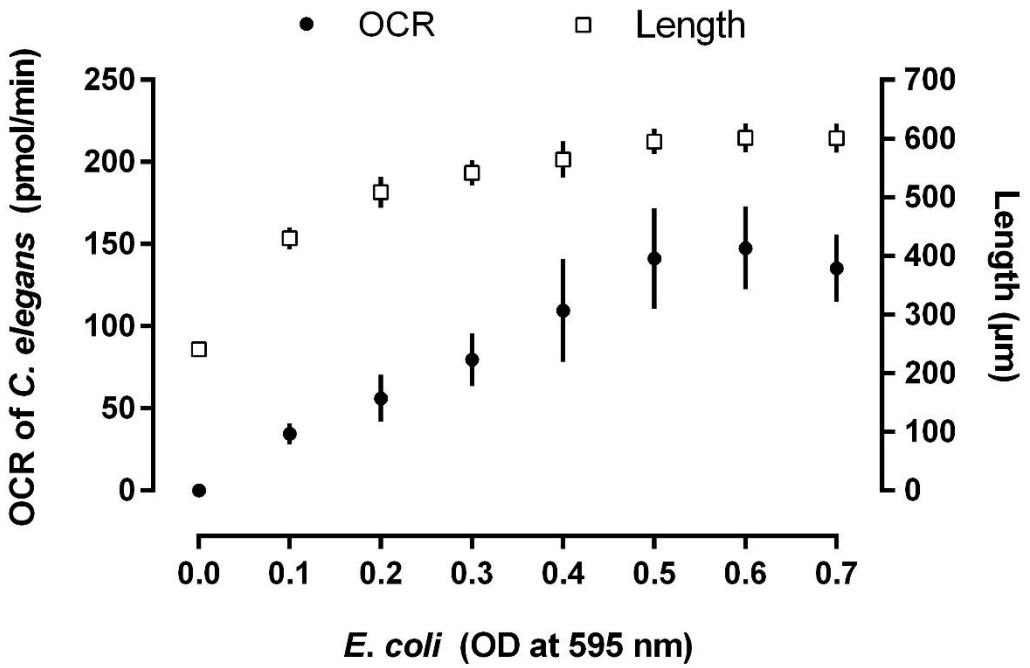


Fig. 4.2. Oxygen consumption rate (OCR) and length of *Caenorhabditis elegans* is considered at different optical densities (at 595 nm) of its bacterial food source (*Escherichia coli*) after 48 h incubation at 20 °C.

An important observation that requires attention is the OCR per nematode as measured after 48 h incubation at 20 °C. The average OCR per nematode (for OD of 0.6) was calculated as 2.95 pmol/min. Koopman et al. (2016), on the other hand, reported an average OCR per L4 nematode of approximately 4 pmol/min. However, although *C. elegans* L1 nematodes are expected to reach L4 after 48 h incubation at 20 °C, the average length (for ODs 0.6 and 0.7) after OCR measurement was 601 µm, which is representative of the developmental stage prior to the third molting (at 640 µm) (Byerly et al., 1976; Hall and Altun, 2008). This is, however, easily explained by the difference in incubation medium as *C. elegans* is known to present reduced development when cultured in liquid media (Braeckman et al., 2002c). Furthermore, Braeckman et al. (2002c) reported that nematodes grown on solid media, as was undertaken by Koopman et al. (2016), present up to 66% higher metabolic activity. Nonetheless, it is evident that a broad OCR range (0 – 148 pmol/min) (Fig. 4.2) per well can be measured.

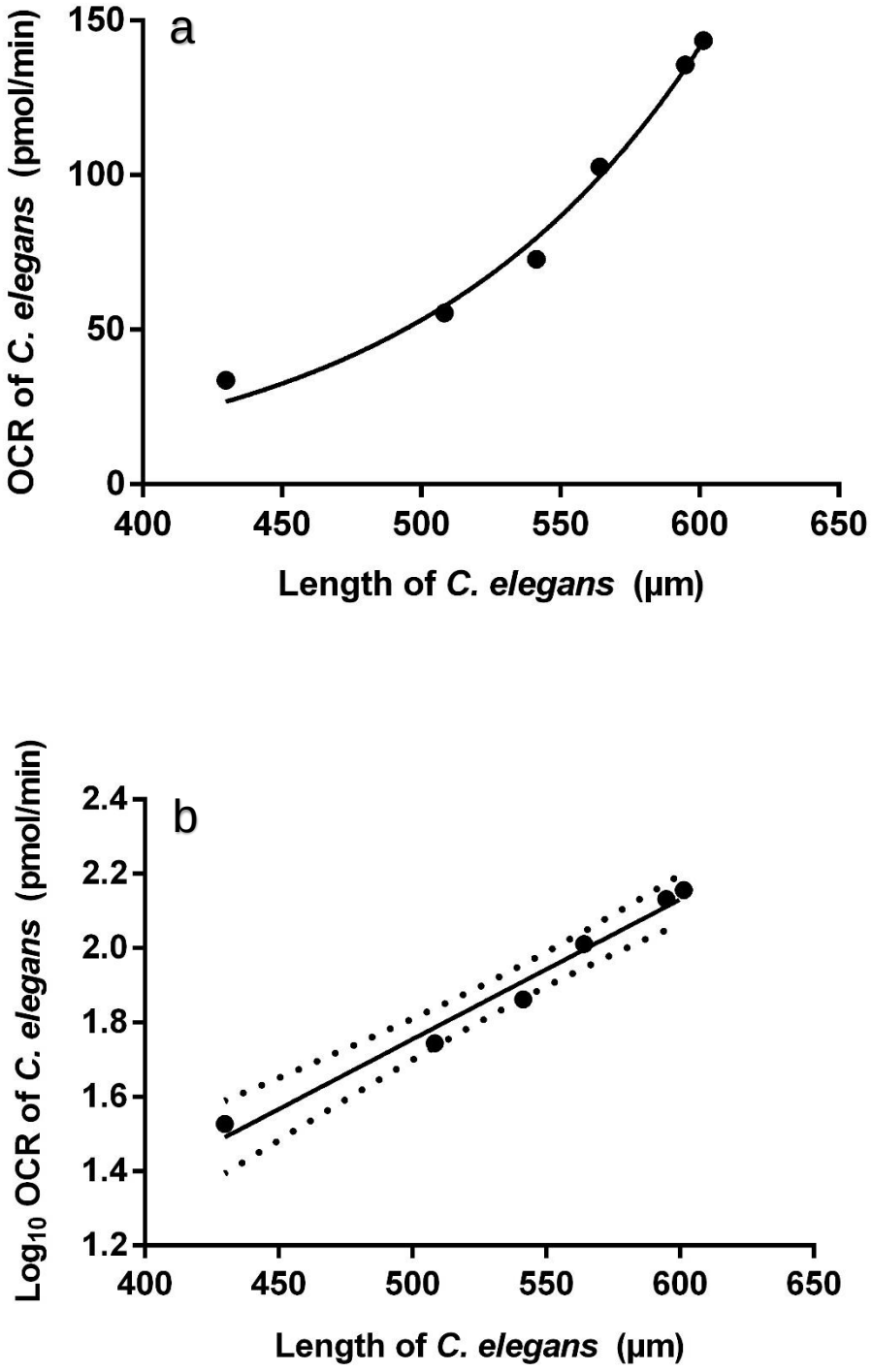


Fig. 4.3. Oxygen consumption rate (OCR) of *Caenorhabditis elegans* is considered against the length of such specimens as an (a) exponential (non-linear) growth curve and (b) $\log_{10}$ transformed linear model (Y=0.003761X-0.1247). The slope of the linear model differed significantly ($P < 0.001$) from zero with nematode length explaining 98% of the OCR variation.

4.6.2 Oxygen consumption rate and growth inhibition of Caenorhabditis elegans following toxicant exposure

The percentage OCR inhibition of *C. elegans* was measured per exposure concentration against a negative control (M9 medium) added to each bioassay plate. Using these data, a concentration-response curve for BAC-C16 (R^2 = 0.93; P < .001) (Fig. 4.4) and Cd (R^2 = 0.98; P < 0.001) (Fig. 4.5) was created.

Caenorhabditis elegans growth inhibition was also investigated for two reasons: 1) a strong relationship, as shown in this study, existed between *C. elegans* OCR and length and 2) *C. elegans* growth inhibition is routinely used as an endpoint of toxicity and can therefore be used to evaluate the sensitivity and validity of *C. elegans* OCR inhibition as a measure of toxicity. Growth inhibition concentration-response curves for BAC-C16 (R^2 = 0.97, P < 0.001) and Cd (R^2 = 0.95, P < 0.001) are illustrated on Fig. 4.6 and Fig.4. 7, respectively. The ECx values for OCR and growth inhibition of *C. elegans* (Table 4.1) were represented by the 10, 20, and 50% inhibition points on the respective curves.

The BAC-C16 concentrations at which 50% OCR and growth inhibition of *C. elegans* occurred were calculated as 8.94 and 9.47 mg/L (Table 4.1), respectively. Benzylcetyldimethylammonium chloride monohydrate is routinely used as a positive control for *C. elegans* growth inhibition assays (Hanna et al., 2016; Höss et al., 2012), while also serving as the positive control in standardised toxicity testing (ISO, 2010). According to ISO (2010) the EC50 for BAC-C16 growth inhibition should range between 8 and 22 mg/L. Furthermore, most studies have reported EC50 values of approximately 15 mg/L (Hanna et al., 2016; Höss et al., 2012). However, Schertzinger et al. (2017) reported EC50 values, for two separate tests, of 9.1 and 10.8 mg/L, respectively, which are similar to results reported in this study. According to Hanna et al. (2016), growth inhibition by BAC-C16 is substantially influenced by food density, with lower densities presenting higher inhibition rates.

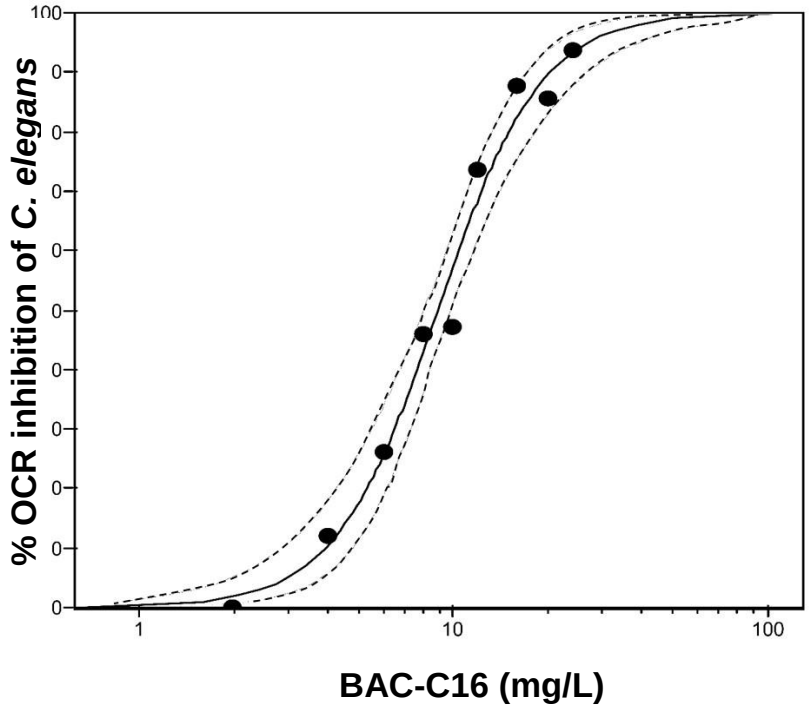


Fig. 4.4. Concentration-response curve of oxygen consumption rate (OCR) inhibition of *Caenorhabditis elegans* following exposure to benzylcetyldimethylammonium chloride monohydrate (BAC-C16). The R^2 value was calculated as 0.93 ($P < 0.001$) and 95% confidence bands indicated as dotted lines.

