HUMAN URINE-DERIVED FERTILIZERS: IMPLICATIONS TO SOIL MICROBIAL ABUNDANCE, CROP GROWTH, PHARMACEUTICAL UPTAKE AND HUMAN HEALTH

by

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Submitted in fulfilment of the academic requirements of

Doctor of Philosophy

in

Crop Science

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South Africa

December 2023

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DECLARATION 2: PUBLICATION

Migeri, S., Lawal, M. A., Hughes, J. C., Badza, T., Abafe, O. A., Martincigh, B. S., & Odindo, A. O. (2023). Uptake of selected antiretrovirals by pepper (*Capsicum annum*), radish (*Raphanus sativus*), and ryegrass (*Lolium perenne*) grown on two contrasting soils and fertilized with human urine-derived fertilizers. *Science of The Total Environment*, https://doi.org/10.1016/j.scitotenv.2023.164551

ABSTRACT

The recovery of nitrogen (N) and phosphorus (P) from human urine and their use for crop production closes the nutrient loop, thus reducing waste and lowering dependency on manufactured mineral fertilizers. However, it is unlikely that human urine-derived fertilizers (HUDF) could be produced in sufficient quantities to meet crop nutrient demands. Therefore, there is a need to develop new ways of using them in combination with mineral fertilizers. Information on using combinations of mineral and HUDF for crop production and their potential interaction effects in soils is limited. Few studies have reported the effect of HUDF on soil microbial abundance. The potential plant uptake of pharmaceuticals in human urine and the risks to human health are poorly understood. This study was undertaken to (i) evaluate the use of different combinations of mineral and HUDF on crop growth and yield and determine their interaction effects on two contrasting soils, (ii) assess the effects of HUDF on soil biological properties as indicators of soil quality, and (iii) determine plant uptake of antiretrovirals on a range of soil-plant systems.

The effects of 15 combinations of mineral and HUDF on maize (*Zea mays*) growth and yield on a high organic matter, clay loam and a sand soil were evaluated in a tunnel study. A laboratory incubation study was conducted to evaluate the effects of stored urine, nitrified urine concentrate (NUC), and dried urine on microbial biomass C and N, carbon dioxide and nitrous oxide emissions, soil enzyme activity and microbial abundance over a 60-day period. A pot experiment was conducted to evaluate the uptake of nine target antiretrovirals (ARVs) by pepper (*Capsicum annum*), ryegrass (*Lolium perenne*) and radish (*Raphanus sativus*) grown in the same two soils and fertilized with stored urine, NUC and struvite. The estimated daily dietary intake (DDI) of ARVs by consumption of the pepper and radish fertilized with stored urine was compared to Threshold of Toxicological Concern (TTC) values based on the Cramer classification tree to assess direct human exposure. Additionally, to identify and reduce risks and optimise benefits associated with the use of HUDFs in agricultural production, the World Health Organization's Sanitation Safety Planning technique was applied in a South African setting.

In the tunnel study, NUC and dried urine as N sources combined with single superphosphate as a P source showed the best performance in both soils with respect to biomass, grain yield, and yield-related variables. The results of the incubation study showed increased soil enzyme activity and microbial abundance in response to the application of dried urine and stored urine while a reduction in enzyme activity was observed in the NUC treatment. In the pot experiment, nevirapine was the only ARV detected in crops grown with NUC and struvite on both soils. Plants fertilized with stored urine absorbed lamivudine, ritonavir, stavudine, emtricitabine,

nevirapine, and didanosine. The calculated daily dietary intake for all ARVs were about 300-3000 times lower than the Threshold of Toxicological Concern values for class III compounds. The sanitation safety plan revealed minimal risk associated with urine collection and treatment and the application of HUDF.

The application of dried and stored HUDF has the potential to improve soil microbial abundance, and the effect of combinations of minerals and HUDF on crop growth is affected by soil type. Plants fertilized with human urine have the potential to absorb ARVs. However, the daily consumption of the tested crops fertilized with stored urine does not pose a health risk to the consumer. Long-term and field-based trials are recommended for future studies to understand the realistic effects of HUDFs on crop growth and soil microbial properties. Additionally, it would be beneficial to create a priority list of pharmaceuticals with the largest plant uptake potential under field settings so that research and assessment may be more focused.

ACKNOWLEDGEMENTS

First and foremost, I want to thank my supervisors Prof A.O. Odindo and Prof J.C. Hughes, who set me up unreasonably well for success in whatever I chose to pursue.

Prof. Bice Martincigh, Dr. Ovokeroye A. Abafe and Muhammad Lawal of the Chemistry Department, University of KwaZulu-Natal are acknowledged for their support and insights regarding pharmaceutical analysis.

Dr Rafaela Conz from Sustainable Agroecosystems ETH – Zürich for her support with microbial abundance analysis and answering my many, many, many questions.

The Water Research Commission for financial support.

The RUNRES project for providing a platform for resource recovery research, and RUNRES colleagues especially Mdoda Zondo, Dr William Musazura, Dr Simon Gwara and Dr Samuel Tenaw for their support.

The Soil and Plant Analytical Services Division, Department of Agriculture, KwaZulu-Natal, Cedara, Pietermaritzburg, for fertilizer recommendation rates and soil analysis.

The postgraduate students in the Department of Crop Science, with special mention of Dr Taruvinga Badza, Dr Nokuthula Hlanga, Dr Tinashe Dirwai, Dr Takudzwa Mandizvo, Mbali Gumede, Simphiwe Hlatshwayo, Anne Linda Chisa and Nqobile Nkomo without whom I'd have spent substantially more time confused and frustrated.

My friends Nqobile Nkomo, Tracy Vongai Mapfumo, Anne Chisa, Rudo Nhemachena, Phyllis Kwenda-Mwadzingeni, Edwich Nsapato-Fynegan, Patience Kumbula, and Dr Patience Shumba, Sue Yolanda Moyo, Tinaishe Moyo whose one-hour phone calls, good humour and unwavering support surround me every day.

My mother, away from home, Mrs Esther Odindo, for the many, many homemade hearty meals, great advice, and a listening ear.

My siblings Tinashe, Tracey and Simbainashe Migeri and sibling-in-law Beauty Marukutira who keep me updated on the coolest apps, videos, movies, latest gossip, and series no matter how busy I think I am.

Finally, I want to thank my husband, Jethro Marukutira. I might have been able to do this without you, but it would have sucked.

DEDICATIONS

I dedicate this work to my late father, Major P. Migeri, and my mother, Mrs O.Migeri, the two people seemingly incapable of doubting my ability to do anything.

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CHAPTER 1: GENERAL INTRODUCTION

Poor soil fertility is a significant challenge for most African farmers, as it can limit crop yields and increase the challenges in building resilient food systems. Fertilizers are one of the most used inputs to address poor soil fertility, but their use in Africa is low compared to other regions. The Africa Fertilizer Summit, held in 2006, aimed to increase fertilizer use from 8 kg to 50 kg of fertilizer nutrients ha^{-1}. Significant progress has been made since then. Africa is the only continent that has consistently increased its fertilizer use over the past ten years. Despite this, its consumption only accounts for 2% of the world's fertilizer use (Histus, 2018). However, the 50 kg fertilizer nutrients ha^{-1} declaration did not consider fertilizer use efficiency or nutrient sources such as organic fertilizers, biofertilizers and other soil amendments. Currently, the African Union, through the fertilizer and soil health summit held in June 2023, has taken the initiative to work with a wide range of stakeholders to reframe the role of fertilizers and soil health in increasing productivity, to advance Africa's food and agricultural systems and foster local innovation and technology (Vanlauwe *et al.*, 2023).

Innovative sanitation technologies that allow the recovery of plant nutrients such as nitrogen (N) and phosphorus (P) from human urine are being developed to minimise sanitation challenges and address the problems of poor soils. This aligns with the United Nation's 2030 Plan for Sustainable Development which emphasises the importance of recycling and sustainable waste management. These treatment technologies include urine storage, struvite production through precipitation, filtration and drying and urine nitrification and distillation for ammonia stabilisation. Critical reviews have summarised and analysed current research on the effects of human urine on crop production (Heinonen-Tanski and van Wijk-Sijbesma, 2005; Karak and Bhattacharyya, 2011; Alemayehu *et al.*, 2020a). The reviews reported crop productivity to be higher or comparable to mineral fertilizers and significantly higher when compared to non-fertilized or control crops. However, it is unlikely that human urine-derived fertilizers (HUDF) could be produced in sufficient quantities to meet crop nutrient demands. Therefore, there is a need to develop new ways of using them in combination with mineral fertilizers. Information on using combinations of mineral and HUDF for crop production and their potential interaction effects in soils is limited.

In addition to the recovered nutrients, pharmaceuticals and, in certain circumstances, pathogens, are not completely inactivated by the developed urine treatment technologies (Duygan *et al.*, 2021). Pharmaceuticals are of major concern to soil microbial properties as they directly inhibit biological activities and are likely to cause inhibitory effects on soil biogeochemical and biodegradation processes. Therefore, one elusive issue is how HUDF impacts soil quality. In addition to soil chemical properties, soil biological properties such as microbial biomass, enzyme activities and microbial abundance are good indicators of soil quality and productivity (Sadet-Bourgeteau *et al.*, 2019). The most sensitive parameters to possible toxicity and general soil environmental changes are soil biological properties. It is important to study these sensitive parameters to maintain agroecosystems' sustainability and ensure agricultural land productivity (Kiani *et al.*, 2017).

The presence of pharmaceuticals in HUDF raises concerns about their potential uptake by plants and their consequent effects on plant growth and development. Previous studies have demonstrated plant uptake of pharmaceuticals from human and veterinary medicines (Herklotz *et al.*, 2010; Kosma *et al.*, 2014; Wu *et al.*, 2015a; de Boer *et al.*, 2018). However, most of these studies involved spiking the growing medium with pharmaceuticals and exposing plants to unrealistic exposure concentrations (Carter *et al.*, 2014). The presence and levels of pharmaceutical compounds and their derivatives could be context-specific. For example, South Africa has the highest HIV treatment programme in the world, accounting for 20% of the global population on HIV therapy (UNAIDS, 2017). One would, therefore, expect that the excretion of antiretrovirals (ARV) and medication prescribed to HIV patients is of major concern in source-separated urine and its derived fertilizers in South Africa. Information on the potential plant uptake of ARVs from HUDF-amended soils in South Africa is limited. The accumulation of pharmaceuticals in food crops grown in human excreta-amended soils is a potential exposure route for consumers. It plays a major role in the perception and acceptance of using HUDF in crop production. Therefore, evaluating the risk associated with the consumption of crops fertilized with HUDF is important.

This study aims to gain deeper insights into the implications of HUDF on crop growth and yield, soil biological properties, plant uptake of ARVs and their potential risk to human health (Figure 1.1).

1.1 Specific objectives

1. To assess the combination effect of N and P in mineral and HUDFs on maize (*Zea mays*) production, on two contrasting soils

2. To investigate the effect of N-based HUDFs application on soil biological properties as soil quality indicators.

3. To determine the uptake of selected ARVs after application of HUDFs by pepper (*Capsicum annum)*, radish (*Raphanus sativus)* and ryegrass (*Lolium perenne)* grown in two contrasting soils and assess the risk associated with consumption of these crops.

1.2 Chapter outline

The structure of this thesis writing is similar to that of sectioned chapters and/or journal papers, so each experimental chapter has four main sections: introduction, material and methods, results and discussion, and conclusion.

Chapter 1: **General Introduction**

This chapter describes the background/context of the study, rationale and problem statement. The chapter outlines the aim, research questions and objectives of the study.

Chapter 2: **Enhancing Sustainable Agriculture: Exploring the Potential and Challenges of Human Urine-Derived Fertilizers in Crop Production**

This chapter describes sanitation technologies that allow the recovery of nutrients for crop production, with particular emphasis on their ability to eliminate pharmaceuticals. It also discusses the effects of HUDFs on crop production and soil properties. The review explains the potential uptake of pharmaceuticals by plants, factors that influence uptake and the potential risk to human health and plant toxicity. Analytical methods for pharmaceutical analysis in soils and plants are described. The chapter concludes by identifying knowledge gaps and recommendations for future research.