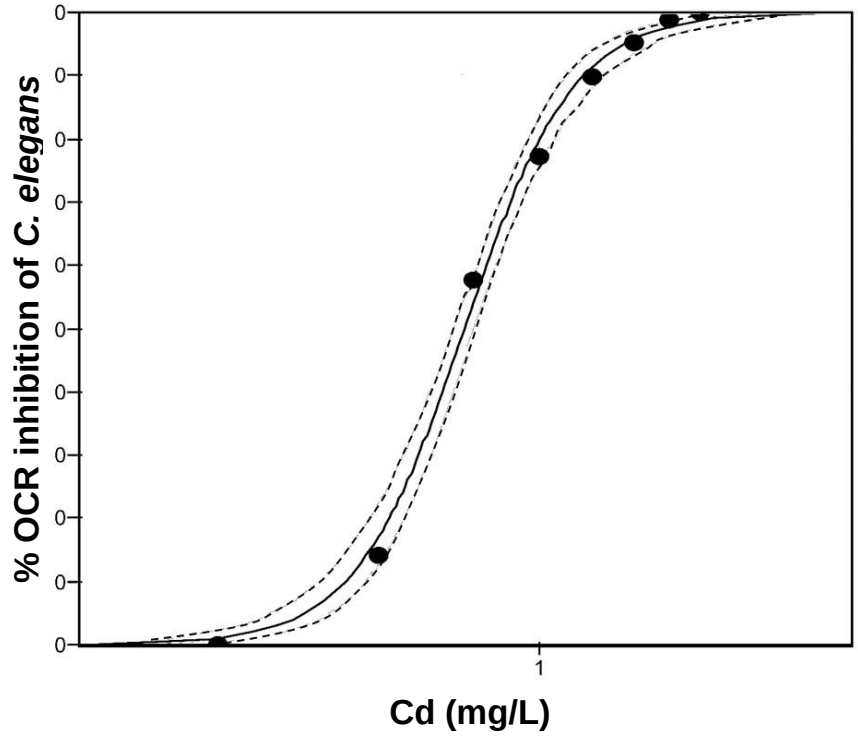


Fig. 4.5. Concentration-response curve of oxygen consumption rate (OCR) inhibition of *Caenorhabditis elegans* following exposure to cadmium (Cd). The R^2 value was calculated as 0.98 ($P < 0.001$) and 95% confidence bands indicated as dotted lines.

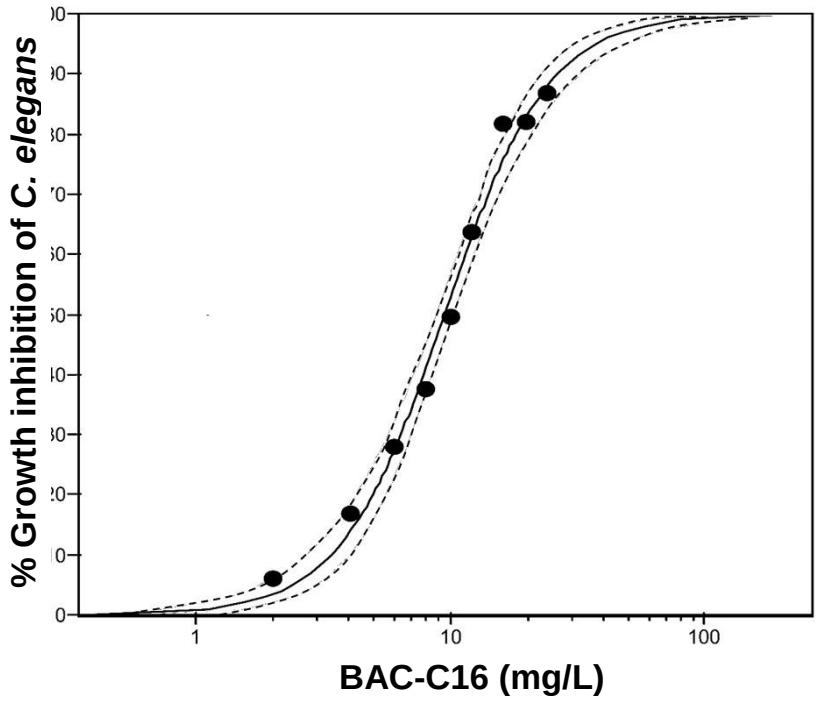


Fig. 4.6. Concentration-response curve of growth inhibition of *Caenorhabditis elegans* following exposure to benzylcetyldimethylammonium chloride monohydrate (BAC-C16). The R^2 value was calculated as 0.97 ($P < 0.001$) and 95% confidence bands indicated as dotted lines.

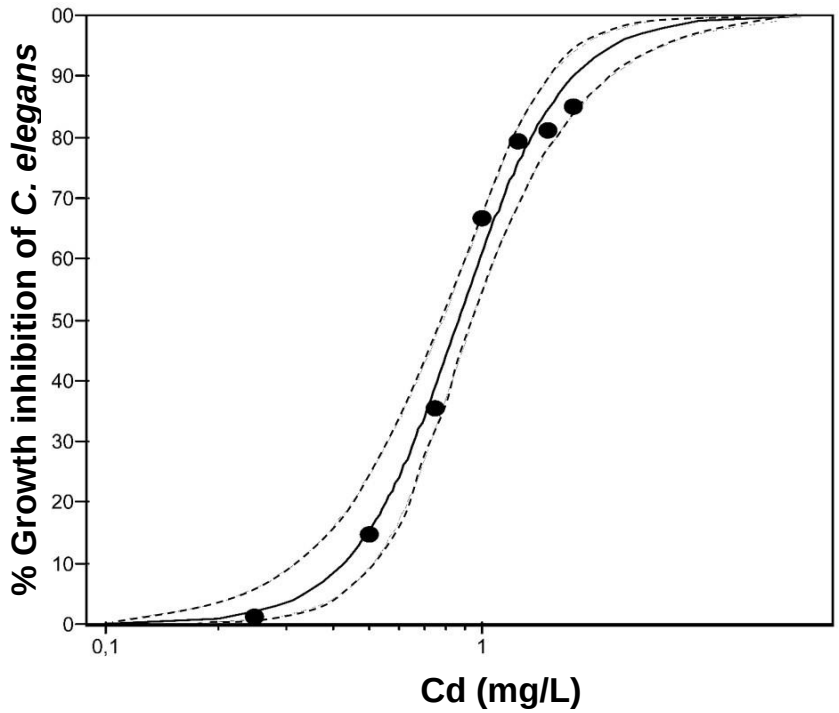


Fig. 4.7. Concentration-response curve of growth inhibition of *Caenorhabditis elegans* following exposure to cadmium (Cd). The R^2 value was calculated as 0.95 ($P < 0.001$) and 95% confidence bands indicated as dotted lines.

The Cd concentrations at which 50% OCR and growth inhibition of *C. elegans* occurred were calculated as 0.73 mg/L and 0.86 mg/L (Table 4.1), respectively. As with BAC-C16, Cd toxicity has been linked to food density, with increasing toxicity as food density decreases (Höss et al. 2011). Höss et al. (2011) attributed this to a decrease in bioavailability of freely dissolved Cd at high bacterial densities. Nonetheless, this was not viewed as a concern during this study as the minimum required amount of food for *C. elegans* was used at a constant density in control and exposure wells of BAC-C16 and Cd bioassays. The toxic effect of Cd on *C. elegans* growth is comparable to the results of other studies. Traunspurger et al. (1997) reported a lowest observed effect concentration (LOEC; 72h exposure) of 0.14 mg/L for the growth of *C. elegans*. This can be compared to the EC10 and EC20 values of 0.43 and 0.56 mg/L, respectively, reported in the present study after 48 h exposure. Van Kessel et al. (1989), on the other hand, showed a substantially higher LOEC (11.2 mg/L) for *C. elegans* after 48 h exposure to Cd. However, this was in the absence of food, which likely lead to lower Cd bioaccessibility as a result of reduced pharyngeal pumping and thus lower ingestion of Cd.

Table 4.1. Effective concentration values at 10, 20 and 50% of the oxygen consumption rates (OCRs) and growth inhibition of *Caenorhabditis elegans* were calculated with the Probit analysis using the linear maximum likelihood regression algorithm. Upper and lower 95% confidence intervals (CI) values, based on Fieller's Theorem, are also provided.

	OCR			**Growth**		
BAC-C16	**EC10**	**EC20**	**EC50**	**EC10**	**EC20**	**EC50**
Value (mg/L)	3.99	5.26	8.94	3.4	4.96	9.47
Lower 95% CI	2.96	4.23	8.07	2.72	4.25	8.8
Upper 95% CI	4.82	6.08	9.83	4	5.58	10.5
Cadmium						
Value (mg/L)	0.44	0.52	0.73	0.43	0.56	0.86
Lower 95% CI	0.39	0.48	0.69	0.32	0.45	0.79
Upper 95% CI	0.47	0.55	0.76	0.51	0.63	0.94

Similar ECx values for OCR and growth inhibition of *C. elegans* were evidenced (Table 4.1). Furthermore, the correlations (R^2 values) between OCR and growth inhibition of this nematode species for BAC-C16 and Cd were 0.93 ($P < 0.001$) and 0.91 ($P < 0.001$), respectively, which is indicative of strong, positive correlations. The similarity in ECx values and strong correlation evidenced between the nematode OCR and growth validate the use of OCR inhibition as an endpoint of toxicity as growth is already accepted and routinely used (Hägerbäumer et al., 2015; ISO, 2010). However, OCR inhibition seems to be slightly less sensitive than the reproduction of *C. elegans* (EC50 of BAC-C16: 7.5 mg L^{-1}; Höss et al. 2012; EC50 of Cd: 0.21 mg L^{-1}; Höss et al. 2011). It should, however, be noted that exposure periods for deriving ECx values of OCR and reproduction differed (48 and 96h, respectively) and thus have to be compared with caution.

Lastly, investigation of the raw Seahorse data revealed that oxygen depletion was never induced during measurements. Furthermore, no significant ($P > 0.05$) difference in OCR was evidenced between 20 °C and 24.5 °C, which supports findings by Koopman et al. (2016). Subsequently, it can be concluded that the increase in temperature during OCR measurement had no effect on the outcome of the bioassays.

4.6.3 Advantages of oxygen consumption rate inhibition as a toxicity endpoint

Although *C. elegans* OCR and growth inhibition presented similar sensitivity, OCR inhibition as an endpoint of toxicity has clear benefits. Firstly, it allows high-throughput assessments with the Seahorse respirometer's 96-well format. Also, OCR measurement is quick and automated, while also serving as a relevant, functional endpoint (Koopman et al., 2016). Lastly, this protocol and the use of *C. elegans* as a test organism can be further studied by, for example, considering the sensitivity of *C. elegans* organismal vs. cellular respiration.

4.7 Final considerations

The data presented in this study show that OCR inhibition of *C. elegans* can be effectively used as an endpoint of toxicity. Together with the high-throughput capability of a Seahorse respirometer, this protocol can be employed to rapidly measure the toxicity of different substances to *C. elegans* in an aqueous media. An extension of this protocol is its use in measuring the toxicity of environmental samples (see Section 4.4.7), which is based on the same methodology employed by ISO (2010). It is recommended to make use of the BAC-C16 EC50 concentration (8.94 mg/L) for OCR inhibition of *C. elegans* as a positive control. However, one important consideration is the 50% dilution effect that occurs as a result of adding food, nematodes, and other reagents. Future studies should explore options of reducing the dilution effect while still producing accurate results.

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4.10 Supplementary material

Segmented model

The segmented model states that for any value of x less than x_0 the expected value of Y is a quadratic function, while for values of x greater than x_0 the mean of Y is the constant c.

$$E[Y|x] = \begin{cases} \alpha + \beta x + \gamma x^2 & if\ x < x_0 \\ c & if\ x \geq x_0 \end{cases}$$

Continuity and smoothness conditions were imposed to the two segments of the model. Firstly, the continuity condition was obtained so that the quadratic and the plateau section meet at x_0. Secondly, the first derivative with respect to x was set to 0 at x0.

Continuity condition

$$E[Y|x_0] = \alpha + \beta x_0 + \gamma x_0^2$$

Smoothness condition

$$\frac{\delta E[Y|x_0]}{\delta x} = \beta x_0 + 2\gamma x_0 \equiv 0$$

Solving the equation for x_0 and substitute into the expression for c, the two conditions are jointly satisfied when:

$$x_0 = -{}^{-\beta}/_{2\gamma}$$

$$c = \alpha - {}^{\beta^2}/_{4\gamma}$$

CHAPTER 5: ARTICLE 4

Water quality and the ecological risk posed to irrigated soils

Prepared for submission to **Environmental Research**.

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5.1 Abstract

The pollution of freshwater resources utilized for irrigation presents a major challenge to the agricultural sector. This not only poses a direct threat to crop production, but also to soil health (quality), which influences crop yield and quality. Traditionally, soil health assessments were based on physico-chemical properties, while biotic attributes were disregarded. Today it is known that soil fauna fulfil important ecosystem functions (e.g. pest control and nutrient cycling) and should therefore be considered in soil health assessments. This can be achieved using the recently standardised soil quality TRIAD approach, which incorporates the chemistry, ecology, and ecotoxicology lines of evidence. This approach was used to evaluate the risk posed by dissolved metals, nutrients, and salts to the health of irrigated soils from selected farmlands associated with the Hartbeespoort, Crocodile (West) and Marico-Bosveld (reference system) irrigation schemes. Each line of evidence was scaled from 0 (no effect) to 1 (maximum effect) and an integrated risk number calculated. Results indicated that irrigation water quality, especially elevated salt and nutrient concentrations, posed a low risk to soil health at the Hartbeespoort Irrigation Scheme. However, it is likely that irrigation water quality induced risk was overshadowed by the adverse effects of agricultural activities (e.g. tillage and fertilizer application). This was most evident in the ecology line of evidence as the studied farmlands, including the reference system, presented disturbed ecosystems. Subsequently, the paucity of information regarding the effect of irrigation water quality on soil health requires further investigation with emphasis placed on accounting for agricultural activities.

Keywords: Conventional agricultural practices; Crocodile (West) Irrigation Scheme; Hartbeespoort Irrigation Scheme; Soil quality TRIAD

5.2 Highlights

- A soil quality TRIAD was used to evaluate the status of irrigated soils.
- *Caenorhabditis elegans* oxygen consumption rate inhibition was used as an endpoint of toxicity.
- None to low risk was evidenced for soils subjected to low quality irrigation water.
- A lack of information remains on the threat posed to soil irrigated with low quality water.

5.3 Introduction

The importance of soil preservation is well appreciated especially since agricultural output is required to double within the next 40 years in order to meet increasing global demands (McBratney et al., 2017). Crop production, however, is threatened by anthropogenic activities and the pollution of freshwater resources utilized for irrigation presents a major challenge (Du Preez et al., 2018; Ma et al., 2015). This not only poses a direct threat to crop production (e.g. leaf burn from chloride [Cl] exposure), but also to soil health (quality), which influences crop yield and quality (Turmel et al., 2015). In order to promote sustainable agriculture, the health status of agricultural soils, especially those subjected to anthropogenic disturbance, needs to be assessed and monitored to facilitate informed intervention.

Traditionally, soil health assessments in agricultural systems were based on physico-chemical (abiotic) properties that influence crop yield and quality, while biotic attributes were mostly disregarded (Haney et al., 2018). However, soils can be viewed as living ecosystems and the associated faunal assemblages fulfil important functions including plant disease, insect, and weed control, carbon transformation, nutrient cycling, and soil structure maintenance (Kibblewhite et al., 2008; Lehman et al., 2015; Neher, 2001). Assessing and monitoring soil health thus requires a holistic approach that integrates both abiotic and biotic measurements. To this end, the soil quality TRIAD approach, which incorporates the chemistry, ecology, and ecotoxicology lines of evidence (LOEs) into an ecological risk assessment (ERA) framework, was recently standardised (ISO, 2017).

This ERA framework generally consists of five steps, namely, **step one**: a desktop study that determines whether a pollution pathway (linkage) exists, **step two**: the design of the practical investigation plan, **step three**: execution of the soil quality TRIAD, **step four**: integration of different LOEs and calculation of final risk, and **step five**: a decision on how to proceed (ISO, 2010). In this context, the chemistry LOE measures the concentration of the constituent(s) of concern, while the ecology LOE is represented by community and/or group specific

assessments used to infer ecosystem health. The ecotoxicology LOE, in turn, considers the toxicity of environmental samples (ISO, 2017; Jensen et al., 2006). Each LOE is represented by one or multiple appropriate tests of which the data are scaled, for example between 0 (no effect) and 1 (maximum effect), and if necessary weighted, allowing the calculation of the integrated (combined) risk. Although originally developed as a measure of sediment quality (Chapman, 1990), the TRIAD approach has been successfully applied in terrestrial environments (Gutiérrez et al., 2015; Jensen et al., 2006; Ribé et al., 2012).

The Crocodile (West) Catchment hosts extensive irrigated lands as part of the Hartbeespoort and Crocodile (West) irrigation schemes, which receive water from the anthropogenically impacted Crocodile (West) River system (Du Preez et al., 2018). Pollutants (e.g. metals, nutrients, and salts) that originate from urban, industrial, and agricultural runoff, sewage effluent, as well as wastewater discharge, have led to the degradation of this freshwater system (Ballot et al., 2014; DEAT, 2005; Du Preez et al., 2018). According to Du Preez et al. (2018) a cause for concern is the evidenced increase in salt and especially nutrient concentrations from over the past decade. The authors also found that electrical conductivity (EC), as well as specific ions (Cl and sodium [Na]) and nutrients (inorganic nitrogen [N] and phosphate as phosphorous [PO_4-P]) concentrations exceeding threshold values provided by the *South African Water Quality Guidelines for Agricultural Use: Irrigation* (DWAF, 1996a) and *Australian and New Zealand Guidelines for Fresh and Marine Water Quality* (ANZECC, 2000). However, as with traditional soil health assessments, these irrigation water quality guidelines evaluate water's suitability for irrigation solely from a physico-chemical perspective. It was hypothesised that the soil quality TRIAD approach can be used to assess the risk that irrigation water quality poses to the health of irrigated soils in the Crocodile (West) and Marico catchments.

5.4 Material and methods

5.4.1 Structure of the soil quality TRIAD

Steps one and two of the ERA framework is reported on in Du Preez et al. (2018) and Chapter 3: Article 2, respectively. This work, in turn, focuses on the soil quality TRIAD assessment (ERA: step three), as well as the calculation of the ecological risk (ERA: step four) and the subsequent implications (ERA: step five). Results of the chemistry and ecology LOE are contained in Chapter 3: Article 2. Therefore, the aim of this study was to report and discuss results from the ecotoxicology LOE, while also providing background on findings from the ecology and chemistry LOEs. Furthermore, this study is aimed at evaluating the integrated ecological risk posed by dissolved constituents of concern (metals, nutrients, and salts) to the health of irrigated soils from selected farmlands associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld (Marico Catchment, reference system) irrigation schemes.

5.4.2 Site description

The study area consisted of eight farmlands associated with the Hartbeespoort (four), Crocodile (West) (two), and Marico-Bosveld (two) irrigation schemes. The Hartbeespoort Irrigation Scheme receives water via a canal system from the Hartbeespoort Dam, a major reservoir of the Crocodile (West) River system. Farmers associated with the Crocodile (West) Irrigation Scheme, in turn, abstract water directly from the Crocodile (West) River. Since the Marico River is subjected to minimal anthropogenic impact (Du Preez et al., 2018; Wolmarans et al., 2017), the Marico-Bosveld Irrigation Scheme, which is supplied with water via a canal system connecting to the Marico-Bosveld Dam, was selected as the reference system. During the first sampling interval (March/April 2016), the selected farmlands were subjected to soybean crop production, while different crops (beetroot [*Beta vulgaris* L.], carrot [*Daucus carota* L.], maize [*Zea mays* L.], soybean [*Glycine max* L. Merrill], and wheat [*Triticum aestivum* L.]) were cultivated during the second sampling interval (September/October 2016)

(Chapter 3: Article 2). The stepwise execution of the TRIAD assessment is schematically illustrated in Fig. 5.1.

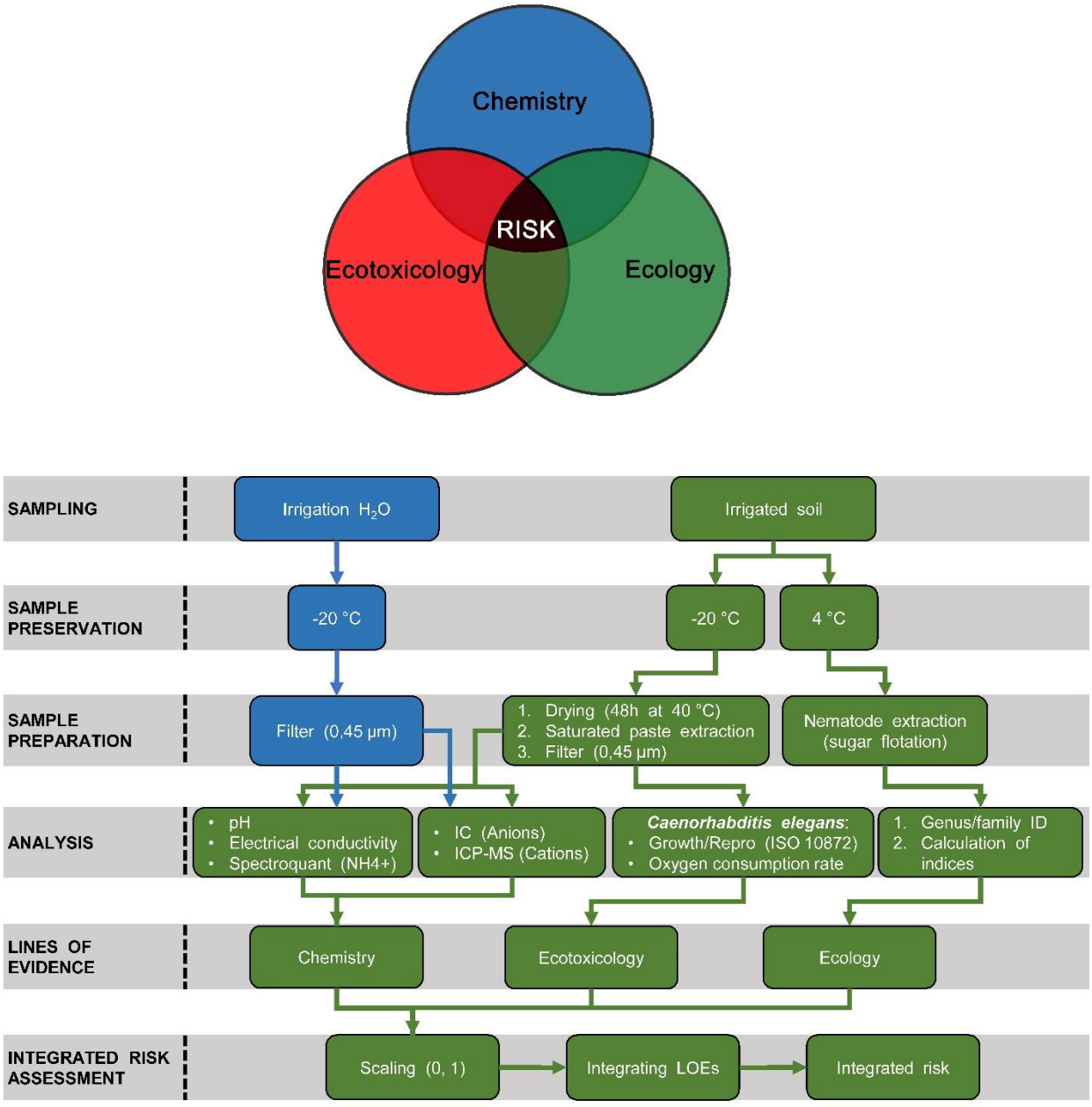

Fig. 5.1. The soil quality TRIAD approach and its stepwise execution. Irrigation water quality data were not integrated into the final risk number.