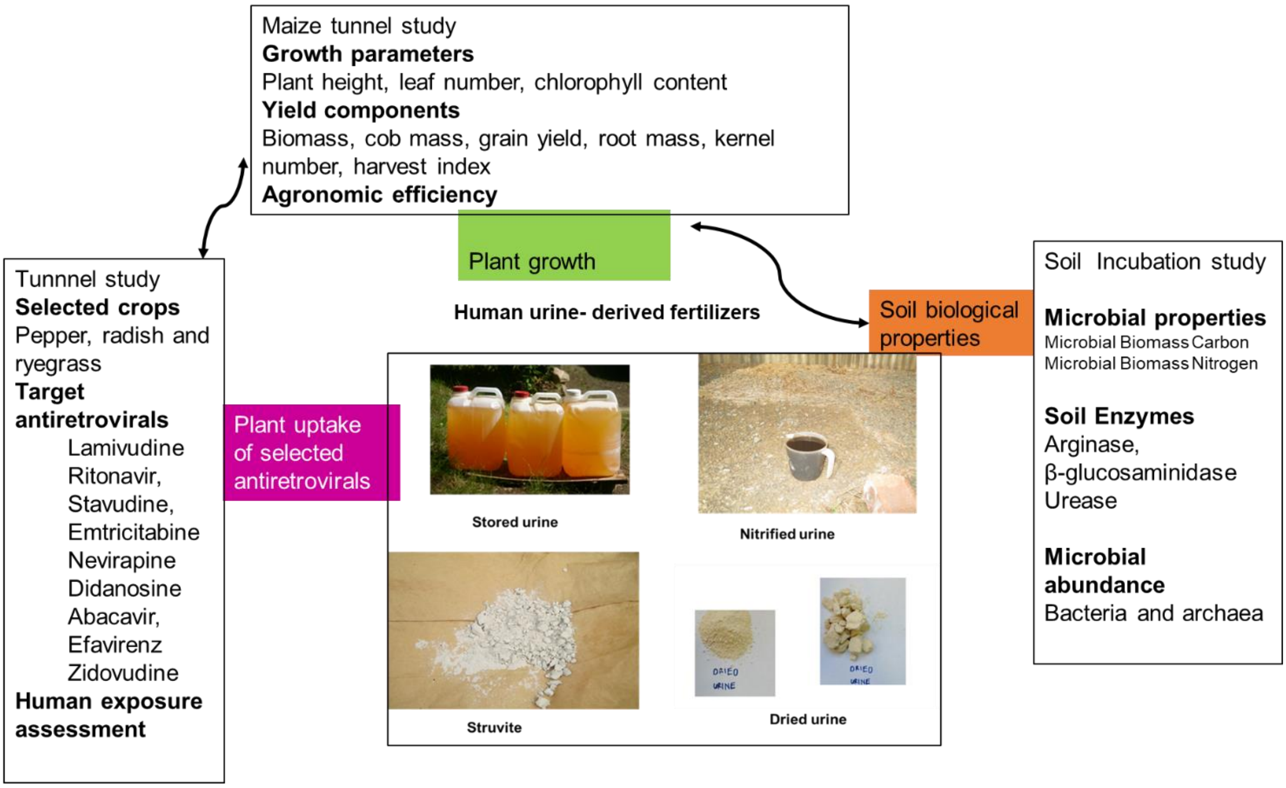

Figure 1. 1 : Outline of the studies undertaken using human urine-derived fertilzers

Chapter 3: Effects of combining human urine-derived and mineral fertilizers on maize (*Zea mays*) growth and yield performance on two contrasting soil types.

This chapter presents and explains the approach and results from a tunnel study on the combination effects of mineral (Lime Ammonium Nitrate, Single Super Phosphate (LAN, SSP) and HUDFs (dried urine, stored urine, NUC, struvite) on maize production on two contrasting soils. The study also determines the agronomic efficiency of HUDF.

Chapter 4: **Impact of Human Urine-Derived Fertilizers on Soil Quality: A Short-Term Study on Microbial Abundance, Enzymatic Activities, and Microbial Biomass Carbon and Nitrogen**

The impact of HUDFs on soil microbial properties as indicators of soil quality is reported in this chapter. An incubation study focused on three N-based urine fertilizers (stored urine, dried urine and NUC). The analysis and results focus on microbial biomass N and carbon, soil enzyme activity and microbial quantification of genes involved in the N cycle.

Chapter 5: **Uptake of selected antiretrovirals by pepper (*Capsicum annum*), radish (*Raphanus sativus*), and ryegrass (*Lolium perenne*) grown on two contrasting soils and fertilized with human urine-derived fertilizers.**

This chapter presents and explains the approach and results from experimental work on the uptake of selected ARVs in soil/plant systems. This includes analysis of target ARVs in HUDFs, urine-treated soils and crops planted in these soils. Human exposure risks associated with the consumption of HUDF-treated crops are also evaluated in this chapter.

Chapter 6: **General discussion and conclusion**

The summary, conclusion, recommendations, and future perspectives on sanitation innovations are highlighted in this chapter.

1.3 Sanitation Safety plan

The Sanitation Safety Planning methodology developed by the World Health Organisation was implemented within a South African context to minimise health risks and maximise the benefits of using HUDFs in crop production. Although this is not the main objective of this study, it was included as an appendix of the thesis to assist users in systematically identifying and managing health risks along the sanitation chain.

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CHAPTER 2: ENHANCING SUSTAINABLE AGRICULTURE: EXPLORING THE POTENTIAL AND CHALLENGES OF HUMAN URINE-DERIVED FERTILIZERS IN CROP PRODUCTION

ABSTRACT

Nutrient recovery from human urine promotes sustainable food production while benefiting the environment by reducing the quantity of untreated waste deposited into the environment. However, in some cases, urine treatment techniques cannot completely remove pharmaceuticals. Therefore, their extensive use in agriculture can pose new and significant challenges. This review (a) synthesizes the current state of knowledge on urine treatment technologies with an emphasis on their ability to remove pharmaceuticals, (b) highlights the effectiveness of different human urine-derived fertilizers (HUDFs) in crop production, (c) discusses the potential plant uptake of pharmaceuticals from various growth media, (d) reports the range of analytical approaches for pharmaceutical determination in plants and (e) provides recommendations for future research. The review highlights the need to combine HUDF and mineral fertilizers to meet crop nutrient requirements and evaluates the agronomic efficiency of HUDF. The presence of pharmaceuticals in HUDF warrants investigation into their impact on soil biological properties as an indicator of soil quality. Plant uptake studies have explored spiked soils, biosolids, and treated wastewater as sources of various pharmaceuticals in soil-plant systems. However, considering the pharmaceutical excretion route, HUDFs are an important source. Antiretroviral drugs (ARV) are a significant pharmaceutical class to consider for plant uptake studies in South Africa because the country has the highest use of ARV therapy in the world. Future research on the impact of HUDFs on plant productivity should focus on pharmaceutical uptake, field trials, and crops other than short-season crops, such as fruit trees. It would be beneficial to create a priority list of pharmaceuticals with the most significant plant uptake potential under realistic field settings so that research and assessment may be more focused.

Keywords: agronomic efficiency, human urine-derived fertilizers, pharmaceuticals

2.1 INTRODUCTION

The use of human excreta for crop production has been a common practice in certain countries for centuries. Most practices and experience using human excreta in agricultural systems are found in India, China, Japan, Korea, Nepal, Vietnam and Thailand (Cooke, 1986). According to Wolgast (1993), one person produces approximately 5.7 kg of nitrogen (N), 0.6 kg of phosphorus (P) and 1.2 kg of potassium (K) per year in the form of excreta. Urine contains about 90% of the N, 50–65% of the P, and 50–80% of the K, while most organic matter is contained in the faeces. The composition of excreta varies depending on diet, physical activities, and body size. The renewed interest in human excreta can be attributed to concepts such as sustainable agriculture, the circular economy and climate change mitigation. In addition, sanitation challenges, especially in developing countries, have provided an opportunity to develop innovative sustainable sanitation technologies linked to agriculture.

Using human urine and its derived products as fertilizers provides sustainable options for managing waste, and minimising environmental pollution and contamination of surface and groundwater bodies. The importance of recycling and sustainable waste management has been emphasised by the United Nations (Leal Filho *et al.*, 2019). Several sanitation technologies to treat human urine for use in agriculture have been developed in line with this global imperative. Urine treatment technologies have been reviewed extensively (Simha and Ganesapillai, 2017; Martin *et al.*, 2020; Patel *et al.*, 2020). The reviews highlight the need for an integrated technology design strategy to ensure nutrient recovery, inactivation of pathogens, and reduction or removal of pharmaceuticals.

Recently, there has been concern over introducing pharmaceuticals to the environment through urine-derived fertilizers. This could result in the uptake and accumulation of pharmaceuticals in plants and soil, posing risks to human and environmental health. Studies have demonstrated the uptake and accumulation of pharmaceuticals by plants. However, in most cases, the plants were exposed to unrealistic concentrations (Carter *et al.*, 2014). Also, differences in experimental design and analytical methodologies make comparing studies difficult. The procedures to measure the uptake of each of the hundreds of pharmaceuticals currently used are expensive. Therefore, models to forecast contaminant accumulation in crops are being developed.

This review aims to (a) synthesize and summarize the current understanding of urine treatment technologies with emphasis on their ability to eliminate pharmaceuticals, (b) highlight the effectiveness of different urine-based fertilizers in crop production, (c) discuss the potential for uptake of pharmaceuticals by plants in different growth media and (d) summarize the range of analytical approaches for pharmaceutical and metabolite determination in plants.

2.2 SANITATION TECHNOLOGIES

Several sanitation systems (both waterborne and waterless) have been designed, providing opportunities for the recovery of nutrients needed for crop production (Udert *et al.*, 2015). These include Urine Diversion Toilets (UDT), the Decentralized Wastewater Treatment System (DEWATS), and new designs tested under the Bill and Melinda Gates Foundation (BMGF) “Reinvent the Toilet Challenge” programme.

The concept of UDT is based on the principle of urine separation from faeces, designed to collect and drain urine from the front area of the toilet while faeces drop through a large opening in the back. The process imitates the human anatomy, where urine and faeces go through different pathways and are excreted separately. These toilets are designed according to anal cleansing processes to ensure that faecal matter does not block the urine area and urine does not spray into the faecal matter compartment. There are two types of UDTs, the urine dry diversion toilet (UDDT) and the urine diversion flush toilet (UDFT). The latter uses water for flushing, while the former does not use water.

In UDDTs, materials such as sawdust, lime, soil, and ash are added to the faecal compartment after defecation to promote drying, and pathogens die off and raise pH (Winblad *et al.*, 2004). There are three types of UDDTs: single vault, double vault and tecpan UDDTs. The single vault is characterised by a single compartment for faecal collection and storage; secondary treatment of the faecal matter is required for this type of UDDT. The double vault has twin-pit compartments, used alternately. When one vault is full of faecal matter, the compartment is sealed, and the other is used. The tecpan has metal sheets as vault chamber doors, exposed to the sun, to absorb and transfer heat into the vaults and speed up the drying process. This is a popular model in Central and West Africa distributed by Centre Régional de l’Eau Potable et l’Assainissement à faible couts (CREPA), an organisation working in Benin, Burkina Faso, Burundi, Cameroon, Central African Republic, Congo, Côte d'Ivoire, Gabon, Guinea Bissau, Guinea, Mali, Mauritania, Niger, Rwanda, Senegal and Togo to improve water, sanitation, and hygiene in poor communities (Laré-Dondarini, 2015).

Sanergy, a Nairobi-based initiative, promotes using UDDTs under the name Fresh Life Toilets (FLT). The facilities allow excreta to be stored in sealed cartridges, ensuring that foul odours and flies are not a problem, and making it hygienic for the surrounding community. Sanergy franchises FLTs to local informal settlement residents who run them as businesses. The enterprise supports FLT owners by providing business training and guaranteed waste collection while the owners are committed to cleaning the toilets and keeping them open. Properly equipped Sanergy staff collect excreta daily. Full cartridges are gathered from facilities using wheelbarrows, handcarts and/or trucks, depending on ease of access. Approximately 1 134 FLTs are in the informal settlements of Nairobi (Auerbach, 2016). This initiative has provided decent sanitation to over 50 000 people, and about 2 500 metric tonnes of waste were safely removed from Nairobi informal settlements in 2017. It has also reduced the use of 'flying toilets', where a plastic bag is used to collect human faeces, tied and discarded on the roadsides or thrown as far away as possible.

The eThekwini municipality in KwaZulu-Natal (KZN), South Africa, has installed approximately 85 000 UDDTs since 2002. These toilets serve about 500 000 residents in the peri-urban areas of Durban, and they are provided by the municipality as permanent assets to households and communities (Roma *et al.*, 2013). The UDDTs were installed due to cost and environmental impact considerations (Gounden *et al.*, 2006).

The UDDT technology is a sustainable approach to sanitation challenges because it can function without water and provide opportunities for nutrient recovery. However, using UDDTs depends on the willingness and acceptance of the residents. Institutions responsible for installing UDDTs should involve the communities in awareness programmes on the proper use and maintenance of UDDTs and waste disposal before installation. This is especially so because the technology is viewed by UDDT users as inferior as it is mainly installed in low-income and informal settlements. Duncker and Matsebe (2008) suggested targeting middle and high-income earners to promote the technology through the eco-village concept to create some "status" for the technology. Furthermore, many UDDT users in South Africa were dissatisfied with the technology due to the householder's responsibility of emptying the toilets (Mkhize *et al.*, 2017).

Human excreta collected from the UDDTs is transported to wastewater treatment facilities, while in most cases, urine is channelled into a soak-away pit constructed below the ground. Sometimes, it is used for crop production or treated to produce plant nutrient sources and soil conditioners. Urine has a higher potential for use as fertilizer because it is usually regarded as sterile; pathogens detected in urine result from contamination from faecal matter or menstrual blood (Carr and Strauss, 2001). The treatment systems and their products are described below, emphasising human urine-derived fertilizers (HUDF).

2.3 HUMAN URINE-DERIVED FERTILIZERS

The use of human excreta in agriculture has recently focused on nutrient recovery from source-separated excreta. The Bill and Melinda Gates Foundation has played a significant role in the rapid increase of sanitation technology studies through the "Reinvent the Toilet Challenge". The Toilet Challenge has been working with leading engineers and scientists since 2011 to develop low-cost toilets that do not need electrical or water connections and can transform human waste into valuable resources. According to Winker *et al.* (2009), human urine is the most researched and promising product of sanitation technologies. Human urine is generally treated by concentrating nutrients by eliminating water from the urine or selectively extracting nutrients. Nutrient concentration techniques include membrane distillation, nitrification distillation, and forward osmosis, whereas nutrient extraction is accomplished through adsorption, ion exchange, stripping, and precipitation (Simha *et al.*, 2020). Scientific developments and technological advancements in safely recovering nutrients from human urine have been described in detail and well summarised (Simha and Ganesapillai, 2017; Alemayehu *et al.*, 2020a). Hence, the following section will focus on the effectiveness of HUDFs in crop production.