5.4.3 Chemistry line of evidence: sampling, processing, and analysis of soil and irrigation water

For assessments associated with the chemistry LOE, 12 composite samples (consisting of five sub-samples each) of rhizosphere soils were collected per farmland. Using a clean hand shovel, soil was sampled up to a depth of 20 cm. Furthermore, three water samples were collected from each irrigation dam using outlet valves during pump operation. In total, 192 soil and 48 water samples were collected. These samples were transported and stored at -20°C until further processing.

Soil samples were homogenised, dried at 40°C for 48 h, and sieved (< 2 mm). Following, soil water (capillary water that occupies soil pores) was extracted using the saturated paste extraction method (FSSA, 2002). Although laborious and time-consuming, this method is generally regarded as the most accurate measure of soil salinity and soil water content under field conditions (Doolittle, 2011; Rhoades et al., 1999). Extracted soil water and collected irrigation dam water samples were vacuum filtered with a 0.45 µm Sartorius CN sterile membrane, which allowed analysis of the dissolved fraction of metals, nutrients, and salts.

Electrical conductivity and pH was measured using WTW Cond 3210 and Mettler Toledo FE20 meters, respectively. Cation (calcium [Ca], magnesium [Mg], phosphorus [P], potassium [K], and Na) and trace element concentrations were measured using an Agilent 7500 CE series ICP-MS, while major anion ([Cl, nitrate [NO_3], nitrite [NO_2], and sulfate [SO_4]) concentrations were quantified with a Metrohm 930 Compact IC Flex. A Pharo 300 Spectroquant was used to measure ammonium (NH_4) concentrations, while total alkalinity (TAL) (pH < 8.2) was quantified by means of titration.

The difference in means of selected metal concentrations was compared between the respective irrigation schemes. Firstly, the D'Agostino and Pearson omnibus test was used to determine whether the data presented a normal distribution. Following, a one-way Analysis of Variance (ANOVA) (parametric data) or Kruskal-Wallis (non-parametric data) test was used

to determine whether the means differed significantly. Following, Tukey's (parametric data) or Dunn's (non-parametric data) test was used for multiple comparisons between the respective irrigation schemes. Results were plotted on bar charts and analyses performed using GraphPad Prism 6 software package. It should be noted that significance for all relevant univariate analyses presented for this LOE was regarded at $P < 0.05$.

5.4.4 Ecology line of evidence: sampling, extraction, and analysis of nematode assemblages

For the characterization of nematode assemblages (ecology LOE), 20 composite samples (consisting of five sub-samples each) of rhizosphere soils were collected per farmland following the same methodology as described for the chemistry LOE. In total, 320 composite samples were collected and stored at 4°C until further processing.

Soil samples were homogenised and nematodes extracted from a 200 g representative aliquot using the decanting and sieving followed by sugar centrifugal flotation method (Marais et al., 2017). Nematodes were stored in 10 ml filtered tap water at 6-8 °C and counted (within two weeks of extraction) using a Nikon Eclipse 50i light microscope (100x magnification). Family level occurrence and abundance data were generated in order to calculate the Maturity Index using the Nematode Indicator Joint Analysis (NINJA) web-based tool (Sieriebriennikov et al., 2014). The Shannon Diversity Index was calculated as follows:

$$H' = -\sum(pi \ \ln pi)$$

where *pi* represents the proportion of the *i*-th taxa in a sample (Neher and Darby, 2009).

5.4.5 Ecotoxicology line of evidence: measuring the toxicity of soil water samples

Due to the co-linearity between EC and all the major ions (data not shown), EC was used as a proxy of salt content. Subsequently, from each field, the sample with the highest EC per sampling interval was selected for investigations. Following ISO (2010), the growth and reproduction of *Caenorhabditis elegans* Maupas, 1900 was determined after exposure (96 h at 20°C) to extracted soil water samples. Four replicates of each of the selected soil water samples were tested. The negative control consisted of M9 medium, while a positive control (benzylcetyldimethylammonium chloride monohydrate [BAC-C16]) was included to ensure the validity of the test results (ISO, 2010). The EC50 value of BAC-C16, a product that inhibits bacterial growth and represented the positive control, was calculated (results not shown) as 16.94 mg/L. According to ISO (2010) the percentage growth inhibition for the positive control should be between 20% and 80% when measured against the negative control.

Secondly, a novel high-throughput approach (Chapter 4: Article 3), which involves measuring the oxygen consumption rate (OCR) of exposed *C. elegans* nematodes using a Seahorse XF^{e}96 Extracellular Flux Analyzer (respirometer), was also used as a sublethal assessment of toxicity. Briefly, a Seahorse bioassay plate was loaded with eight 100 µL replicates of each of the selected soil water samples. Following, heat-killed *Escherichia coli* OP50 (food source), as well as penicillin-streptomycin (prevent bacterial growth) and cholesterol (promote nematode development) was added to each well. Lastly, 50 larval stage one nematodes were added and the final well volume brought to 200 µL with M9 medium. As before, a negative (M9 medium) and positive (BAC-C16) control for OCR inhibition was included. The EC50 value (positive control concentration) of BAC-C16 for OCR inhibition (measured against the negative control) was calculated as 8.94 mg/L. The plate was sealed and incubated for 48 h at 20°C. After incubation, OCR was measured using the Seahorse respirometer.

The growth, reproduction, and OCR results were expressed as the percentage inhibition (against the negative control [M9 medium]) per farmland/irrigation scheme as follows:

$$\%\ Inhibition = \left(100 - \frac{\bar{x}_S}{\bar{x}_C}\right) \times 100$$

where $\bar{x}_S$ and $\bar{x}_C$ represents the mean of the parameter at a farmland/irrigation scheme and the negative control (M9 medium), respectively. Furthermore, the data were tested for normality (as previously described) after which the unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) was used to test for significant differences between the means. For parametric data with an unequal number of replicates, Welch's correction was applied. It should be noted that significance for all relevant univariate analyses presented for this LOE was regarded at $P < 0.05$.

5.4.6 Scaling, weighting, and integration of TRIAD results

Based on the criteria listed in Table 5.1, scaling from 0 (no effect) to 1 (maximum effect) and weighting of results were first applied within each LOE after which the integrated risk was calculated. It should be noted that if any of the tests presented risk lower than the reference site, a risk value of 0 was assigned (Ribé et al., 2012).

Table 5.1. Ecological risk assessment analysis and criteria for each line of evidence as implemented in the integrated risk assessment (ISO, 2017).

Line of evidence	Analysis	Criteria	Scaling (0 - 1)
Chemistry	Irrigation water content (dissolved)	**1. Metals**: TWQR (Target water quality range as listed in the *South African Water Quality Guidelines: Aquatic Ecosystems*) and reference system **2. Nutrient and salts:** Reference system	**1. Metals:** Ratio to TWQR value and background correct (reference system) **2. Nutrient and salts:** Site hazard quotient calculation based on ratio-to-reference approach with significant variance integration. Assignment of hazard classes to equal ranges in 0 – 1 scale.
	Soil water (capillary water that occupies soil pores) content (dissolved)	**1. Metal content**: TWQR (Target water quality range as listed in the *South African Water Quality Guidelines: Aquatic Ecosystems*) and reference system **2. Nutrient and salt content:** Reference system	**1. Metals:** Ratio to TWQR value and background correct (reference site) **2. Nutrient and salts:** Site hazard quotient calculation based on ratio-to-reference approach. Assignment of hazard classes to ranges in 0 – 1 scale.
Ecotoxicology	*Caenorhabditis elegans*: growth and reproduction inhibition/stimulation	Reference system	Integration using BKX (“bodemkwaliteitsindex”) method with background correct (reference system)
	Caenorhabditis elegans: oxygen consumption rate inhibition/stimulation	Reference system	
Ecology	Maturity Index	Nematode-specific index ranging from 1 (disturbed) to 5 (undisturbed)	Integration using BKX (“bodemkwaliteitsindex”) method with background correct (reference system)
	Shannon Diversity Index	Lower diversity = greater disturbance	

For the chemistry LOE, the concentration of metals, nutrients, and salts were considered. Metals including aluminium (Al), arsenic (As), chromium (Cr), copper (Cu), manganese (Mn), selenium (Se), uranium (U), and zinc (Zn) were selected based on the availability of target water quality range (TWQR) criteria. The latter were sourced from the *South African Water Quality Guidelines: Aquatic Ecosystems* (DWAF, 1996b) as no criteria exist for soil water extracted using the saturated paste method. Nonetheless, these guidelines have been developed by considering the toxic effect of dissolved metals to faunal assemblages (DWAF, 1996b) and therefore were considered appropriate for use in this study. However, to compensate for uncertainty that originate from the use of these guidelines, scaled result values were weighted (see below). This method was used for both irrigation and soil water samples collected from the respective farmlands. The former, however, were not integrated into the final risk number, but were used to investigate differences between the irrigation and soil water environments. The concentration of each metal (averaged per farmland) was scaled as follows (Jensen et al., 2006):

$$R_1 = 1 - \left(1/\left(1 + \left(\frac{m}{TWQR}\right)\right)\right)$$

$$R_2 = \frac{R_1 m - R_1 ref}{1 - R_1 ref}$$

where *m* and *ref* represents the concentration of the metal at the study and reference sites, respectively. R_1 and R_2 denote the first and second step of the scaling approach, respectively. The combined risk presented by the selected metals at each site was calculated as follows:

$$Risk = \left(1 - ((1 - R_2)_1 \times (1 - R_2)_2 \times (1 - R_2)_3 \ldots (1 - R_2)_n)^{\frac{1}{n}}\right) \times Z$$

where *n* represents the number of metals and *Z* the weighting factor (of 0.8), which accounts for the uncertainty associated with the use of the specified target water quality range (ISO, 2017).

The risk posed by nutrients (inorganic N [NO_2 + NO_3 + NH_4] and P) and salt ions (Cl, SO4, Ca, K, Mg, and Na), however, were calculated differently. Since DWAF (1996b) does not provide TWQR values for most salts, the combined risk was calculated based on the ratio-to-reference (RTF) method as implemented by Piva et al. (2011) and Li et al. (2018) as follows:

$$RTF = \frac{C_{site}}{C_{ref}} \times Z$$

Where C_{site} and C_{ref} refer to the concentration of the constituent (nutrient or salt ion) at the study and reference sites, respectively. *Z* represents the statistical significance (*P* value) between the means of the study and reference sites as determined using an ANOVA test. *Z* equals 1 [if $P < 0.05$], 3.5 – (50 x *p*) [if $0.05 \leq P \leq 0.06$], or 0.2 x $p^{-0.3257}$ [if $0.06 < P \leq 1$]. Analysis of Variance tests were performed using Graphpad Prism 6 software package. Following, the hazard quotient (HQ) was calculated per site as follows:

$$HQ_{nutrients+salts} = (\%param_{RTF<1.3} \times 1) + (\%param_{1.3 \leq RTF < 2.6} \times 3) +$$

$$(\%param_{2.6 \leq RTF < 6.5} \times 9) + (\%param_{6.5 \leq RTF < 13} \times 27) + (\%param_{RTF \geq 13} \times 81)$$

where $\%param_{RTF}$ is the percentage of RTF values within the specified range to the total number. Based on this assessment, each site's hazard level can be categorized in one of five classes, namely, Absent (HQ = 100), Slight (100 < HQ < 300), Moderate (300 ≤ HQ < 900), Major (900 ≤ HQ < 2700), and Severe (2700 ≤ HQ ≤ 8100) (Li et al., 2018). However, in order to integrate these results into the risk assessment, each class was assigned an equal range between 0 and 1 as follows: Absent, (0 - 0.2), Slight (0.21 – 0.4), Moderate (0.41 – 0.6), Major (0.61 – 0.8), and Severe (0.81 – 1). This was achieved by setting the limits of each HQ class to represent the limits of the corresponding scaled class and adjusting the values accordingly.

The risk results of the 1) metals and 2) nutrients and salts assessments were integrated into a single risk number per site per sampling interval as follows:

$$Risk = 1 - \left((1 - R_{metals}) \times (1 - R_{nutrients + salts})\right)^{1/2}$$

The ecology (Maturity and Shannon Diversity indices) and ecotoxicology (*C. elegans* growth, reproduction, and OCR inhibition) LOEs were separately scaled using the BKX ("bodemkwaliteitsindex") method as this allows results from different tests to be integrated, while both lower and higher than reference values can be used (Jensen et al., 2006). The following equation was applied:

$$BKX = 1 - 10^{\left((-\sum|logX_n|)/n\right)}$$

where *x* is the ratio between the study and reference sites and *n* the number of results (toxicity endpoints).

The integrated risk number between the chemistry, ecology, and ecotoxicology LOEs per site per sampling interval was calculated as follows:

$$Risk = 1 - \left(\left(1 - R_{chemistry}\right) \times \left(1 - R_{ecology}\right) \times \left(1 - R_{ecotoxicology}\right)\right)^{1/3}$$

The integrated risk number per irrigation scheme was calculated using the same equation by adjusting the power number to equal the number of risk numbers to be integrated. Equal weights (of 1) were assigned to risk numbers calculated for each LOE. Lastly, the standard deviation between the LOEs was calculated in order to evaluate the concordance of the calculated risks (ISO, 2017; Jensen et al., 2006).

5.5 Results

5.5.1 TRIAD assessment

Results from the chemistry and ecology LOEs are available in a separate report (Chapter 3: Article 2). Briefly, the Hartbeespoort and Crocodile (West) irrigation schemes were subjected to irrigation water characterized by elevated salt and nutrient (inorganic N and P) concentrations. However, a clear link between irrigation water quality and soil health was not evidenced as both the studied and reference systems presented disturbed soil ecosystems. A redundancy analysis triplot was used to show that a strong correlation existed between inorganic N, crop production (and associated agricultural activities), and r-strategist nematodes.

While in Chapter 3 (Article 2) emphasis was placed on the individual studied farmlands, this work further combined results per irrigation scheme, which were also used for calculating the ecological risk. Therefore, the EC (measurement of salinity) of irrigation water associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld (reference system) irrigation schemes were studied. Electrical conductivity readings of irrigation water (Fig. 5.2) at the Hartbeespoort and Crocodile (West) irrigation schemes were significantly ($P < 0.05$) higher than at the reference system during both sampling intervals. Similarly, as illustrated in Fig. 5.3, the EC of soil water samples were significantly ($P < 0.05$) lower at the reference system during the first sampling interval. However, during the second sampling interval, the reference system presented soil EC values significantly ($P < 0.05$) higher than the Crocodile (West) Irrigation Scheme, while no significant ($P > 0.05$) difference was recorded between the Hartbeespoort Irrigation Scheme and the reference system. Concentrations of the selected metals in irrigation (Fig. 5A) and soil (Fig. 5B) water associated with the respective irrigation schemes are illustrated on supplementary graphs.

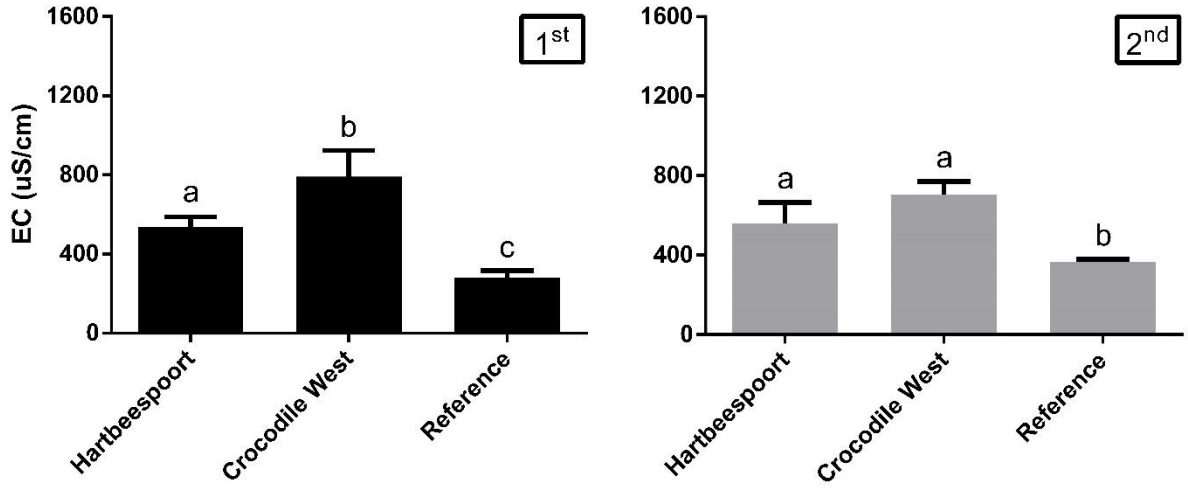

Fig. 5.2. Electrical conductivity (EC) of irrigation water associated with the Hartbeespoort and Crocodile (West) irrigation schemes, as well as the reference system (Marico-Bosveld Irrigation Scheme), during the first (1st) and second (2nd) sampling intervals. Bars with common superscript do not differ significantly ($P > 0.05$).

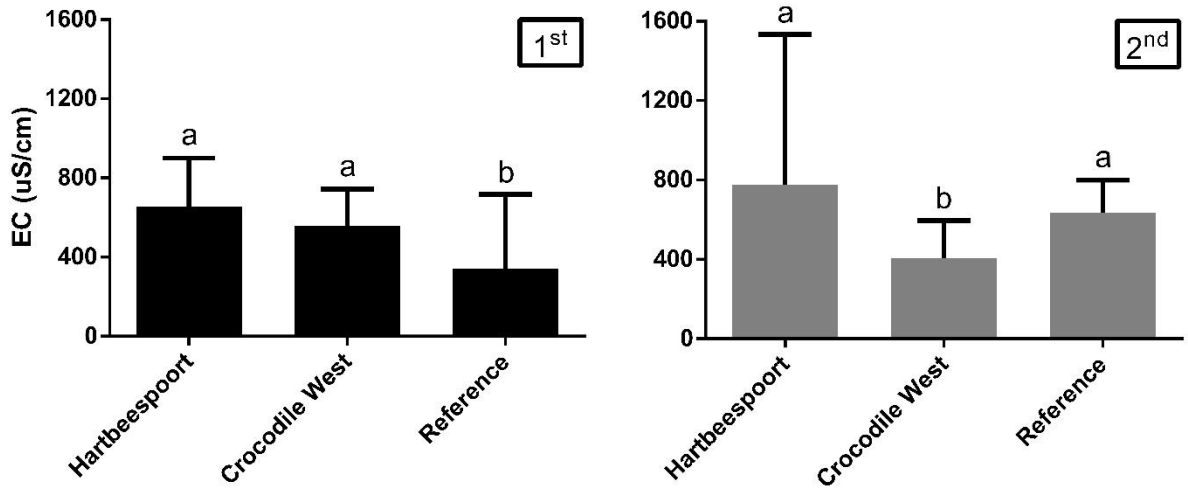

Fig. 5.3. Electrical conductivity (EC) of soil water (capillary water that occupies soil pores) associated with the Hartbeespoort and Crocodile (West) irrigation schemes, as well as the reference system (Marico-Bosveld Irrigation Scheme), during the first (1st) and second (2nd) sampling intervals. Bars with common superscript do not differ significantly ($P > 0.05$).

Results from the ecotoxicology LOE are presented as the percentage inhibition (against the negative control) of *C. elegans* growth, reproduction, and OCR (Table 5.2). The percentage inhibition for the positive control, also measured against the negative control, was calculated as 55.3% and 58% for growth and OCR, respectively; the tests were thus valid. The results indicate that variation occurred between the studied farmlands associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes, as well as between the respective sampling intervals. Significant ($P < 0.05$) inhibition of growth was observed for HB 2 (5.8%), HB 3 (5%), and HB 4 (8.2%) during the first sampling interval. During the second sampling interval, significant ($P < 0.05$) growth inhibition was observed for HB 1 (9%) and HB 4 (6.7%), while significant ($P < 0.05$) stimulation was observed for HB 3 (-7.7%). Reproduction of *C. elegans* was significantly ($P < 0.05$) inhibited, during the first sampling interval, at all farmlands associated with the Hartbeespoort Irrigation Scheme, as well as MB 8 (19.5%). During the same sampling interval, *C. elegans* reproduction was significantly ($P < 0.05$) stimulated for CW 6 (-44.4%) and MB 7 (-31.6%). Furthermore, during the second sampling interval, HB 1 (31.1%), CW 6 (26.6%), and MB 7 (38.4%) presented significant ($P < 0.05$) reproduction inhibition, while HB 3 (-37.7%) and CW 5 (-42.1%) presented significant ($P < 0.05$) reproduction stimulation. *Caenorhabditis elegans* OCR was significantly ($P < 0.05$) inhibited at MB 8 (93.5%) during the first sampling interval, and significantly ($P < 0.05$) inhibited at HB 1 (25.1%), HB 4 (81.3%), CW 5 (24.4%), and MB 8 (33.8%) during the second sampling interval.

When considering the ecotoxicology results per irrigation scheme, however, only growth (4.9%) and reproduction (16.9%) of *C. elegans* were significantly ($P < 0.05$) inhibited (Hartbeespoort Irrigation Scheme) during the first sampling interval. During the second sampling interval, only reproduction of *C. elegans* at the Hartbeespoort (34.3%) and Marico-Bosveld (28.2%) irrigation schemes were significantly ($P < 0.05$) inhibited. It should be noted that the reference system (farmlands and irrigation scheme) presented some of the highest *C. elegans* reproduction and OCR inhibition and stimulation results.

Table 5.2. Percentage inhibition (positive values) and stimulation (negative values) of sublethal toxicity tests (growth, reproduction, and oxygen consumption rate [OCR]) at the studied farmlands associated with the Hartbeespoort (HB), Crocodile (West) (CW), and Marico-Bosveld (MB) irrigation schemes. The percentage inhibition per toxicity test was also calculated per irrigation scheme.

	HB 1	**HB 2**	**HB 3**	**HB 4**	**CW 5**	**CW 6**	**MB 7**	**MB 8**	**HB**	**CW**	**MB**
						First sampling interval					
Growth	0.7	5.8*****	5*****	8.2*****	-2.6	-0.2	-4	0.3	4.9*****	-1.4	-1.9
Reproduction	14.5*****	18.6*****	11.2*****	23.3*****	7.9	-44.4*****	-31.6*****	19.5*****	16.9*****	-18.3	-6.1
OCR	-10.5	6.8	-17.6	-2.9	-14	12.8	-17.4	93.5*****	-6.1	-0.6	38.1
						Second sampling interval					
Growth	9.0*****	-2.5	-7.7*****	6.7*****	-1.3	0.9	-0.2	1.5	1.4	-0.2	0.7
Reproduction	31.1*****	-11.2	-37.7*****	25.8	-42.1*****	26.6*****	38.4*****	-6.5	2	-7.8	16
OCR	25.1*****	14.4	16.5	81.3*****	24.4*****	6.9	18.6	33.8*****	34.3*****	15.7	28.2*****

5.5.2 Integrated risk assessment

The results from the three LOEs were scaled and integrated into an ERA (Table 5.3) per farmland per sampling interval, as well as per irrigation scheme per sampling interval. For each farmland/irrigation scheme the final risk number, which represents the integrated risk for the combined LOEs, was also calculated. Although the calculated risk posed by irrigation water quality was also listed, it was not integrated into the final risk number. Following Jensen et al. (2006), each risk number was categorized as presenting either no, low, moderate, or high risk.