2.3.1 Stored urine

Urine collected from the UDDTs intended for crop production must be appropriately stored for pathogen die-off. The World Health Organisation recommends storage for at least six months at 20 °C or higher for safe use in crop production (World Health Organisation, 2006). During urine storage, the pH increases due to urea hydrolysis, increasing the ammonia concentration. Ammonia, a biocide against pathogenic microorganisms, inactivates viruses by attacking the viral genome (Decrey *et al.*, 2015). However, most pharmaceuticals do not hydrolyse, even though urine storage inactivates some pathogens (Bischel *et al.*, 2015b). Moreover, long-term urine storage results in a foul smell and loss of nutrients through volatilization.

The use of urine in crop production has been the focus of several studies in different geographical locations (Table 2.1), with most studies conducted in Finland, South Africa, Ethiopia, Ghana, Nepal, and Nigeria. Some studies have also been conducted in Brazil, India, Turkey, Uganda, the USA, and Zimbabwe. These studies represent various environmental conditions and, most importantly, populations with different diets that affect urine nutrient content. The database includes field studies (58%) and pot trials (42%), with the majority (66%) of studies published between 2010 and 2020. The studies that present soil information were conducted on soils with a wide range of soil pH (4.7-7.3) with most conducted in the pH range of 6.1-7.1. Some studies have excluded soil information (AdeOluwa and Cofie, 2012;

Alemayehu *et al*., 2020b; Heinonen-Tanski and van Wijk-Sijbesma, 2005; Kocatürk *et al*., 2012; Ranasinghe *et al*., 2016). The most common plants used were vegetables (cucumber, cabbage, tomato, sweet pepper, amaranth, okra, beetroot, and spinach) due to their high nutrient demand and short growing season.

Table 2.1 shows that several urine sources have been investigated, including household UDTs, male urinals, and mobile public toilets. Educational institutions (primary schools and universities) were significant urine sources because of the large population densities in a confined area. Therefore, larger quantities of urine could be collected in a short period. The storage time for the urine ranged from one week to 6 months. These studies show that human urine significantly increases plant productivity compared to non-fertilized soils. However, most of these studies lack information on one or more soil conditions, urine quality, and application rates, making comparing them difficult. Furthermore, information on the potential crop uptake of pharmaceuticals from soils treated with urine is limited.

Table 2. 1: Studies on the effects of human urine on plant productivity under different conditions

Country of study	Urine source	Crop	Application rate	Urine composition	Study type	Soil information	Effect on crop productivity	Reference
Brazil	University of São Paulo male urinals, stored 1 week	Lettuce Maize	125 mL pot^{-1} 400 mL pot^{-1}		Greenhouse	pH: 6.5	Urine treatment substantially improved growth and leaf development in maize and lettuce experiments compared to control plants.	(Chrispim *et al.*, 2017)
Ethiopia	Kotebe Metropolitan University (KMU) toilets, stored for 3 months	Cabbage	1.5 L pot^{-1} 0.75 L pot^{-1} 0.36L pot^{-1} 0.31 L pot^{-1}	N: 2.8 g L^{-1} P: 0.18 g L^{-1} K: 1.27 g L^{-1}	Greenhouse	Sa:21%, Si: 23.1%, Cl: 56.6%, pH: 6.2	Urine, when diluted three times, provides nutrients that can be used to replace synthetic fertilizer.	(Kassa *et al.*, 2018)
Ethiopia	UDDTs	Maize	500 mL pot^{-1} 800 mL pot^{-1} 1000 mL pot^{-1} 1200 mL pot^{-1}		Greenhouse	N/A	Using urine as a fertilizer improved maize production while raising the bottom soil's salt content.	(Alemayehu *et al.*, 2020b)
Finland	Household UDTs, stored for 6 months	Cucumber	9.7 L m^{-2}	N: 2.4 g L^{-1} P: 2.3 g L^{-1} K: 0.6 g L^{-1}	Field	Clay loam	Urine-fertilized cucumber yield was slightly higher than the chemical fertilizer-treated crops.	(Heinonen-Tanski *et al.*, 2007)
Finland	Household UDTs, stored for 6 months	Cabbage	10.2 L m^{-2}	N: 0.93 g L^{-1} P: 0.63 g L^{-1} K: 0.36 g L^{-1}	Field	Silty Clay loam pH: 6.4	Maximum growth was achieved earlier in the urine-fertilized crops	(Pradhan *et al.*, 2007)
Finland	Household UDTs, stored for 6 months	Tomato	81 mL pot^{-1}	N:8.4 g L^{-1} P:0.7 g L^{-1} K:2.0 g L^{-1}	Greenhouse	Peat: Sand mix pH: 6.6	Tomato fruit yield in mineral-fertilized plants was the same as the urine-fertilized plants.	(Pradhan *et al.*, 2009)
Finland	Household UDTs, stored for 3 months	Pumpkin		N: 8.17 g L^{-1} P: 0.65 g L^{-1}	Field	pH: 7.1	In contrast to mineral-fertilized plots, urine-fertilized plots produced lower pumpkin yields.	(Pradhan *et al.*, 2010b)
Finland	Household UDTs, stored for 6 months	Red beet	0.14 L m^{-2}	N: 8.4 g L^{-1} P: 2.3 g L^{-1} K: 3.0 g L^{-1}	Field	pH:7.3	The growth rate and biomass of mineral-fertilized plants and urine-fertilized plants did not differ statistically.	(Pradhan *et al.*, 2010a)
Finland	Male urinals and household UDT, stored for 6 months	Barley	19200 L ha^{-1} 37200 L ha^{-1}	N: 2.8 g L^{-1} P: 0.16 g L^{-1} K: 0.47g L^{-1}	Field	pH: 6.5	The barley grain and straw yield grown with urine fertilizer was equivalent to the yield in mineral fertilized plots.	(Viskari *et al.*, 2018)
Ghana	Male urinals in Accra, stored for 1 month	Cabbage			Field	pH: 7.1	Urine application can improve cabbage yields, nutrient absorption, and	(Amoah *et al.*, 2017)

							nitrogen and phosphorous content in the soil.	
Ghana	Valley View University urinals	Sorghum		N: 4.8 g L^{-1} P: 0.1 g L^{-1} K: 1.4 g L^{-1}	Field	Sa:67.3%, Si:23.3%, Cl:9.4%, pH: 4.7	In rain-fed grain sorghum cultivation, a combination of urine, TSP, and KCl will replace NPK compound fertilizers.	(Germer *et al.*, 2011)
India	N/A	Banana	N/A		Field	N/A	The urine-fertilized banana yield was lower than the mineral-fertilized plants.	(Sridevi *et al.*, 2009)
Nepal	Mobile public toilets	Sweet pepper	N/A	N:0.73% P:0.42% K:2.0%	Field	Sandy loam pH: 5.7, OC:6.0%	Yield from mineral and urine treatments was comparable	Shrestha et al.2013
Nepal	Shree Gyan Jyoti Primary School urinals and household urinals, stored for 2 weeks	Radish Potato Mustard Cauliflower Cabbage		N: 2.9 g L^{-1} P: 3.1 g L^{-1} K: 9.5 g L^{-1}	Field	Sa:10.9%, Si:9.9%, C: 79.3%, pH: 4.7	Urine fertilizer generated significantly higher mustard biomass and marginally higher or the equivalent of other crops than manure.	Pradhan et al. (2011)
Nigeria	University of Ibadan male urinals	Amaranth			Field	pH:4.8	In both primary and residual plantings, 100% urine worked better as a fertilizer.	(AdeOluwa and Cofie, 2012)
Nigeria	ECOSAN toilets are stored for 2months	Okra	10 000 L ha^{-1} 15 000 L ha^{-1} 20 000 L ha^{-1}	N:4.4 g L^{-1} P:0.23 g L^{-1} K:0.94 g L^{-1}	Greenhouse and Field	Site A: Sa 81%, Si 14.7%, Cl 4%, pH 6.0, OC 0.92% Site B: S 81%, Si 13.7%, C 4%, pH 6.4, OC ,1.16%	Urine and mineral fertilizer treatments enhanced pod yield relative to control. Yield attributes of okra in 20,000 L urine/ha treated plots outperformed mineral fertilizer treated plots.	(Akpan-Idiok *et al.*, 2012)
Sri Lanka	Male urinals	Bushita bean			Greenhouse		In crop production, urine can be used as an efficient nitrogen fertilizer substitute.	(Ranasinghe *et al.*, 2016)
South Africa	University of Fort Hare male hostel urinals	Beetroot Carrot Maize Tomato	20 mL pot^{-1} 40 mL pot^{-1} 80 mL pot^{-1} 160 mL pot^{-1}		Greenhouse	Sandy clay loam pH:6.3	Using high rates of human urine resulted in high sodium accumulation in plant tissues.	(Mnkeni *et al.*, 2008)
South Africa	University of Fort Hare male hostel urinals	Spinach	80 mL pot^{-1}	N:0.74% P:0.03% K:1.62%	Greenhouse	Sandy clay loam pH:6.3	Application of urine increased fresh and dry matter yields of spinach as a result of higher N and P uptake.	(Kutu *et al.*, 2011)
South Africa	Community UDTs	Rye grass	15.25 mL pot^{-1}	N:0.465 g L^{-1} P:0.231 g L^{-1}	Greenhouse	Soil A: Cl 11%, pH: 4.0, OC 0.5% Soil B: Cl 23%, pH: 4.11, OC 6%	Urine-treated rye grass had significantly higher dry matter than the control but was comparable to mineral-treated plants.	(Mchunu N *et al.*, 2018)
Turkey	Source-separated urine samples were	Mixed grass	2560 mL pot^{-1} 317 mL pot^{-1} 113 mL pot^{-1}		Greenhouse	Peat and perlite	Urine-treated pots had significantly lower yields than mineral fertilizer-treated pots.	(Kocatürk and Baykal, 2012)

	collected from 20 individuals.							
Uganda	Households UDTs, stored for 2 weeks	Maize	32 608 L ha^{-1}	N:2.3 g L^{-1}	Field	N/A	The use of urine has a beneficial effect on maize yields.	(Andersson, 2015)
USA	UDTs, stored for 1 month	Beans Turnip	18 797 L ha^{-1} 19645 L ha^{-1}	N: 5.9 g L^{-1} P: 0.43 g L^{-1} K: 1.96 g L^{-1}	Field	pH: 5.50	Snap beans and turnips can be grown with urine, with better yields over the no-fertilizer and comparable yields to synthetic fertilizer.	(Pandorf *et al.*, 2019)
Zimbabwe	EcoSan toilets	Maize	N/A	N/A	Field	N/A	Urine improves maize crop production and water productivity in rainfed agriculture.	(Guzha *et al.*, 2005)

N/A: Not available in manuscript: Cl: Clay, S: Silt, Sa: Sand, OC: Organic carbon, UDT: Urine diversion toilet, N: Nitrogen, P: Phosphorus, K: Potassium, TSP: Triple superphosphate, KCl: Potassium chloride

2.3.2 Alkaline dehydrated urine

Senecal and Vinnerås (2017) developed a method for increasing N concentration (from 0.6 to > 6 %) in urine by dehydrating it, resulting in a dry powder with 7.8 % N, 2.5 % P, and 10.9 % K by weight. The method is intended for use in a container-based sanitation system that collects, processes, and decreases the volume of urine within the container. At 35 °C, fresh urine is added to wood ash at varied intervals to alkalise and inhibit the enzyme urease from hydrolyzing urea to ammonia. Alkalised biochar can also be used for the same purpose and to increase the pH of biochar to ≥12.5, potassium hydroxide pellets and biochar are mixed in a weight ratio of 1:4, deionised water is added, and the mixture is set aside for 1 h to ensure uniform dissolution of the pellets. However, using alkalised biochar could affect the financial sustainability of the system and its implementation on a larger scale. Simha *et al.* (2020) suggested using an anion exchanger to stabilize the urine before passing it through wood ash or alkalised biochar. Sanitation 360, a Swedish company, has partnered with Scandinavian Water and Sanitation and Sanitation Ambassadors to assess the market potential of manufacturing and selling urine-drying technology in South Africa. The project is running until May 2024. The long-term objective is to have a certified product via a process that enables a circular economy to be built and monitored within the sanitation sector.

2.3.3 Struvite

Struvite is a magnesium, ammonium phosphate ($MgNH_4PO_4.6H_2O$) produced through urine precipitation, filtration and drying (Udert *et al.*, 2003; Li *et al.*, 2019a). Struvite crystals form after adding a magnesium source, followed by filtration to separate the crystals from the liquid and, finally, dry the crystals to remove all the liquid. About 91% of the P but less than 4% of the N is precipitated from the urine, which makes struvite a P source (Etter *et al.*, 2015). Ostara, a struvite-producing company in Belgium, uses the same technology to make struvite from wastewater. During controlled struvite precipitation, P is recovered. Magnesium and dewatering liquors are added to the reactor to produce a pure fertilizer marketed as Crystal Green (Nagy *et al.*, 2019b). Multiform Harvest Inc. in the United States of America, NuReSys in Belgium, Sustec and Paques in the Netherlands, and Suez and Veolia in France are other struvite producers.

A meta-analysis on the agricultural potential of struvite as a P fertilizer (Hertzberger *et al.*, 2020) identified 59 publications, most of which were conducted in greenhouses. The meta-analysis included studies that produced struvite from various sources, including human urine, cow urine, wastewater treatment plants, and swine aerobic reactors. The study concluded that the effectiveness of struvite as a P fertilizer is influenced by soil pH and its contribution to total

N. This was in line with a more focused review on using struvite made from human urine in agriculture (Nagy *et al.*, 2019b), which concluded that struvite is an effective slow-release fertilizer. Antonini *et al.* (2012) conducted an agronomic study with six urine-derived struvite fertilizers. The fertilizers differed in P percentage, the temperature at which the precipitates were dried, and the sources where urine was collected before treatment. The study concluded that urine-derived struvite is a valuable P source that promotes substantially higher P uptake and plant biomass than mineral fertilizers (Cedaran phosphate fertilizer).

A study conducted in the Netherlands on the efficacy of struvite for maize growth found that struvite is an efficient P fertilizer (Gell *et al.*, 2011). A nutrient uptake study conducted by Bonvin *et al.* (2015) on struvite processed from synthetic urine, labeled with ^{33}P radioisotope and stable ^{15}N isotope, determined that ryegrass recovered 26% of the P and 75% of the N from the applied struvite. Given the observed positive effects of struvite, urine-based struvite has a high potential to reduce crop production reliance on finite phosphate rock-derived fertilizers. A study by Ronteltap *et al.* (2007) showed that 98% of each of the seven pharmaceuticals analysed during struvite production remained in the urine after precipitation. Pharmaceuticals that remain after precipitation are not treated during the filtration and drying (Bischel *et al.*, 2015b). Therefore, pharmaceuticals in struvite used for crop production remain a potential threat to human and environmental health.

2.3.4 Nitrified urine concentrate

Nitrified urine concentrate (NUC) is produced through biological nitrification and distillation processes. The nitrification process stabilizes the collected urine, oxidising it into non-volatile nitrate (NO_3^-); the other is stabilized as non-volatile NH_4^+ (Udert *et al.*, 2016). Distillation removes Water from the nitrified urine, resulting in a concentrated nutrient stream. There has been little evaluation of NUC as a fertilizer due to its recent development. Bonvin *et al.* (2015) determined N and P uptake of ryegrass from NUC processed from synthetic urine and labelled with ^{33}P and ^{15}N. The ryegrass recovered 75% of the applied N, and the study concluded that NUC is a valuable N source. A significant increase in biomass was reported in a more realistic study where NUC processed from real human urine was used for ryegrass production, primarily when a split application was used (Mchunu N *et al.*, 2018). Nitrified urine concentrate has also been used in hydroponic studies to cultivate lettuce (Mauerer *et al.*, 2018) and tomatoes (Magwaza *et al.*, 2020). Tomato plants fertilized with NUC showed increased chlorophyll content and nutrient uptake, while lettuce yield was comparable to plants with mineral fertilizer-treated plants.

The survey results in Msunduzi, KZN, South Africa, were used to determine public approval of NUC use (Wilde *et al.*, 2019). The results showed that people were much more optimistic about using NUC as a soil amendment than raw urine. In 2018, the Swiss Federal Office of Agriculture released a permit to distribute NUC as a fertilizer for edible plants. It is being marketed as Aurin by VUNA GmBh, a Swiss Federal Institute of Aquatic Science and Technology (EAWAG) spinoff (EAWAG, 2020). Experiments conducted by Duygan *et al.* (2021) on the fate of pharmaceuticals during urine treatment revealed that of the 12 pharmaceuticals studied, only atazanir, clarithromycin, darunavir and ritonavir were completely inactivated in the nitrification tank. The addition of powdered activated carbon as a post-nitrification process has been successfully tested to remove approximately 90% of pharmaceuticals (Köpping *et al.*, 2020).

2.4 AGRONOMIC EFFICIENCY OF HUMAN URINE-DERIVED FERTILIZERS

In addition to the increase in crop yield, agronomic efficiency (AE), the increase in crop yield per unit of fertilizer applied (Fairhurst, 2012), is another key indicator of fertilizer use efficiency. It is calculated to assess the efficiency of the applied N and P sources. Agronomic efficiency comprises capture efficiency (the percentage of nutrients absorbed) and conversion efficiency (the yield produced per amount of nutrients absorbed). Factors influencing capture efficiency include sufficient nutrients and plant requirements such as soil moisture, aeration and physical support, while genotypic properties regulate conversion efficiency (Giller *et al.*, 2006). A meta-analysis conducted by (Vanlauwe *et al.*, 2011) evaluated the impact of the Integrated Soil Fertility Management (ISFM) system on the AE of fertilizer N (N-AE) applied in maize-based systems in sub-Saharan Africa (SSA). The study concluded that N-AE is improved through different ISFM components and management practices such as proper fertilizer management, improved varieties, the combined application of organic inputs and fertilizer, and adaptation of input application rates to within-farm soil fertility gradients, where these are important. The agronomic efficiency of P (P-AE) is doubled by applying N and using organic resources such as *crotalaria, tithonia and sesbania* that can replace mineral fertilizer N application and improve the use efficiency of the added P fertilizer. Although several studies have demonstrated the ability of HUDFs to increase crop yield, there is limited information on their AE.

2.5 EFFECTS OF HUMAN URINE-DERIVED FERTILIZERS ON SOIL PROPERTIES

Regardless of the high levels of nutrients in HUDF, it has been reported that pharmaceuticals and, in certain circumstances, pathogens, are not completely inactivated by the developed urine treatment technologies (Bischel *et al.*, 2015b). Pharmaceuticals are of major concern to soil microbial properties as they directly inhibit biological activities and are likely to cause inhibitory effects on biogeochemical and biodegradation processes (Patel *et al.*, 2019).

The most sensitive parameters to possible toxicity and general soil environmental changes are soil microbial properties. It is important to study these properties to maintain agroecosystems' sustainability and ensure agricultural land productivity (Kiani et al., 2017). The significance of soil microorganisms in residue breakdown, crop development and regulating biogeochemical processes, particularly the N cycle, is well established (Macdonald *et al*., 2011, Xu *et al*., 2020). The N cycle includes transformation processes such as N-fixation, denitrification, and nitrification, and is a crucial biogeochemical cycle for supporting plant growth (Stein and Klotz, 2016; Kuypers *et al.*, 2018). Ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB), particularly *Nitrobacter* and *Nitrospira*, have been reported to play a principal role in nitrification (Könneke *et al.*, 2005) while denitrification is driven by nitrite and nitrous oxide reducers (Zumft, 1997). The abundance and diversity of the microbial communities responsible for specific N transformation processes are frequently described using functional gene markers (Ouyang *et al.*, 2018). The genes encoding nitrogenase reductase (*nifH*), ammonia monooxygenase (amoA), nirK and nirS (encoding nitrite reductase) (Braker *et al.*, 1998) and nitrous oxide reductase (*nosZ*) are among the most frequently studied N cycling marker genes (Henry *et al.*, 2006).

Several factors affect microbial abundance, composition and activity in agricultural soils. Soil properties such as soil type and texture (Naveed *et al.*, 2016), moisture, pH and temperature (Szukics *et al.*, 2010) impact microbial community composition. Microbial communities are also influenced by agricultural practices such as crop rotation, tillage, and the use of cover crops and fertilizer (Sommermann *et al.*, 2018). The abundance and structure of soil microbial communities are impacted by applying both organic and mineral fertilizers (El Azhari *et al*., 2012). The chemical composition of the organic matter, particularly the C:N ratio, the quantity of organic material applied and the community present in the soil before amendment application determines the response of the microbial community structure and function (Siedt *et al.*, 2021). However, organic matter short-term experiments may not show significant changes in soil microbial structure. On the other hand, a study by Allison and Martiny (2008) revealed that 84% of the evaluated 38 studies showed a significant sensitivity of soil microbial community composition and structure to the application of mineral fertilizers.

Application of P fertilizers has a less significant impact on soil microbial communities than N fertilization. Therefore, research has focused on the effects of N fertilization (Li *et al.*, 2020). The changes in NH_4^+-N and pH induced by N fertilization are two of the most important factors affecting the structure and diversity of microbial communities (Zeng *et al.*, 2016). A meta-analysis of the long-term effects of mineral fertilizers on soil microorganisms reported that repeated application of inorganic N fertilizers increased soil microbial biomass C by 15% (Geisseler and Scow, 2014). Additionally, an increase in AOB and nosZ abundance but no effect of other genes was reported due to the application of inorganic N based on 157 observations from 47 peer-reviewed studies (Ouyang *et al.*, 2018). Soil biological properties such as microbial biomass, enzyme activities and microbial abundance are good indicators of soil quality and productivity. (Sadet-Bourgeteau et al., 2018). Therefore, it is important to understand the effects of HUDF application on soil biological properties.

2.6 PHARMACEUTICALS IN THE ENVIRONMENT

The adoption of HUDF aligns with principles of circular economy and resource efficiency, as it closes the nutrient loop by returning valuable nutrients to the soil in a decentralized and cost-effective manner. However, because treatment techniques cannot remove pharmaceuticals completely, their widespread agricultural application faces a new barrier. Pharmaceuticals are compounds manufactured for medicinal purposes. They are categorised according to their function, including but not limited to analgesics, antibiotics, anti-neoplastics, antihistamines, anti-inflammatory substances, and X-ray contrast media. Active pharmaceutical ingredients are the most important components of pharmaceuticals. According to the World Health Organisation (2011), they can be defined as substances used in a finished pharmaceutical product that are intended to furnish pharmacological activity, to have a direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease or to have a direct effect in restoring, correcting or modifying physiological functions in human beings. They are complex molecules with different physiochemical and biological properties and functionalities. This makes their behaviour in the environmental complex.

Since the 1960s, environmental pollution research has focused on compounds that are toxic to, or persistent in, the environment due to the harmful effects of some synthetic chemicals. These include organochlorine pesticides such as dichlorodiphenyltrichloroethane, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans. These synthetic chemicals were included in the Stockholm Convection (2001) and legislations such as Regulation 850/2004/EC of the European Parliament and the European Union list of carcinogenic substances that were intended to eliminate or restrict the production and use of such persistent organic pollutants. The concern over the occurrence of pharmaceuticals and personal care products (PPCP) in the environment has increased over the past two decades. Although the detected concentrations of PPCPs in the environment are below the human therapeutic doses, the effects of long-term exposure to these low concentrations on human health and development are unknown. However, these low concentrations may have adverse effects on plant microbiota and soil organisms, affecting plant-microorganism symbiosis.

According to Biel-Maeso *et al.* (2019), the most significant entry routes of PPCPs into soils are related to the application of treated sewage sludge as fertilizer and the reuse of reclaimed water for irrigation of agricultural soils. No legislation currently controls the use of biosolids on agricultural land with respect to the concentration of PPCPs in most countries. Pharmaceuticals are of greater concern in HUDF than personal care products due to their excretion route from the human body. A meta-analysis of the excretion pathways of 212 pharmaceuticals from the Swiss Pharmaceutical Compendium showed that 64% of each pharmaceutical was excreted via urine while 35% was via faeces (Lienert *et al.*, 2007a). This is important for HUDFs produced from source-separated urine.

Most pharmaceuticals are transformed within the human or animal body and are broken down into metabolites. These metabolites differ in taxological and pharmacological properties from their parent pharmaceutical. It is important to note that the term metabolite has various definitions. In some cases, it is defined as a product of biochemical transformations of the parent pharmaceutical. In others, it means compounds that result from biotic or non-biotic alterations in wastewater treatment systems or the environment (Klassen *et al.*, 2017).

2.6.1 Pharmaceutical uptake and distribution in plants

Pharmaceuticals are often designed to cross biological membranes. Therefore, there is concern that they could enter the food chain after plant uptake and pose potential risks to human and animal health. Pharmaceuticals are transported through the soil to the root cortex by mass flow or diffusion (Zheng and Guo, 2021). The Casparian strip in the cell wall prevents water and pharmaceuticals from entering the plant root's endodermis due to suberin's hydrophobic nature. Therefore, the pharmaceuticals cross the cell membrane through the symplastic pathway and enter the xylem vessels. The movement of pharmaceuticals within the plant is driven by transpiration and diffusion (Sauvêtre and Schröder, 2015). According to Goldstein *et al.* (2014), hydrophobic pharmaceuticals are partitioned into lipids and retained in the roots, hydrophilic ones are taken up through the xylem, and non-ionic pharmaceuticals accumulate in the leaves. The negatively charged cell wall repels ionic pharmaceuticals and they accumulate in the fruits. The bioconcentration factor (BCF; the ratio of the pharmaceutical concentration in a plant to that in the bulk soil) is the standard way to assess pharmaceutical distribution in soil-water-plant systems (Pan *et al.*, 2014; Wu *et al.*, 2015a; Keerthanan *et al.*, 2021). However, pharmaceuticals in soil pore water and those absorbed by soil may have different bioavailability to plant uptake. As a result of the varied affinities of pharmaceuticals to the soil, BCFs estimated based on pharmaceutical concentration in bulk soil are not necessarily comparable between investigations utilizing different soils.

2.6.2 Factors affecting plant uptake of pharmaceuticals

The uptake of pharmaceuticals by plants depends on the physicochemical properties of the pharmaceuticals, the plant species, and the soil properties. The pharmaceutical physicochemical properties influencing plant uptake include molecular mass and hydrophobicity. According to Zhang *et al.* (2017), plants easily take up pharmaceuticals when the molecular mass is less than 1000. The octanol-water partition coefficient (log K_{ow}) is a major quantitative hydrophobicity parameter influencing pharmaceuticals' plant uptake. The log K_{ow} is the ratio of the concentration of a substance in a mixture of two solvents, *n*-octanol and water. It is used as a relative indicator of the tendency of an organic compound to adsorb to soil. Plants more easily take up organic compounds with a low log K_{ow}than compound with a high log K_{ow}. Calderón-Preciado *et al.* (2013); (Pérez *et al.*, 2022). However, using log K_{ow} as the key factor affecting the uptake of pharmaceuticals should only be considered for neutral pharmaceuticals (Colon and Toor, 2016). Additional parameters, such as electrical attraction, should be considered for ionic pharmaceuticals (Zhang *et al.*, 2017). The chemical stability of pharmaceuticals within the soil is another physicochemical factor influencing plant uptake of pharmaceuticals. This is because degradation of less stable compounds (half-life < 14 days) may occur before plant uptake (O'Connor, 1996). A study conducted by (Carter *et al.*, 2014) found that concentrations of diclofenac and sulfamethazine were undetectable in the soil after 3 days of exposure, which was likely due to their corresponding half-lives of 0.5 and 0.99 days, respectively. Another important property that could affect the behaviour of a compound in the environment is its pKa value (Herklotz *et al.*, 2010; Goldstein *et al.*, 2014). The pKa is the negative base-10 logarithm of the acid dissociation constant. It estimates a pH value at which a weak acid would ionize.