Irrigation water presented either moderate or high risk as a result of nutrient and salt content at all of the farmlands/irrigation schemes during both sampling intervals. In contrast, no risk, with the exception of HB 2 (low risk) during the second sampling interval, was evidenced for metal content. Although most metals exceeded the target water quality range, the reference site values were similar to the studied sites, which resulted in reduced risk numbers after background correction was applied.

Table 5.3. Integrated risk assessment of the chemistry, ecology, and ecotoxicology lines of evidence (LOEs) per farmland/irrigation scheme per sampling interval. Risk numbers are classified (and colour coded) as presenting either no, low, moderate, or high risk according to Jensen et al. (2006).

	First sampling interval						Second sampling interval						Per irrigation scheme			
	HB 1	HB 2	HB 3	HB 4	CW 5	CW 6	HB 1	HB 2	HB 3	HB 4	CW 5	CW 6	HB (1st)	CW (1st)	HB (2nd)	CW (2nd)
Chemistry (Irrigation water)																
Dissolved nutrients and salts	0.81	0.79	0.85	0.81	0.83	0.83	0.84	0.84	0.81	0.71	0.74	0.74	0.82	0.83	0.8	0.74
Dissolved metals	0.05	0.02	0.03	0.25	0.04	0.12	0.06	0.49	0.18	0.01	0.01	0.02	0.09	0.08	0.21	0.01
Chemistry (Soil water) LOE																
Nutrients and salts in solution	0.34	0.65	0.51	0.54	0.36	0.49	0	0.26	0	0.49	0	0.24	0.52	0.43	0.21	0.13
Metals in solution	0.08	0.1	0.22	0.26	0.04	0.25	0	0.03	0.05	0.14	0	0.06	0.17	0.15	0.06	0.03
Ecology LOE																
Maturity and Shannon Diversity indices	0.17	0.13	0.1	0.21	0.09	0.19	0.22	0.13	0.02	0.14	0.09	0.06	0.15	0.14	0.13	0.08
Ecotoxicology LOE																
C elegans: growth, reproduction, and oxygen consumption rate	0.1	0.18	0.08	0.17	0.06	0.19	0.07	0.16	0.23	0.4	0.2	0.14	0.13	0.13	0.22	0.17
Chemistry LOE	0.22	0.44	0.38	0.41	0.22	0.38	0	0.15	0.02	0.34	0	0.15	0.37	0.3	0.14	0.08
Ecology LOE	0.17	0.13	0.1	0.21	0.09	0.19	0.22	0.13	0.02	0.14	0.09	0.06	0.15	0.14	0.13	0.08
Ecotoxicology LOE	0.1	0.18	0.08	0.17	0.06	0.19	0.07	0.16	0.23	0.4	0.2	0.14	0.13	0.13	0.22	0.17
Integrated Risk (IR)	**0.17**	**0.26**	**0.2**	**0.27**	**0.13**	**0.26**	**0.1**	**0.15**	**0.1**	**0.3**	**0.1**	**0.12**	**0.23**	**0.2**	**0.16**	**0.11**
Deviation	0.06	0.17	0.17	0.13	0.08	0.11	0.11	0.02	0.12	0.14	0.1	0.05	0.13	0.1	0.05	0.05

Risk indicators:				
0.00 ≤ IR ≤ 0.20	**no risk**	0.21 ≤ IR ≤ 0.50	**low risk**
0.51 ≤ IR ≤ 0.75	**moderate risk**	0.76 ≤ IR ≤ 1.00	**high risk**

The TRIAD tests associated with the farmland soils, in turn, were mostly classified as presenting either no or low risk, while nutrients and salts at the Hartbeespoort Irrigation Scheme presented moderate risk during the first sampling interval. However, metals presented no or low risk at all the farmlands/irrigation schemes, which resulted in the chemistry LOE presenting low risk during the first sampling interval and no risk, with the exception of HB 4 (low risk), during the second sampling interval. The integrated risk was highest (0.3) at HB 4 (second sampling interval). Other integrated risk numbers classified as low were recorded at HB 2 (0.26), HB 4 (0.27), and CW 6 (0.26) during the first sampling interval. Also, the Hartbeespoort Irrigation Scheme presented low integrated risk during the same sampling interval. The standard deviation in risk between the LOEs was low (≤ 0.17).

5.6 Discussion

5.6.1 Irrigation water quality

The spatial variation in irrigation water quality recorded during this study supports findings by Du Preez et al. (2018). The latter authors evidenced, over a period of 10 years (2005 – 2015), significant differences in specific ion (Cl, SO4, Ca, K, Mg, and Na) and nutrient (inorganic N and PO4-P) concentrations between the same systems. Differences were further demonstrated by the risk assessment as irrigation water associated with the Hartbeespoort and Crocodile (West) irrigation schemes presented either moderate or high risk for nutrient and salt content when related to the reference system (Marico-Bosveld Irrigation Scheme).

5.6.2 Ecotoxicology line of evidence

The ecotoxicology results indicated that most of the studied farmlands presented substantial toxic variability between the executed tests, as well as between sampling intervals. Reproduction data of *C. elegans* also presented substantially larger percentage ranges than growth, which indicate that this endpoint is likely more sensitive. Also, the reproduction of target organisms is also regarded as being more ecologically relevant than growth (Höss and Williams, 2009). Oxygen consumption rate inhibition also presented large percentage ranges despite a strong correlation being evidenced in Chapter 4: Article 3 between OCR and growth inhibition of *C. elegans*. The reason for this remains unknown, however, it is possible that the mixture of constituents present in the environmental samples had a more substantial influence on the OCR of *C. elegans* than evidenced in Chapter 4: Article 3 with BAC-C16 and cadmium toxicity testing.

A number of farmlands, although not evidenced per irrigation scheme, presented significant stimulation of especially *C. elegans* reproduction, which may be a resulting toxic response (e.g. hormesis) (Álvarez et al., 2005). Furthermore, the contrasting difference (inhibition vs. stimulation) between toxicity tests observed for some farmlands might be linked to the different physiological mechanisms (e.g. reproduction vs. metabolic activity of the target nematode species, *C. elegans*) involved. This represents one of the key advantages of *C. elegans* toxicity testing since a whole (intact) organism with functioning physiological systems (e.g. digestive and reproductive) is exposed (Hunt, 2017).

5.6.3 Ecological risk posed to irrigated soils

The moderate risk posed to irrigated soils (Hartbeespoort Irrigation Scheme) by nutrient and salt content during the first sampling interval likely related to the low quality irrigation water sourced from the polluted Hartbeespoort Dam (Ballot et al., 2014; DEAT, 2005; Du Preez et al., 2018). During the same sampling interval, low risk was also evidenced at HB 4 for the

ecology LOE, which indicates a resulting disruption of the associated faunal assemblages. Although not necessarily evidenced in the calculated risk for the ecotoxicology LOE, increased soil salinity can inhibit microbial growth (Rath et al., 2016; Yuan et al., 2007), while specific ions can present toxicity induced effects (Rath et al., 2016; Šalamún et al., 2014). Šalamún et al. (2014), for example, reported that nematode faunal assemblages were adversely affected, as was indicated by a lack of sensitive species, following Mg pollution. Furthermore, increased nutrient levels, although serving as a food source to soil communities, can alter food web structures (Gruzdeva et al., 2007; Hu et al., 2017; Liang et al., 2009). According to Hu et al. (2017) the application of nutrients (as fertilizers) can increase the abundance of especially bacterivore nematodes, while elevated levels of N and P may reduce soil biodiversity. Similarly, Sarathchandra et al. (2001) reported a reduction in faunal diversity as a result of N application. These are important considerations since irrigating with water containing nitrogen levels of 30 mg/L, as was recorded at the Hartbeespoort Irrigation Scheme (Chapter 3: Article 2), are equal to 300 kg/ha of N when 1000 mm of water is applied (DWAF, 1996a).

During the second sampling interval, however, only low risk was evidenced for soil water nutrient and salt content. This indicates that irrigation water quality did not substantially influence, at least during this period, soil water salinity. It is likely, however, that other factors altered the salt and nutrient content in solution (also at the reference system), which resulted in either no or low risk being recorded at the Hartbeespoort and Crocodile (West) irrigation schemes. Factors that may have impacted soil water quality include agricultural activities such as the application of fertilizers (Parker, 2010; Shrivastava and Kumar, 2015) and water application rates (Aragüés et al., 2015), as well as climatic conditions (e.g. rainfall) (Schofield and Kirkby, 2003).

Furthermore, it is important to consider that the farmlands associated with the Hartbeespoort and Crocodile (West) irrigation schemes, as well as the reference system, presented disturbed ecosystems, which has also been attributed to the disruptive effects of agricultural activities (e.g. tillage) (Chapter 2: Article 3). Similarly, toxicity induced effects were recorded for soil

water samples from the reference system. Subsequently, in the present study, minimal ecological risk was evidenced for these LOEs following background (reference site) correction.

A last consideration is the relatively low standard deviation of the integrated risk numbers, which are indicative of low uncertainty relating to the execution of TRIAD tests and integration of the LOEs (ISO, 2017). According to Mesman et al. (2011) the maximum proposed deviation value is 0.4, well above values evidenced during this study.

5.7 Conclusion

Following the soil quality TRIAD assessment, it is concluded that irrigation water quality posed a low risk to the health of irrigated soils. However, in order to more accurately investigate the potential treat that anthropogenic pollution poses to irrigated soil health, the seemingly substantial impact induced by agricultural activities will have to be accounted for.

There remains a paucity of information regarding the risk that irrigation water quality poses to the health of soils. More specifically, the threat posed to faunal assemblages that fulfil important ecosystem functions requires further investigation.