Plant characteristics influencing the uptake of pharmaceuticals include the root system, shape and size of leaves, and lipid content, which vary from species to species. The primary plant biological characteristics that strongly influence the uptake of pharmaceuticals from external media to roots include the lipid and carbohydrate content of root cell walls and the permeability of root cell membranes (Chen *et al.*, 2009). The type of growth media also affects plant uptake of pharmaceuticals. Hydroponics has demonstrated the ability of plants to take up higher concentrations of pharmaceuticals than plants grown in soil (Wu *et al.*, 2013; Zhang *et al.*, 2017; Ravichandran and Philip, 2021). According to Wu *et al.* (2015a), the antidepressant fluoxetine accumulated in plants grown under hydroponic conditions but not in plants grown in soil. Soil chemical and physical properties have been shown to influence plant uptake of pharmaceuticals. High organic matter content in soils has been reported to reduce the plant uptake of organic pollutants because ionized compounds might be strongly bound to soil organic matter (Trapp *et al.*, 1994).

2.6.3 Plant uptake studies

Studies on agricultural applications of biosolids containing human pharmaceuticals are dominated in the literature by livestock manure containing veterinary pharmaceuticals. However, human pharmaceutical studies have grown significantly for a wide range of therapeutic groups over the last decade. Therefore, studies focusing on plant uptake of human pharmaceuticals from soil were selected for a scoping analysis. Considering this selection criterion, very few studies were found that have investigated HUDF as a source of pharmaceuticals for plant uptake. Ten studies were identified, with eight conducted under controlled conditions and two in the field (Table 2.2).

Table 2. 2 : Pharmaceutical plant uptake from selected studies

Source of pharmaceutical	Therapeutic class	Pharmaceutical	Concentration applied	Plant	Type of Study	Comment	Reference
Spiked soil	Analgesics	Acetamiphen	1µg/g	*Raphanus raphanistrum* (radish)	Greenhouse Soil: Sa 73%, Si 12.6%, Cl 2.8% OM 2.8%	Pharmaceuticals in pore water represent more effective fractions for plant uptake than those sorbed by soil	Li *et al.* (2019b)
	Anticonvulsant	Carbamazepine	1µg/g				
	Sulfa antibiotic	Sulfadiazine	1µg/g				
	Antibiotic	Sulfamethoxazole	1µg/g				
	Anticonvulsant	Lamotrigine	1µg/g				
	Estrogen	Estrone	1µg/g				
	Antibiotic	Lincomycin	1µg/g				
Spiked irrigation water	Bronchodilators	Salbultamol	4.6 ng/L	*Eruca sativa* (rocket) *Zea mays* (maize)	Green house Peat mixture: 50% OM, pH 7.1	Normal growth of plants was observed	Marsoni *et al.* (2014)
	Beta blocker	Atenolol	241 ng/L				
	Antibiotic	Lincomycin	249 ng/L				
	Anticonvulsant	Carbamazepine	33 ng/L				
	Antilipemic	Bezafibriate	57 ng/L				
	Antibiotic	Ofloxacin	150 ng/L				
	Histamine antagonist	Ranitidine	39 ng/L				
	Antineoplastic	Cyclophosphamide	33 ng/L				
Biosolids and TWW	Anticonvulsant	Carbamazepine	29.04 µg/kg 2.24 µg/kg	*Solanum lycopersicum* (tomato) *Lactuca sativa* (lettuce) *Triticum aestivum* (wheat)	Green house Soils used Soil A: Sa 47.5%, Si 12.5%, Cl 40% pH 7.81, OM 0.48 Soil B: Sa 87.5%,Si 0%, Cl 12%, pH 7.67, OM 0.41 Soil C (15%C, 12.5%S, 72.5%Sa) pH 7.80, OM 1.3	Introduction of polar pharmaceuticals to the soil via biosolids is lower than via treated wastewater	Mordechay *et al.* (2018)
	Metabolite	Dihydroxy-CBZ	2.24 µg/kg				
	Metabolite	Epoxide-CBZ	< LOQ				

Source of pharmaceutical	Therapeutic class	Pharmaceutical	Concentration applied	Plant	Type of study	Comment	Reference
Spiked urine for struvite production	Anticonvulsant	Carbamazepine	234 µg/L	*Solanum lycopersicum* (tomato)	Green house Mixture of sand and potting soil: OM n/a, pH n/a	No pharmaceuticals were detected in tomato fruits	de Boer *et al.* (2018)
	Beta blocker	Propranolol-HCl	14 µg/L				
	NSAID	Diclofenac	640 µg/L				
	Antibiotic	Sulfamethaxazole	630 µg/L				
	NSAID	Ibuprofen	27 µg/L				
Spiked soil	Anticonvulsant	Carbamazepine	1 mg/kg	*Lolium perenne* (ryegrass) *Raphanus raphanistrum* (radish	Green house Soil: Sa 89%, Si 3%, Cl 3%, pH 6.25, OM 1%	Uptake of pharmaceuticals by ryegrass and radish from spiked soil was observed	Carter *et al.* (2014)
	Beta blocker	Propranolol	1 mg/kg				
	NSAID	Diclofenac	1 mg/kg				
	Antibiotic	Sulfamethazine	1 mg/kg				
	Antidepressant	Fluoxetine	1 mg/kg				
Spiked TWW and freshwater	Hyperlipidemia	Bezafibrate	0.86 µg/L	*Cucumis sativus* (cucumber) *Solanum lycopersicum* (tomato)	Green house Soils Aeolian :Sa 82%, Si 7.5%, Cl 12.5%, pH 7.94, OM 0.73% Alluvial : Sa 22%, Si 37.5%, Cl 40%pH 7.92,OM1.78% Sand: Sa 92%, Si 0.5%, Cl 7.5%) pH7.57, OM 0.39%	Concentration of most pharmaceuticals was higher in the cucumber fruit than the tomato fruit	Goldstein *et al.* (2014)
	Stimulant	Caffeine	1.31 µg/L				
	Anticonvulsant	Carbamazepine	0.90 µg/L				
	Hyperlipidemia	Clofibric acid	1.03 µg/L				
	Hyperlipidemia	Gemifibrozil	0.87 µg/L				
	NSAID	Ibuprofen	0.67 µg/L				
	NSAID	Ketoprofen	0.98 µg/L				
	Anticonvulsant	Lamotrigine	0.97 µg/L				
	blocker	Metoprolol	1.07 µg/L				
	NSAID	Naproxen	0.85 µg/L				
	Vasoactive-agent	Sildenafil	0.49 µg/L				
TWW	Hyperlipidemia	Bezafibrate	0.35 µg/L	*Daucus carota* (carrot) *Ipomoea batatas* (sweet potato)	Field study with use of lysimeters Soils Site A: Sa 47.5%, Si 12.5%, Cl 40%,pH 8.1, OM 0.49% Site B: Sa 87.5%, Cl 12.5%, pH 8.0, OM 0.41% Site C: Sa 72.5%, Cl 15%, Si 12.5%, pH 8.2, OM 1.50%	No pharmaceuticals were detected in the edible parts of the crops	Malchi *et al.* (2014)
	Stimulant	Caffeine	0.30 µg/L				
	Anticonvulsant	Carbamazepine	0.54 µg/L				
	Hyperlipidemia	Clofibric acid	0.01 µg/L				
	Hyperlipidemia	Gemifibrozil	0.12 µg/L				
	NSAID	Ibuprofen	0.05 µg/L				
	NSAID	Ketoprofen	0.17 µg/L				
	Anticonvulsant	Lamotrigine	0.82 µg/L				
	blocker	Metoprolol	0.01 µg/L				
	NSAID	Naproxen	0.06 µg/L				
	Vasoactive agent	Sildenafil	0.01 µg/L				

Spiked soil	Anticonvulsant	Carbamazepine	0.05-10 mg/kg	*Cucurbita pepo* (zucchini)	Greenhouse Soil:, Sa 78.7%,Si 16.5%, Cl 4.8%pH 7.8, OM 0.46%	Carbamazepine concentrations < 4mg/kg resulted in burnt leaf edges and reduction of photosynthetic pigment	Carter *et al.* (2015)
	Calcium-channel blockers	Verapamil	0.05-10 mg/kg				
Biosolids Applied at the rate of 14.8t/ha	NSAID	Naproxen	n.d	*Glycine max* (Soybean) *Triticum aestivium* (wheat)	Greenhouse study Soil: peat potting soil Sa 3%,Cl 4%, pH 6.5, OM 95%	No pharmaceuticals were detected in the plant tissue	Cortés *et al.* (2013)
	NSAID	Ketoprofen	n.d				
	NSAID	Ibuprofen	217 ng/g				
	NSAID	Diclofenac	22 ng/g				
Dewatered municipal biosolids Applied at the rate of 8t/ha	Antibiotic	Ciprofloxacin	5870 ng/g	*Daucus carota* (carrot), *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Zea mays* (sweet corn)	Field study Soil :Sa 18%, Si 67%, Cl 15%, pH 7.5, OM 3.4%	Some analytes detected in plant material were not detected in the biosolids	Sabourin *et al.* (2012)
	Antibiotic	Norfloxacin	1750 ng/g				
	NSAID	Ibuprofen	167 ng/g				
	Steroid hormone	Progesterone	130 ng/g				
	Anticonvulsant	Carbamazepine	94.5 ng/g				

n/a: Not available in manuscript, n.d: not detected, TWW: Treated Wastewater, Cl: Clay, Si: Silt, Sa: Sand, OM: Organic matter, < LOQ below limit of quantification

Pharmaceuticals reported in the evaluated studies (Table 2.2) were applied to the soil via spiking soil, spiking irrigation water, biosolids, and treated wastewater (TWW). Spiking soil is an experimental procedure involving dissolving the target pharmaceuticals in acetone and adding this to 25% of the soil sample. The acetone is allowed to evaporate before adding the remaining 75% of the soil to achieve the desired concentration (Brinch *et al.*, 2002). Spiking soil is an unrealistic exposure method to study plant uptake of pharmaceuticals. However, it gives a baseline on the fate of pharmaceuticals in soil. Freshwater is spiked with a cocktail of pharmaceuticals to mimic TWW which has become an important source of irrigation, especially in semi-arid areas. Goldstein *et al.* (2014) compared the uptake of pharmaceuticals from spiked freshwater and TWW. They observed that crops grown using the former exhibited higher concentrations of pharmaceuticals than crops grown with TWW. This could be attributed to the better water quality of the freshwater that influenced the plant uptake of the pharmaceuticals.

Several studies have explored the application of biosolids as a source of pharmaceuticals for plant uptake studies in both field and greenhouse studies (Table 2.2). Application of biosolids and TWW exhibit a more realistic exposure of pharmaceuticals to soil for plant uptake studies. Biosolids have been reported to display an inhibitory effect on plant uptake of pharmaceuticals and increase their residence time in the soil (Sharma *et al.*, 2017; Mordechay *et al.*, 2018). Organic matter content increases with the application of biosolids, thus increasing sorptive sites and surface area for adsorption. This results in increased sorption of pharmaceuticals to soil and decreased plant bioavailability. de Boer *et al.* (2018) investigated the application of struvite as a source of pharmaceuticals for tomatoes (*Solanum lycopersicum*). However, the urine used to produce the struvite was spiked with a cocktail of pharmaceuticals to mimic source-separated urine. Struvite was combined with an adsorbent mineral, zeolite, and biochar to allow for N recovery. Tomato grown with the struvite and zeolite combination exhibited the lowest pharmaceutical uptake.

The evaluated studies explored various pharmaceutical therapeutic groups for plant uptake studies. The targeted pharmaceuticals and their therapeutic groups were based on their detection frequency in TWW. Anticonvulsants, specifically carbamazepine, are the most assessed pharmaceutical group for plant uptake studies (Table 2.2). Carbamazepine has been reported to be frequently detected in TWW and biosolids (McClellan and Halden, 2010; Jelic *et al.*, 2012), and it is relatively persistent in the environment (Mordechay *et al.*, 2018). However, it is important to note that the prevalence of pharmaceuticals is geographically specific, consequently determining the type of pharmaceuticals detected in wastewater treatment plants (WWTP). Antiretrovirals (ARV) are a significant pharmaceutical class to consider for plant uptake studies in South Africa, bearing in mind that the country has the highest ARV therapy programme globally, with approximately 4.6 million people being treated. Despite persistent challenges, including stigma, healthcare disparities, and drug resistance, the widespread availability of ART has transformed the landscape of HIV/AIDS in the country. Antiretroviral drugs are classified into several categories based on their mechanisms of action and targets within the HIV replication cycle. The main classes of ARVs used in South Africa include Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs): NRTIs interfere with the activity of reverse transcriptase, an enzyme essential for HIV replication. These drugs mimic nucleosides or nucleotides, the building blocks of DNA, and when incorporated into the viral DNA chain, they terminate its synthesis. Common NRTIs used in South Africa include zidovudine, lamivudine, tenofovir disoproxil fumarate, and emtricitabine. Tenofovir and emtricitabine are often co-formulated into a single pill known as Truvada, which is used for pre-exposure prophylaxis (PrEP) to prevent HIV transmission in high-risk individuals. Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), bind directly to reverse transcriptase, causing a conformational change that inhibits its activity. These drugs are particularly effective against HIV-1, the predominant strain of the virus worldwide. Examples of NNRTIs used in South Africa include efavirenz (EFV), nevirapine (NVP), and etravirine. Protease Inhibitors (PI), block the activity of HIV protease, an enzyme necessary for the maturation of viral particles. By inhibiting protease, these drugs prevent the cleavage of viral polyproteins into functional components, thereby halting the production of mature infectious virions. Commonly prescribed PIs in South Africa include lopinavir/ritonavir and atazanavir. Integrase Strand Transfer Inhibitors (INSTIs): INSTIs are a newer class of antiretrovirals that target integrase, an enzyme responsible for integrating viral DNA into the host cell genome. By blocking integrase activity, these drugs prevent the establishment of viral reservoirs and halt the progression of HIV infection. In South Africa, dolutegravir and raltegravir are among the INSTIs commonly used in HIV treatment regimens. Entry Inhibitors, interfere with the initial stages of HIV infection by targeting viral entry receptors or co-receptors on host cells. One example is maraviroc (MVC), a CCR5 antagonist that blocks HIV entry into CD4+ T cells by binding to the CCR5 co-receptor.

The presence of ARVs has been reported in South African WWTPs (influent and effluent) (Abafe *et al.*, 2018) and surface water (Wood *et al.*, 2015). Nevirapine and efavirenz were detected in effluent sampled from WWTP in Gauteng province (Schoeman *et al.*, 2017). Abafe *et al.* (2018) reported the persistence of atazanavir, efavirenz, lopinavir and nevirapine in effluent from selected WWTP in KZN, the province with the highest (40%) reported HIV/AIDS cases (Satoh and Boyer, 2019). The fixed-dose combination (FDC) was rolled out in 2015 as the first line of treatment for HIV-positive patients. The FDC is a combination of two or more pharmaceuticals in a single pill; in this case, the pill contains efavirenz 600 mg, emtricitabine 200 mg and tenofovir disoproxil fumarate 300 mg. Efivarenz ($C_{14}H_9ClF_3NO_2$) is a synthetic non-nucleoside reverse transcriptase inhibitor with a molecular mass of 315.14 g/mol, a water solubility of 0.00855 mg/ml and a half-life of 40-45 days. According to Bang and Goa (2003), nearly all urinary excretion is in the form of metabolites. Emtricitabine ($C_8H_{10}FN_3O_3S$) is also a synthetic nucleoside reverse transcriptase inhibitor with a molecular mass of 247.248 g/mol. It is predominantly eliminated from the body unchanged through urine (Nakatani-Freshwater and Taft, 2008). Tenofovir disoproxil fumerate ($C_9H_{14}N_5O_4P$) is excreted unchanged in the urine via a combination of glomerular filtration and active tubular secretion.

Different plant species have been used for pharmaceutical uptake studies. Most of the evaluated studies focused on vegetables, including carrots, cucumber, lettuce, radish, tomato, and zucchini (Table 2.2). The focus on vegetables could be that they are mostly consumed raw, posing a greater risk to human health. However, sweet potato, maize, wheat, and soybean have also been assessed for pharmaceutical uptake studies (Table 2.2). The type of plant species determines the concentration of pharmaceuticals taken up and thus is important in estimating the potential risk to human health (Colon and Toor, 2016). In a study by Goldstein *et al.* (2014), tomatoes and cucumbers were exposed to the same growing conditions and pharmaceuticals. The results showed that cucumber fruits had a higher concentration of pharmaceuticals than tomatoes. The risk uptake of PPCPs is anticipated to be higher than some other crops due to their high-water uptake.

2.6.4 Toxicity of pharmaceuticals to plants

Negative effects of pharmaceuticals on plant biochemical processes and phenotypic appearances have been reported (Guruge *et al.*, 2019). The plant species, pharmaceutical type, and concentration determine the specific effects on the exposed plant (Bartrons and Penuelas, 2017). Andrioli *et al.* (2014) studied the effects of pharmaceutical formulations containing griseofluvin on the mitotic machinery of onion (*Allium cepa*) meristematic cells. The results showed that griseofluvin inhibited mitotic index and increased chromosome fragmentation, disorganized anaphase, and caused nuclear abnormalities in the interphase of the plants. Currently, studies on the effects of pharmaceuticals on cell function are limited and are overshadowed by studies on other organic compounds such as pesticides. In addition to influencing growth and development, several antibiotics are susceptible to biotransformation inside plants, creating highly toxic chemicals. For example, in spring onion (Allium fistulosum), nitrofuran was converted into genotoxic hydrazine-containing metabolites (Di Marco *et al.*, 2014).

However, several studies have been carried out to demonstrate the negative phenotypic effects of pharmaceuticals. Decreased efficiency of photosystem 2 was reported in sorghum (*Sorghum bicolor*) when exposed to spiked soil with 83 mg kg^{-1} of the anti-inflammatory drug ibuprofen (González-Naranjo *et al.*, 2015). The mature leaves of zucchini (*Cucurbita pepo*) were burnt and showed reduced photosynthetic pigment when exposed to 4 mg kg^{-1} of carbamazepine (Carter *et al.*, 2015). In addition, exposure of duckweed (*Lemma minor*) to 323 nmol L^{-1} of the anti-depressant fluoxine for 21 days decreased root growth and asexual reproduction (Amy-Sagers *et al.*, 2017). In a study conducted by Marsoni *et al.* (2014) water spiked with a mixture of salbutamol, a bronchodilator (4.6 ng L^{-1}), atenolol, a beta-blocker (241 ng L^{-1}), lincomycin, an antibiotic (249 ng L^{-1}), cyclophosphamide, an antineoplastic (10 ng L^{-1}), carbamazepine (33 ng L^{-1}), bezafibrate, a lipid-lowering agent (33 ng L^{-1}), ofloxacin, an antibiotic (150 ng L^{-1}) and ranitidine, a histamine blocker (39 ng L^{-1}) was used to irrigate maize (*Zea mays*), and a decrease in root length was observed in the seedlings but there was no effect on germination.

2.6.5 Potential risk to human health

The potential accumulation of pharmaceuticals in food crops grown in human excreta-amended soils poses health challenges to consumers. It plays a major role in the perception and acceptance of the use of human excreta in crop production. Human exposure risk can be assessed by 1) the acceptable daily intake (ADI) that is computed by multiplying the no observed adverse effect threshold obtained from the most sensitive experimental testing by a 100-fold safety or uncertainty factor, and (2) the threshold of toxicological concern (TTC) that is based on the Cramer classification system and is used to calculate exposure threshold values for compounds with known structures (Bhatia *et al.*, 2015). A proof of concept study conducted by Paltiel *et al.* (2016) in Israel revealed that the consumption of fresh produce (carrots, cucumber, lettuce and pepper) irrigated with reclaimed wastewater resulted in excretion of carbamazepine and its metabolites. Prosser *et al.* (2014) conducted a review that reported residues of PPCPs in food crops grown in soils amended with biosolids or irrigated with wastewater. These residues were used to estimate daily intake for adults and toddlers and compared to the ADI to determine whether PPCPs in the plant tissue posed a hazard to human health. The study concluded that a minimal *de minimis* risk to human health existed. However, unintentional human pharmaceutical exposure requires further studies. Additionally, different classes of pharmaceuticals should be considered for risk assessment studies.

2.6.6 Potential risk to the environment

There are currently several uncertainties associated with the ecotoxicology and sub-lethal effects of pharmaceuticals due to a lack of knowledge about their fate in the environment, their uptake, metabolism, and excretion (pharmacokinetics) by wildlife, and their target affinity and functional effects (pharmacodynamics) (Arnold *et al.*, 2014). Environmental exposure concentrations depend on the volume of usage, degree of release to the environment, and persistence once in the environment. Natural microbial communities in soil and water are critical for controlling the quality of ecosystems and regulating the fate of xenobiotics, including pharmaceuticals, released into the environment (Ebele *et al.*, 2017). Most xenobiotics are eliminated through biodegradation in metabolic or co-metabolic pathways, whereby biodegradation uses a chemical for energy, carbon, nitrogen, or other nutrients. At the same time, the unintentional breakdown of a contaminant by an enzyme or cofactor generated during the microbial metabolism of another substance is known as co-metabolism (Doukani *et al.*, 2022).

Impacts of concern on microbial ecology include any compromise of key ecosystem services, such as nutrient and biogeochemical cycling. Antibiotics can alter the composition of soil microbial communities, reduce soil respiration and nitrification rates, and decrease the degradation of other PPCPs in rare circumstances. Predictive models, such as the University of Minnesota Pathway Prediction System (UM-PPS), can estimate compound susceptibility to microbial degradation based on contaminant structure and known microbial degradation pathways (Knutson *et al.*, 2021). A review of the impacts of agricultural practices, including the use of pesticides, on the relationship between biodiversity and ecosystem function shows that some changes in microbial communities can be harmful to ecosystem function, while others are functionally neutral due to the versatility of their metabolic and biosynthetic systems (Chagnon *et al.*, 2015). However, one elusive issue is how soil biological communities can absorb pesticide impacts before ecosystem function is impaired.

2.7 PHARMACEUTICAL ANALYTICAL METHODS IN PLANTS

Researchers are interested in studying the uptake of pharmaceuticals by plants, leading to the development of various analytical methods. The presence of pigments, fats, and waxy material in plants poses challenges to detection and quantification. Analytical procedures involve extraction, clean-up, and detection using different methods and instruments. Targeted analysis identifies specific pharmaceuticals for quantification, effectively optimising sample preparation. Untargeted analysis aims to comprehensively detect known pharmaceuticals but may face challenges in optimizing sample pre-treatment.

The extraction techniques include solid-liquid extraction (SLE), sonication, and accelerated solvent extraction (ASE). SLE is considered inefficient, while sonication is preferred for its low cost and better efficiency. ASE combines high temperatures and pressures with liquid solvents. Clean-up methods such as liquid-liquid extraction (LLE) and solid-liquid extraction (SLE) isolate, concentrate, and purify samples. Hydrophilic-lipophilic balanced cartridges in solid-phase extraction (SPE) are a preferred clean-up method.

Identification and quantification involve instrument analysis, with liquid chromatography–tandem mass spectrometry (LC-MS/MS) receiving significant attention. LC-MS/MS combines liquid chromatography and mass spectrometry for separation and structural identification with high sensitivity. Gas chromatography-mass spectrometry (GC-MS) is also used, but LC-MS/MS is preferred for its ability to identify a wide range of pharmaceutical classes and higher throughput. The addition of formic acid or acetic acid improves LC-MS/MS performance. Analytical approaches are expensive, leading to the development of predictive models to forecast pharmaceutical accumulation in plants.

2.7.1 Predictive models

Models for plant uptake of pharmaceuticals range from simple correlations with a single parameter to more complex mechanistic models. Single parameter correlations are commonly based on properties of the compounds of interest, such as the log Kow, molecular mass, and, in certain cases, root lipid content. However, empirical single parameter correlations have primarily been developed for neutral organic compounds and are unlikely to apply to the many pharmaceuticals that ionize at pH values that are significant to the environment (Miller *et al.*, 2016)

Contrary to single-parameter models, compartmental models consider plant and environmental properties such as plant type, root lipid fraction, soil organic matter, and chemical properties, thereby providing more detailed predictions. Examples include the Dynamic Plant Uptake (DPU) model and the Biosolids-amended Soil Level IV (BASL4) model. However, compartmental models have been used less frequently due to the more demanding data requirements, such as root volume and rate constants for plant growth, compound metabolism, and contaminant loss from soil due to processes other than plant uptake, as well as the need to adapt them to the conditions of the particular study area (Prosser *et al.*, 2014). Furthermore, these models have been reported to overestimate plant uptake concentrations.

Collins and Finnegan (2010) compared projections to experimental study data intended to reflect a range of pharmaceutical chemical characteristics and uptake mechanisms to test nine compartmental models for non-ionizable pollutants and revealed that most models over-predicted root concentrations by at least an order of magnitude. Current model inaccuracies are primarily related to underlying conceptual uncertainty related to pharmaceutical plant uptake.

2.8 ENVIRONMENTAL IMPLICATIONS

Environmental benefits associated with using source-separated human urine for crop production have been demonstrated through the Life Cycle Assessment (LCA) methodology (ISO 14040/44, 2006) (Tidåker *et al.*, 2007; Remy and Jekel, 2008; Spångberg *et al.*, 2014). The scenarios compared the environmental benefits of urine separation systems to traditional wastewater treatment systems. All these studies showed that using urine as fertilizer saves energy and chemicals in WWTPs by reducing the amount of nutrients that need to be removed.

Additionally, urine contributes more than 50% of P and 80 % of N mass load to municipal wastewater despite accounting for less than 1% of the influent volumetric flow at WWTPs. Therefore, reducing its entry into the treatment system flows through bulking and subsequently recovering nutrients eventually leads to improved sustainable environmental health. Also, decreased greenhouse gas emissions have been reported when human urine is considered a resource rather than a waste (Tidåker, 2003).

Using source-separated urine as fertilizer also protects water sources since micropollutants such as PPCPs may be eliminated more efficiently from urine than after it is diluted into wastewater. Through electrodialysis with ozonation (Lamichhane and Babcock, 2012) or ion exchange treatment (Landry and Boyer, 2013), pharmaceuticals could be targeted for removal before mixing with other waste streams. Additionally, the consumption of energy and non-renewable resources for chemical fertilizers can be reduced by recycling nutrients in human urine to agricultural land as fertilizer. On average, about 5.7 kg of nitrogen is produced per person per year through urine. In contrast, in the chemical fertilizer industry, approximately 10 kg of fossil fuel oil is required to make the same amount of ammonium using the Haber – Bosch synthesis process (Heinonen-Tanski and van Wijk-Sijbesma, 2005). As a result, the recovery and reuse of HUDF would promote sustainable development globally.