5.8 Acknowledgements

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5.11 Supplementary material

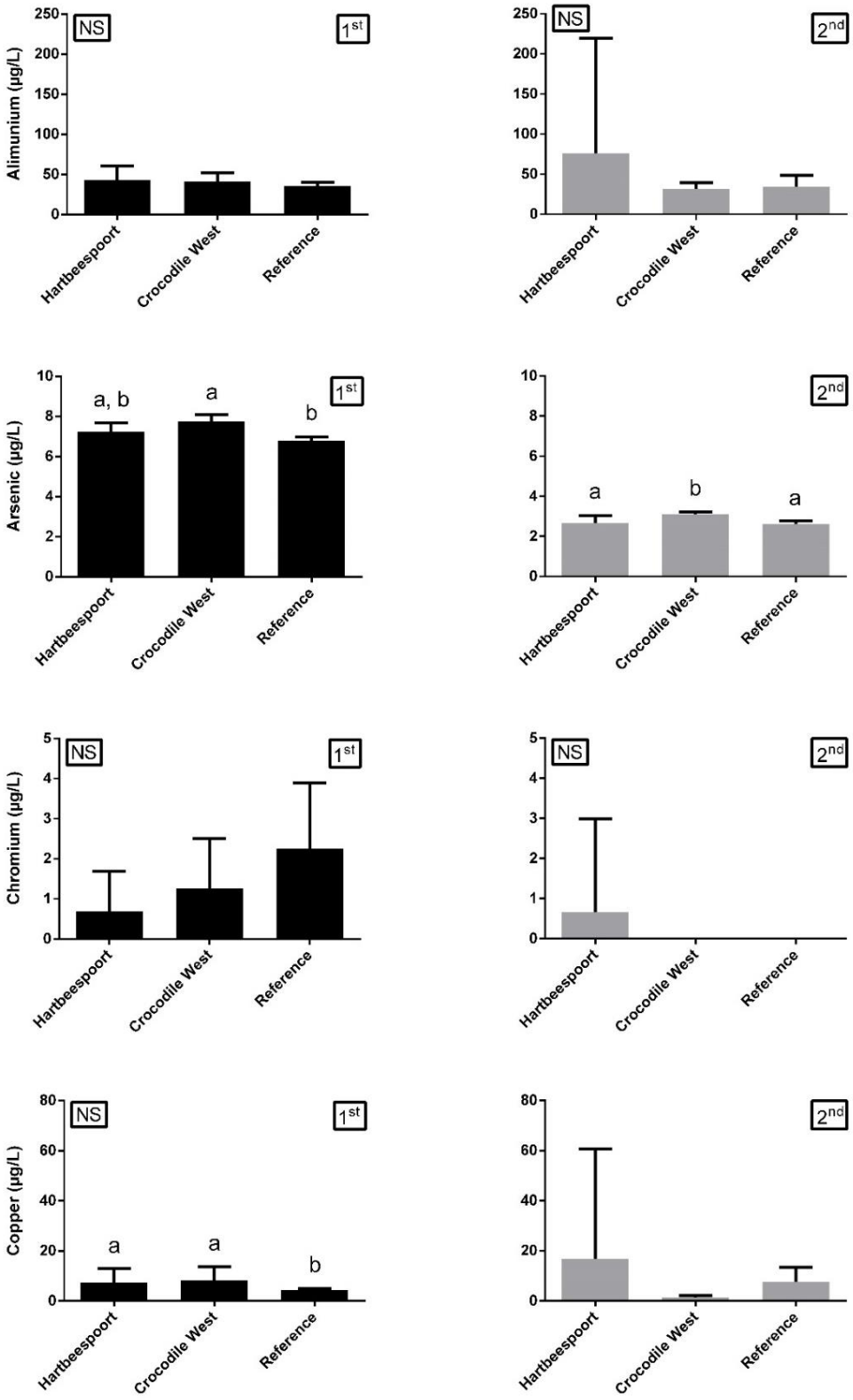

Fig. 5A. Metal concentrations in irrigation water associated with the Hartbeespoort and Crocodile (West) irrigation schemes, as well as the reference system (Marico-Bosveld Irrigation Scheme), during the first and second sampling intervals. Not significant (NS) differences between means are noted, while bars with common superscript do not differ significantly. Significance was regarded at $P < 0.05$.

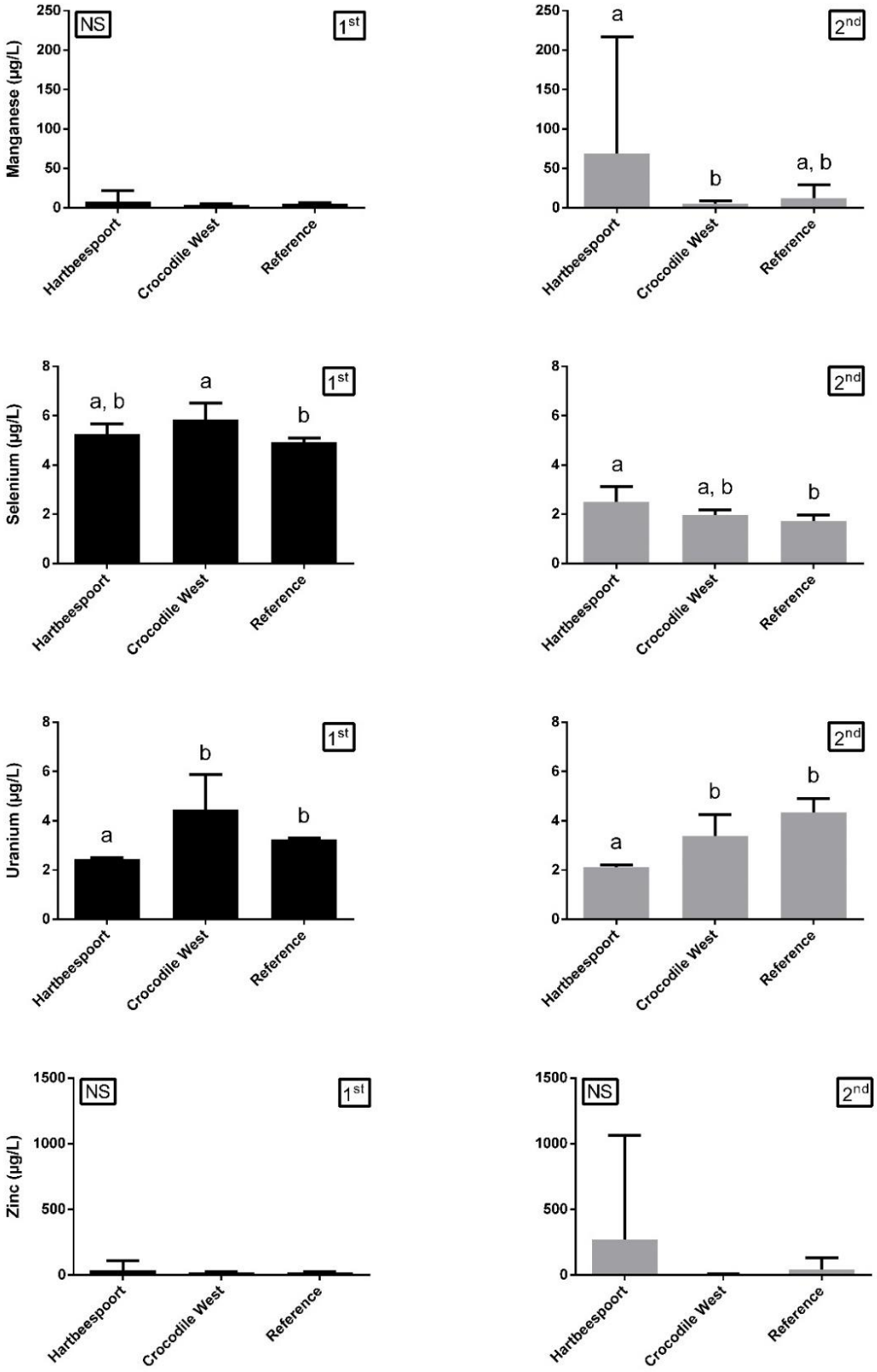

Fig. 5A. Continued.

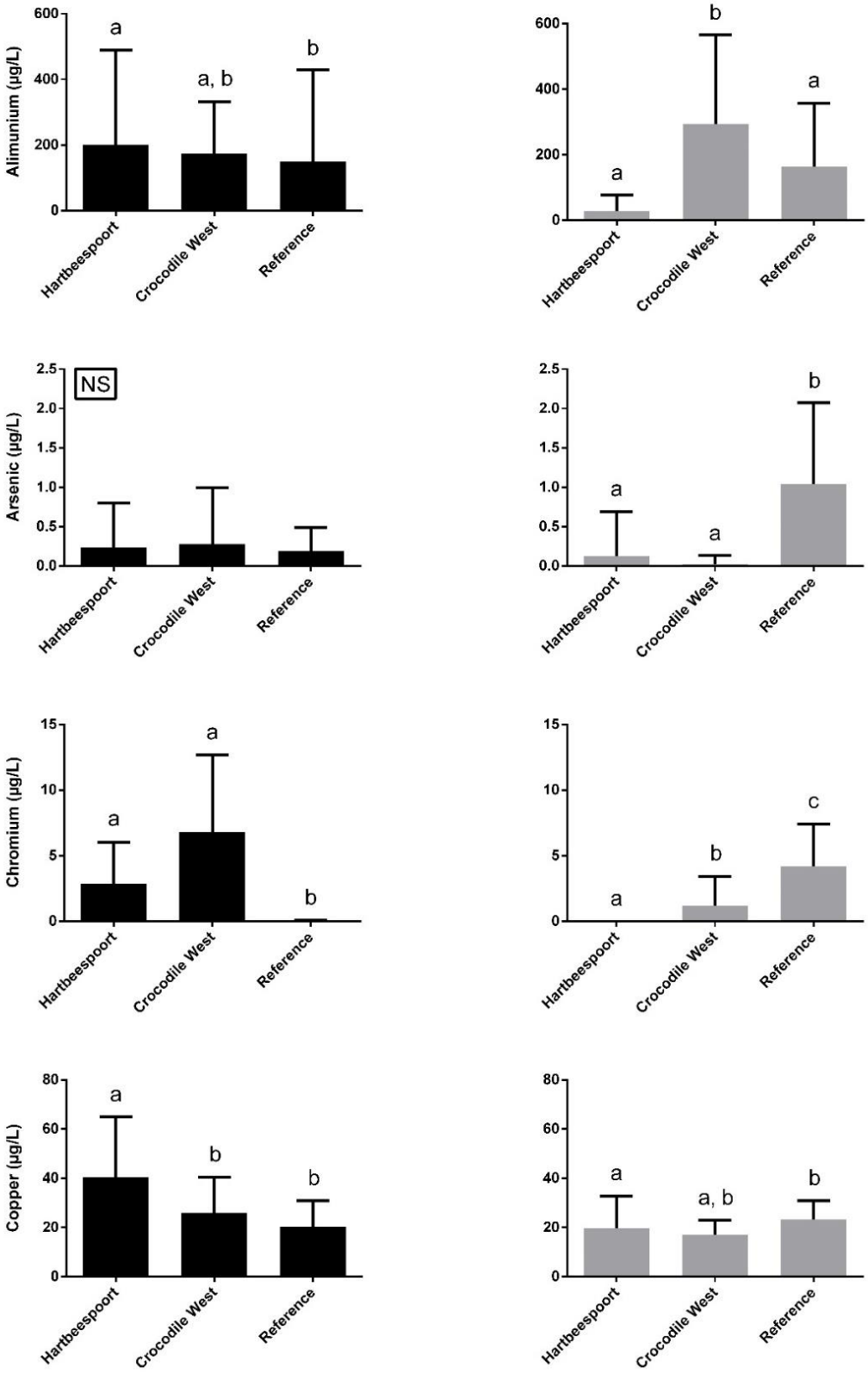

Fig. 5B. Metal concentration of soil water (capillary water that occupies soil pores) associated with the Hartbeespoort and Crocodile (West) irrigation schemes, as well as the reference system (Marico-Bosveld Irrigation Scheme), during the first and second sampling intervals. Not significant (NS) differences between means are noted, while bars with common superscript do not differ significantly. Significance was regarded at $P < 0.05$.