Sustainable wastewater treatment has been prioritized as a result of policy changes around the world. In Germany, for example, the German Sewage Sludge Ordinance currently requires WWTPs to recover P from sewage sludge. In Sweden, Switzerland, and Austria, P recovery at WWTPs is also mandatory (Santos *et al.*, 2021). Such waste and wastewater treatment trends reflect a global paradigm shift towards sustainable development where excreta is viewed as a resource rather than a waste. Therefore, with all these benefits, promoting recovery and reuse of excreta materials should be supported at national, regional, and global levels.

Regardless of these and similar advances in other countries, such progress is still lacking in most developing countries, limiting the expansion of resource recovery innovations. Furthermore, most wastewater professionals consider source-separating technology immature and risky (McConville *et al.*, 2017). This is due to configuration flaws and significant ambiguity about its functioning, stability, and cost-effectiveness in the coming years (Truffer *et al.*, 2013). Despite technological difficulties, on-site systems are a significant addition to the well-established centralized sanitation systems and their ability to provide sanitation management services. As a result, source-separating sanitation technologies are a potentially disruptive innovation for the existing industry.

2.9 CONCLUSION

Environmental benefits associated with HUDF application support their use for crop production. The existing literature highlighted the need for an integrated technology design strategy to ensure nutrient recovery, inactivation of pathogens, and pharmaceutical reduction in urine treatment technologies. Given that most pharmaceuticals are excreted through urine, concern over the potential plant uptake of pharmaceuticals has been raised. Some patterns in their uptake by plants can be derived from current studies, but most did not disclose critical environmental characteristics that would allow a more thorough analysis. Pharmaceutical physiochemical properties, nature of the soils, and plant characteristics were found to significantly affect uptake. Throughout this review, recommendations for future research were made; a summary of these gaps and recommendations is given below.

- Producing HUDF in quantities adequate to meet crop nutrient requirements is improbable. Therefore, there is a need to develop ways of applying them in combination with mineral fertilizers. Due to the recent development of alkaline dehydrated urine (dried urine) there are limited studies on its effectiveness for crops. Furthermore, there is a need to assess the AE of all HUDFs.

- Recycling human urine as fertilizer for agricultural use enhances soil quality through soil fertility improvement. However, besides the reported high levels of nutrients, HUDF also contains pharmaceuticals. Therefore, applying HUDF to agricultural lands may alter the soil's physicochemical and biological properties. It is important to understand the effects of HUDF application on soil properties, especially the biological properties, since they are the most sensitive parameters to possible toxicity and general soil environmental changes.
- Despite various pharmaceuticals being studied in terms of their uptake and metabolization in plants, there is still much work to be done as significant groups of pharmaceuticals have either not been studied or have been only poorly investigated. Antiretrovirals are a significant pharmaceutical class to consider for plant uptake studies in South Africa because the country has the highest ARV therapy programme in the world, these include efavirenz , nevirapine , and etravirine, lopinavir/ritonavir and atazanavir, dolutegravir and raltegravir. Additionally, researchers should consider different classes of pharmaceuticals for risk assessment studies to evaluate the risk associated with consuming food crops fertilized with HUDF.

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CHAPTER 3: EFFECTS OF COMBINING HUMAN URINE-DERIVED AND MINERAL FERTILIZERS ON MAIZE (*ZEA MAYS*) GROWTH AND YIELD PERFORMANCE ON TWO CONTRASTING SOIL TYPES.

ABSTRACT

Plant nutrient recovery from human urine can promote sustainable food production and benefits society by limiting the amount of waste disposed of into the environment. Although the potential of human urine-derived fertilizers (HUDF) has been recognized, information on interaction effects of both HUDF nitrogen (N) and phosphorus (P) sources and combinations of mineral and HUD fertilizers have not been explored. A greenhouse study was conducted to evaluate 15 N x P treatment combinations of HUD and mineral fertilizers on the growth and yield of maize (*Zea mays*) in a high organic matter loam and a leached sandy loam. Data on growth (plant height, leaf number and chlorophyll content) and yield components (biomass, cob mass, grain yield, root mass, kernel number, thousand seed weight and harvest index) were collected. The agronomic efficiency of the N and P fertilizers was calculated. The response of maize in both growth and yield components varied significantly with soil type and the N and P fertilizer combinations. The most effective N x P treatments on the loam in terms of biomass, grain yield, cob mass, and kernel number were nitrified urine concentrate (NUC) x single superphosphate (SSP), dried urine x struvite, dried urine x SSP, limestone ammonium nitrate (LAN) x struvite, and LAN x SSP. In the sandy loam NUC x struvite, dried urine x SSP, LAN x struvite and LAN x SSP treatments gave the best results in terms of yield components. Agronomic efficiency results indicated that dried urine and struvite, and LAN and SSP were the most effective N and P sources in the loam and sandy loam, respectively. The importance of urine treatment technologies for nutrient recovery was shown by the consistently lower yield in the stored urine treatment when applied alone.

Keywords: agronomic efficiency, human urine, maize growth, maize yield components

3.1 INTRODUCTION

Human excreta is increasingly being recognised as a source of essential nutrients necessary for plant growth. On average, excreta each year from one individual contains 5.7 kg of nitrogen (N), 0.6 kg of phosphorus (P), and 1.2 kg of potassium (K) (Wolgast, 1993). The majority of the organic matter is found in the faeces, while urine contains 90% of the N, 50-65% of the P, and 50-80% of the K.

Human urine has been studied extensively and is a promising excreta-derived product from sanitation technologies, due to its low heavy metal content (Vinnerås and Jönsson, 2002), higher nutrient content (Winker *et al.*, 2009) and lower pathogen load (Carr and Strauss, 2001) compared to faecal matter. The treatment process is by either concentrating nutrients through eliminating water from the urine or selectively extracting nutrients. Nutrient concentration techniques include membrane distillation, nitrification distillation, and forward osmosis, whereas nutrient extraction is accomplished through adsorption, ion exchange, stripping, and precipitation (Simha *et al.*, 2020). Scientific developments and technological advances in the safe recovery of nutrients from human urine have been reported comprehensively in the literature (Alemayehu *et al.*, 2020a; Martin *et al.*, 2020; Simha *et al.*, 2020).

Struvite ($MgNH_4PO_4.6H_2O$) precipitation from urine is a favoured approach among researchers (Simha and Ganesapillai, 2017). Hertzberger *et al.* (2020) in a meta-analysis identified 59 publications on the potential of struvite as a P fertilizer. Urine nitrification distillation was developed for the recovery of N in the form of NO_3^- and NH_4^+ to produce nitrified urine concentrate (NUC) (Udert *et al.*, 2016). Several studies that have explored the effectiveness of NUC for crop production (Bonvin *et al.*, 2015; Mauerer *et al.*, 2018; Mchunu N *et al.*, 2018; Wilde *et al.*, 2019; Magwaza *et al.*, 2020) have shown an increase in chlorophyll content, and nutrient uptake with comparable yields to mineral fertilized plants. Senecal and Vinnerås (2017) developed a method for increasing the N concentration in urine (from 0.6 to > 6 %) by dehydration, resulting in a dry powder with 7.8% N, 2.5% P and 10.9% K by mass. This dried urine is a relatively new product and so not many studies on its effectiveness in crop production have been done.

Most of the existing technologies are unable to achieve integrated nutrient recovery as they were designed to optimize certain characteristics (Maurer *et al.*, 2006). Combining mineral and human urine-derived (HUD) fertilizers is potentially a viable option to meet crop nutrient requirements. An assessment of the use of combinations of mineral and HUD fertilizers has not been explored. Therefore, this study aimed to (i) evaluate N x P treatment combinations of HUD and mineral fertilizers on growth and yield of maize (*Zea mays*) in two contrasting soils and (ii) determine the agronomic efficiency (AE) of HUDF.

3.2 MATERIALS AND METHODS

3.2.1 Soils

Bulk samples were collected from the 0 - 30 cm depth of a loam (Inanda (Ia) form; Rhodic Hapludox) and a sandy loam (Catref (Cf) form; Typic Haplaquept) (Soil Classification Working Group, 2019; USDA Soil Taxonomy classification). The Ia was collected from Worlds View, Pietermaritzburg, South Africa (29º 35’ S; 30º 19’ E) under a pine plantation and the Cf from Kwadinabakubo, South Africa (29º 44’ S; 30º 51’ E) under natural grassland. Undisturbed soil cores for the determination of bulk density were collected following the method described by (Blake, 1965). Some of the bulk soils were air-dried and sieved to < 2 mm for analysis. The hydrometer method outlined by (Huluka and Miller, 2014) was used for the analysis of particle size distribution. Organic carbon was estimated by near-infrared reflectance, using the air-dry, 2mm milled soil samples. Phosphorus was extracted using Ambic-2 solution and quantified by the molybdenum blue procedure (Murphy and Riley, 1962). Soil total inorganic N (NH_4^+-N + NO_3^--N) was extracted from the freshly collected soils in a 1:5 soil :2M KCl suspension followed by filtering through Whatman® No. 2 paper according to (Van der Merwe *et al.*, 1984).The filtrates were then analysed using a Nova 60 Spectroquant® (Merck Millipore, Germany) according to standard methods (APHA, 2005). Methods recommended by The Non-Affiliated Soil Analysis Work Committee (Committe, 1990) were followed for the other soil analyses given in Table 3.1.

Table 3. 1: Selected chemical and physical properties of Inanda and Catref *soils*

Property	Inanda	Catref
pH (KCl)	4.11	5.21
Organic C (%)	6.0	0.5
Total N (%)	0.56	0.05
Extractable P (mg kg^{-1})	12.0	0.7
Acid saturation (%)	30	18
Exchangeable acidity ($cmol_c$ kg^{-1})	1.80	0.18
Exchangeable K ($cmol_c$ kg^{-1})	0.07	0.01
Exchangeable Ca ($cmol_c$ kg^{-1})	3.2	0.4
Exchangeable Mg ($cmol_c$ kg^{-1})	0.0	0.4
Total cations ($cmol_c$ kg^{-1})	5.9	1.2
Bulk density (g cm^{-3})	0.80	1.43
Clay (%)	23	12
Silt (%)	48	15
Sand (%)	29	73

3.2.2 Human urine-derived fertilizers

Urine used for this study was collected from household urine diversion toilets in KwaMpumuza, Pietermaritzburg, KwaZulu-Natal, South Africa (29°40'41.4"S ;30°09'46.5"E). The urine was stored for approximately 2 months at a temperature range of 20 – 25 °C before use. The struvite and NUC were produced at Newlands Mashu research facility, Durban, South Africa (29°46′3.94″S;30°58′44.16″ E), following procedures described by Udert et al. (2016). Alkalinized dehydrated urine (dried urine) was obtained from the Swedish University of Agricultural Sciences. Stored urine and NUC were analysed for chemical properties following standard methods for water and wastewater analysis (Rice *et al*., 2012). Total N was analysed using a NOVA 60 Spectroquant® (Merck Millipore, Germany). Struvite and dried urine were analysed following methods from The Non-Affiliated Soil Analysis Work Committee (1990). Selected properties of the HUD fertilizers are given in Table 3.2.

Table 3. 2: Selected total elements, pH and electrical conductivity (EC) of stored urine, nitrified urine concentrate (NUC), dried urine and struvite ± is standard deviation

Parameter	Stored urine ($mg\ L^{-1}$)	NUC ($mg\ L^{-1}$)	Dried urine ($mg\ g^{-1}$)	Struvite ($mg\ g^{-1}$)
Nitrogen	1 790 ± 53	22 600± 342.98	854.8± 23.84	45.2± 2.03
Phosphorus	108.7 ± 9.22	2 610± 8.9	274± 7.1	114± 9.7
Potassium	897± 18.3	9 710± 53.7	1041.2± 87.16	15.1± 1.8
Calcium	14.07± 2.6	97± 5.9	193± 15.3	215.5± 1.2
Magnesium	25 ± 1.6	8.7± 1.2	463± 35.2	645± 15.4
Sodium	966± 27.8	25 386± 265.9	35.3± 4.2	58± 4.2
Chloride	1 830± 13.4	5 226± 63.4	16.9± 7.1	26.2±1.4
pH	8.8 ± 0.2	4.2± 0.37	12.9± 0.8	9.4± 1.56
EC ($mS\ cm^{-1}$)	15.3 ± 4.0	27.6± 0.05	6.2± 1.5	8.6± 0.37

3.2.3 Trial establishment

The experiment was conducted in a plastic tunnel at the University of KwaZulu-Natal, Pietermaritzburg (29° 37' 32.9" S; 30° 24' 18.8" E) under local environmental conditions, with a mean daily temperature of 25.5°C. The soils were air-dried, sieved through an 8 mm mesh, and packed into 20-L pots. Application rates for N and P (Ia :120 kg N ha^{-1}, 80 kg P ha^{-1}; Cf :200 kg N ha^{-1}, 60 kg P ha^{-1}) were recommended by the Soil Fertility and Analytical Services Division, Department of Agriculture, Cedara, KwaZulu-Natal, South Africa. Stored urine, dried urine, NUC and limestone ammonium nitrate (LAN) were applied based on the maize N requirement while struvite and single superphosphate (SSP) were based on the maize P requirement. The liquid fertilizers (NUC and stored urine) were diluted with 1 L of water before application. Struvite, dried urine and mineral fertilizers were applied directly to the soil and mixed. After fertilizer application, two maize seeds (*Zea mays* var. Colorado) were planted in each pot, and later thinned to one plant per pot. Plants were watered with tap water throughout the experiment.