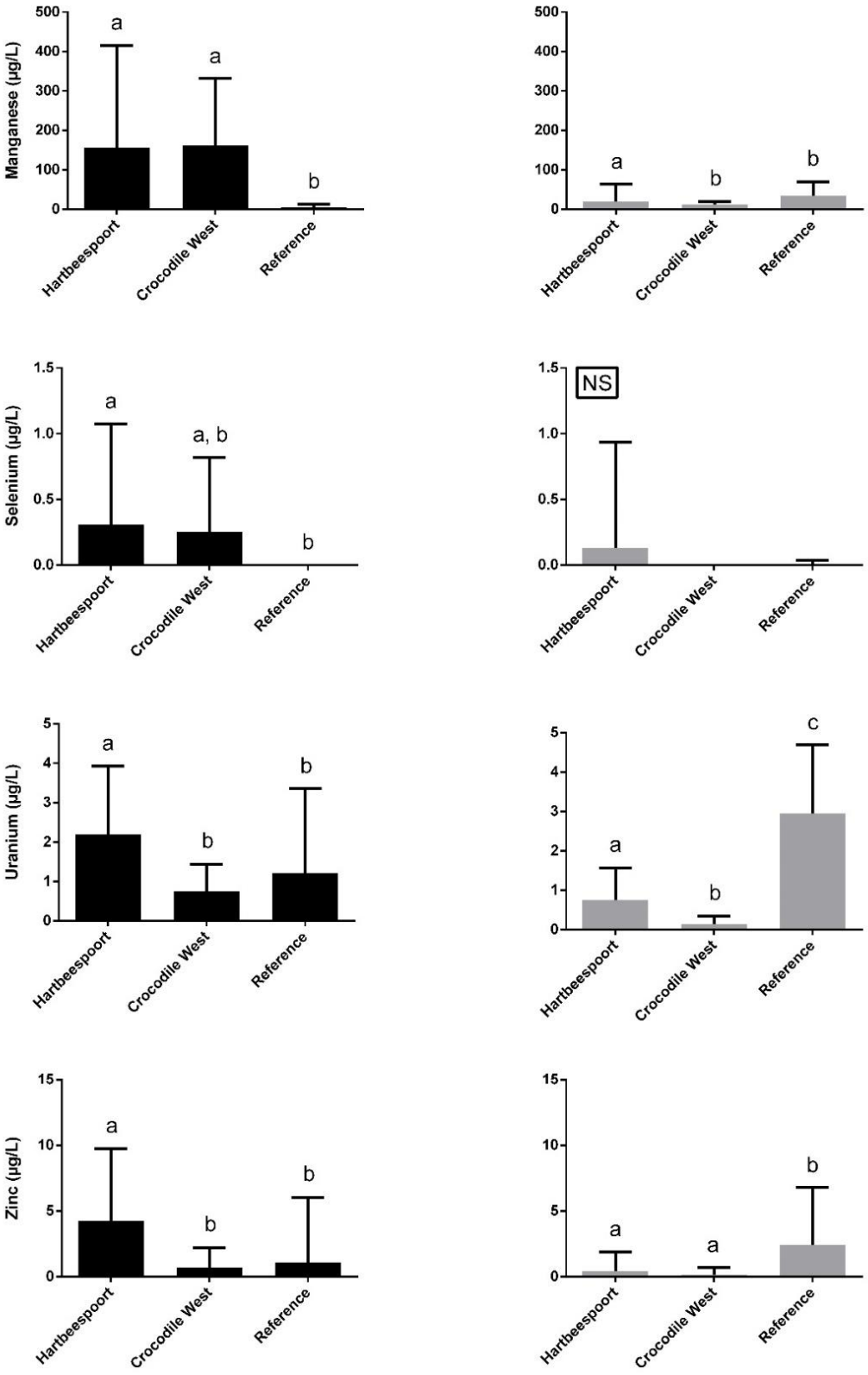

Fig. 5B. Continued.

CHAPTER 6

Conclusions and future trends

6.1 Testing of hypotheses

The aims of this study were to 1) evaluate the quality of irrigation water utilised in selected irrigation schemes associated with the Crocodile (West) and Marico catchments, 2) develop a high-throughput assessment method for evaluating the toxicity of spiked and environmental (aqueous) samples, and 3) assess the subsequent threat to the health of irrigated soils following the TRIAD approach, as part of a site-specific ecological risk assessment (ERA), with nematodes as bioindicators. This study was successful in achieving the aims and completing the associated objectives. The outcome of this study is summarised per hypothesis:

I) The Crocodile (West) Catchment has historically been subjected to anthropogenic pollution that posed a risk to crop production (yield and quality, and sustainability). The Marico Catchment, in turn, was subjected to minimal anthropogenic disturbance.

From literature (Chapter 1) and results (Du Preez et al., 2018) (Chapter 2: Article 1) reported in this study, it can be concluded that the Crocodile (West) River system (Crocodile [West] Catchment) has historically been subjected to anthropogenic pollution. Du Preez et al. (2018) showed that the Hartbeespoort and Crocodile (West) irrigation schemes were exposed to calcium sulfate enrichment and that significant differences in water quality parameters occurred between these irrigation schemes and the reference system (Marico-Bosveld Irrigation Scheme). Furthermore, specific salt ions and nutrients concentrations exceeded threshold values provided by the *South African Water Quality Guidelines for Agricultural Use: Irrigation* (DWAF, 1996) and *Australian and New Zealand Guidelines for Fresh and Marine Water Quality* (ANZECC, 2000), thus posing a risk to crop production. Therefore, the stated hypothesis is accepted.

II) The oxygen consumption rate of the bacterivore nematode *Caenorhabditis elegans* can be used as an endpoint of toxicity in high-throughput assessments.

Chapter 4 (Article 3) reported on the design and testing of a new high-throughput protocol to assess the toxic effect of specific toxicants or mixtures (aqueous environmental samples) by measuring the oxygen consumption rate (OCR) of *Caenorhabditis elegans* Maupas, 1900 after 48 h of exposure. Results produced significant concentration-response relationships following benzylcetyldimethylammonium chloride monohydrate (BAC-C16) and cadmium (Cd) exposure, respectively, allowing the calculation of effective concentration values. Furthermore, a strong, positive correlation was evidenced between *C. elegans* OCR and growth inhibition, validating oxygen consumption as a sublethal endpoint of toxicity. Subsequently, *C. elegans* OCR inhibition was used to measure the toxicity of aqueous environmental samples from the studied farmlands, which evidenced a broad OCR inhibition range. The stated hypothesis is therefore accepted.

III) Farmlands in the Crocodile (West) Catchment are at risk of soil health degradation as a result of being subjected to low quality irrigation water.

The soil quality TRIAD, as part of an ERA (Chapter 5: Article 4), consisted of the chemistry (Chapter 4: Article 2), ecology (Chapter 4: Article 2), and ecotoxicology (Chapter 5: Article 4) lines of evidence (LOEs). This approach was used to assess the risk presented to soil ecosystems associated with the Hartbeespoort, Crocodile (West), and Marico-Bosveld irrigation schemes as a result of anthropogenic disturbance. The ecology LOE, for example, utilized terrestrial, non-parasitic (beneficial) nematodes as bioindicators of soil health and showed that all the studied farmlands presented either disturbed or disrupted ecosystems. Also, inorganic nitrogen (N) content, likely influenced by the application of fertilizers, presented a strong, positive correlation to the abundance and diversity of beneficial nematodes that are indicative of enriched soils. Results from the three LOEs were integrated into the ERA framework, which concluded that irrigation water quality posed only a low risk at some of the

studied farmlands. This is largely attributed to agricultural activities resulting in soil ecosystem disturbance, enrichment of inorganic N, and soils presenting toxicity at the reference system, which was used for background correction in the calculation of risk numbers. For this reason, the stated hypothesis is rejected.

6.2 Conclusions

This study highlighted the state of irrigation water quality in the Crocodile (West) Catchment, as well as the paucity of information relating to the health of soils subjected to irrigation water in this catchment. More specifically, the lack of holistic soil health considerations in the formulation of irrigation water quality guidelines poses a threat to the health of agroecosystems and the sustainability and profitability of crop production. Although sufficient evidence was provided on the adverse impact of anthropogenic activities on irrigation water quality in the Crocodile (West) Catchment, it remained difficult to elucidate the subsequent effects on soil health. This was attributed to agricultural activities (e.g. tillage and fertilizer application) (Briar et al., 2007; Zhong et al., 2017), which likely resulted in an even greater disruption, as well as interactions between numerous abiotic and biotic factors that are impossible to measure in a single study.

Nonetheless, an important consideration is the deterioration of irrigation water quality evidenced from 2005 until 2015 at the Hartbeespoort and Crocodile (West) irrigation schemes (Du Preez et al., 2018). With a positive population growth rate (Cilliers, 2015) and the loss of high-potential arable land (Van den Burg et al., 2012), the deterioration of freshwater resources furthermore poses a severe threat to food security in South Africa. Also, severe water shortages (Donnenfeld et al., 2018) and the ineffective governance structure of the Department of Water and Sanitation do not provide the assurances needed to ensure a substantial increase in South Africa's agricultural outputs, which is vital in avoiding a food deficit (Goldblatt, 2011).

It is thus concluded that emphasis should be placed on 1) promoting the conservation and sustainable use of freshwater resources, 2) better management of human, urban, industrial, and agricultural wastes and runoffs, and 3) employing better soil health practices and monitoring systems in agricultural landscapes to promote crop yield and quality and ensure sustainable crop production for a growing population. Lastly, irrigation water quality guidelines should be adjusted to also include soil ecosystem health as a measured endpoint.

6.3 Future trends

The farmlands studied as part of this research undertaking were subjected to conventional agricultural practices, which likely led to the evidenced disruption of soil ecosystems (Ito et al., 2015; Zhong et al., 2017). Conservation agriculture, in turn, has been shown to promote both physical (Swanepoel et al., 2017) and ecosystem soil structure (Zhang et al., 2017; Zhong et al., 2017). Therefore, future studies aimed at investigating the effect of water quality on irrigated soil health should consider identifying farmlands subjected strictly, for an extended period of time, to conservation agricultural practices. This will possibly allow the effect of water quality on irrigated soil health to be studied with minimal additional factors contributing to ecosystem disturbance. Furthermore, a multi-season comparative study during which fields under conventional and conservation agricultural practices are subjected to low quality irrigation water and the subsequent effect on soil health studied, would likely be the best scenario.

Furthermore, elucidating the effect of irrigation water quality can be investigated in farmlands or trials subjected to a specific type and/or source of pollutant. The soil quality TRIAD approach, for example, has been successfully used to study the effect of metals sourced from metal industry sites on soil health (Li et al., 2018; Ribé et al., 2012). A suitable area for such an undertaking is the Delmas, Ogies, and Leandra districts (Mpumalanga, South Africa), which host some of South Africa's most arable soils where maize is dominantly grown, while also being exploited for coal mining (Delport et al., 2015; Van den Burg et al., 2012). When this

area had been studied using satellite imagery, it became clear that many irrigated farmlands are located directly adjacent to coal mines. According to Van den Burg et al. (2012) a subsequent cause for concern is the effect of soil acidification and pollution as a result of the utilization of acidified (and metal polluted) irrigation water, as well as the deposition of coal dust. Farmers have reported typical maize yield reductions of 1.5 – 2 t/ha at farmlands adjacent to coal mines (Van den Burg et al., 2012). By first conducting preliminary abiotic tests on the irrigation water, it will be possible to identify and determine the concentrations of pollutants applied to soils. An additional consideration is to test the efficacy of phytoremediation (via hyperaccumulator plant species) (Mahar et al., 2016) and chemophytostabilisation (following addition of sewage sludge and/or inorganic additives) (Grobelak and Napora, 2015) in order to lower pollutant concentrations and bioavailability, ultimately improving soil health.

A final consideration is the transport and availability of a pollutant (e.g. metal) from irrigation water to the soil environment. Although soil water (capillary water that occupies soil pores) is regarded as being representative of the bioavailable fraction of pollutants, other fractions can also be analysed. For example, total metal concentrations will provide additional information on the extent to which a pollutant has been anthropogenically enriched. Another benefit of total extractions is the availability of soil screening values, which can then be used, as part of an ERA, for lower tier assessments (ISO, 2017; Jensen et al., 2006). Soil screening values are also available for mild solvent extraction methods (Jensen et al., 2006).

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APPENDIX A

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Cancer Research UK, 1975. Cancer statistics reports for the UK. http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/ (accessed 13 March 2003).

Reference to a dataset:

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APPENDIX C

Proof of submission (Article 2)

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APPENDIX D

Declaration of language editing

Language editing statement

To whom this may concern,

I, Prof. Koos Janse van Rensburg, hereby declare that the thesis titled: 'Nematodes as bioindicators of irrigated soil health in the Crocodile (West) and Marico catchments' by GC du Preez has been edited for language correctness and spelling. No changes were made to the academic content or structure of this work.

Prof. Koos Janse van Rensburg

Prof. Driekie Fourie

24 May 2018

Date