3.2.3 Experimental design

The study was designed as a 5 x 3 x 2 factorial experiment, with the following factors: N fertilizer – five sources (N0 – no nitrogen, N1 – NUC, N2 – store urine, N3 – dried urine, N4 – LAN), P fertilizer – three sources (P0 – no phosphorus, P1 – struvite, P2 – SSP), and soil – two types (Inanda – Ia, Catref – Cf) giving a total of 30 treatment combinations replicated four times, totalling 120 experimental units (20-L pots). The pots were rearranged during the trial to equalise exposure to light.

3.2.4 Data collection

Maize growth parameters (plant height and leaf number) were measured after final seedling emergence; at three weeks after planting (WAP) until, five weeks after planting, seven weeks after planting; and nine weeks after planting. Chlorophyll content reported as the chlorophyll content index (CCI) was recorded using a chlorophyll content meter (OPTI-SCIENCES CCM – 200 plus). The chlorophyll content was measured at 6 WAP and after tasselling on the uppermost, fully expanded leaf of each plant. At physiological maturity (approximately 12 WAP) all ears on all plants in all pots were hand harvested and air dried to a moisture content of approximately 14%. Plants were cut approximately 5cm above the ground, roots were removed from the soil through hand rinsing roots from the soil, followed by air drying. Dry yield parameters (above ground biomass, grain yield, cob mass and root mass) were determined using an electronic balance (Adam AAA 100L, Adam Equipment, South Africa). Thousand seed weight (TSW) was determined following procedures of the International Rules for Seed Testing (ISTA, 2018). Harvest index (HI) was calculated using Equation 3.1(Huetsch and Schubert, 2017):

HI = grain yield (dw)/total above-ground biomass at harvest maturity(dw)…Equation 3.1

3.2.4.1 Agronomic efficiency

Agronomic efficiency (AE) is the increase in grain yield per unit of fertilizer applied (Fairhurst, 2012). It was calculated to assess the efficiency of the applied N and P sources using Equation 3.2: Nitrogen agronomic efficiency (N-AE) was calculated for NUC, stored urine, dried urine and LAN while phosphorus agronomic efficiency (P-AE) was calculated for struvite and SSP in both soils.

$AE = (Y_F - Y_C)/ F_{appl}$ ……………………………………Equation 3.2

Where: Y_F is grain yield from fertilized pots, Y_C is grain yield from control pots, and F_{appl} is the amount of N or P applied per pot.

3.2.5 Data analysis

Data were subjected to analysis of variance (ANOVA) using Genstat version 20 (VSN International, Hemel Hempstead, UK). Treatment means were separated using Fischer's Least Significant Difference (LSD) test at 5% level of significance. Means of significantly different variables were separated using LSD at P = 0.05. Principal component analysis (PCA) and biplot diagrams were used to visualize the association between yield parameters and the different nutrient sources using Origin Pro 2022b (OriginLab Corporation, Northampton, MA, USA).

RESULTS

3.3.1 Effects of nitrogen and phosphorus interactions on maize growth

Plant height increased in both Ia and Cf soils with all treatments over the growth period (Figure 3.1 A and B). At 3 WAP plant height in all treatments was not significantly different in both soils. Plant height significantly increased at 5 WAP, with the zero P treatments having the lowest plant height while the tallest was observed in N4P2 (58 ± 10 cm and 64 ± 8.5 cm on the Ia and Cf, respectively). Treatments N1P2 and N4P2 had the highest plant height at 7 and 9 WAP in both soils.

Plant leaf number at different growth stages in the Ia and Cf soils is shown in Figures 3.1C and 3.1D, respectively. Leaf number differed significantly (P=0.02 Ia; P=0.03 Cf) between N and P fertilizer combinations. Leaf number was lowest in the N0P0 treatment and highest in the N1P2 treatment throughout the growth period on the Cf. In contrast, the Ia exhibited the highest leaf number in the N2P1 treatment except at 9 WAP where the highest leaf number was measured in the N1P2 treatment. Generally maize growth in terms of plant height and leaf number was higher on the Cf than the Ia.

Chlorophyll content significantly differed (P=0.04) between N and P combinations on the Ia soil (Figure 3.2A). Treatments lacking P (N1P0, N2P0, and N3P0) exhibited significantly lower CCI. However, the N4P0 treatment did not differ significantly from treatments where P was applied (N4P1, N4P2). Maize grown on the Ia soil and treated with NUC, and struvite (N1P1) exhibited the highest CCI. In contrast, plants grown on the Cf soil did not show a significant difference between N and P combinations with regards to the CCI (Figure 3.2B). Maize treated with N sources and SSP exhibited the highest CCI (N0P2, N1P2, N2P2, and N3P2), except the LAN treatment where the highest CCI was observed in the N4P1 treatment.

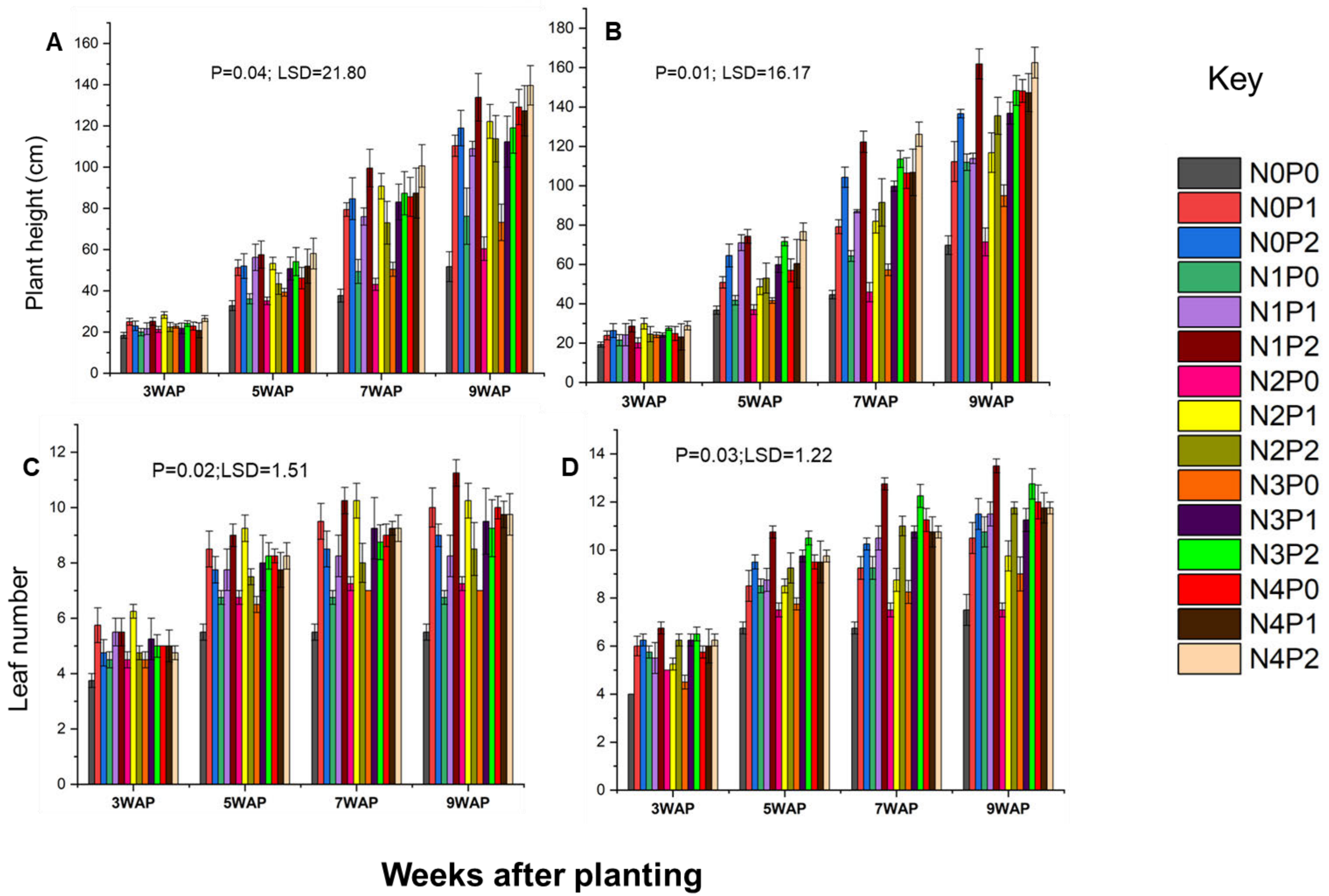

Figure 3. 1 : Plant height (A and B) and leaf number (C and D) of maize grown in Inanda and Catref *soils, respectively, treated with different nitrogen and phosphorus fertilizer sources. N0: Control (no nitrogen); N1: Nitrified urine concentrate; N2: Stored urine; N3 Dried urine; N4: Limestone ammonium nitrate; P0: Control (no phosphorus); P1: Struvite; P2: Single superphosphate*

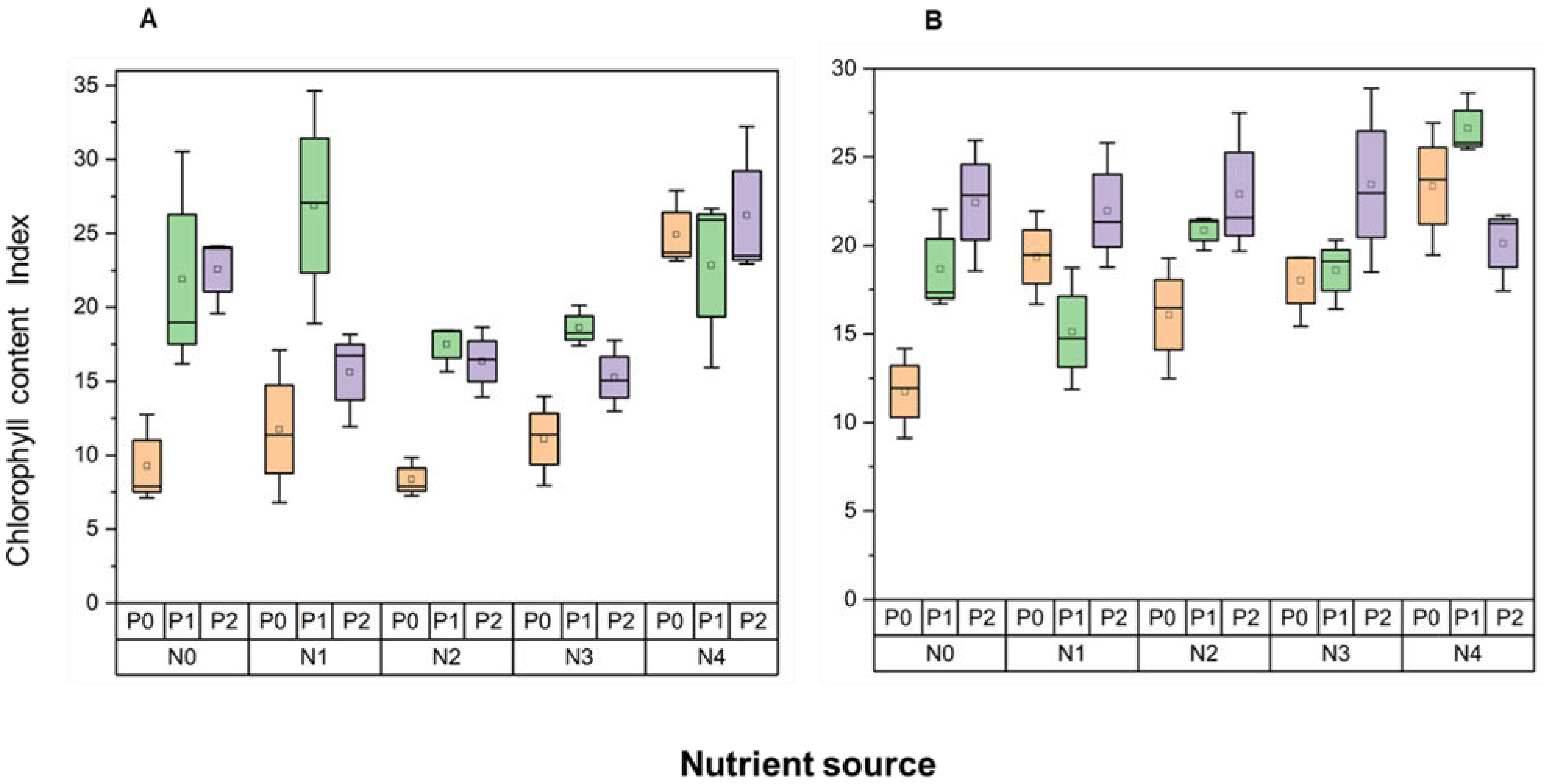

Figure 3. 2 : Chlorophyll content index of maize plants treated with different nitrogen and phosphorus sources grown on A Inanda and B Catref *soil. Results are presented as box plots; average and median data are represented by the solid line and small boxes, respectively. Bars represent range of values; the top and bottom of each box represents the first and third quartiles, respectively. N0: Control (no nitrogen); N1: Nitrified urine concentrate; N2: Stored urine; N3 Dried urine; N4: Limestone ammonium nitrate; P0: Control (no phosphorus); P1: Struvite; P2: Single superphosphate*

3.3.2 Effects of nitrogen and phosphorus interactions on yield components

The analysis of variance (ANOVA) for evaluated maize yield components indicated that the effects of N, P, soil, and their interactions were significantly different for most of the measured yield components (Table 3.3).

Table 3. 3 :Analysis of variance showing mean squares and significance for yield parameters of maize fertilized with nitrogen and phosphorus on Inanda and Catref *soils*

Source of variance	**df**	**BM**	**CB**	**GY**	**RM**	**KN**	**TSW**	**HI**
Nitrogen	4	9104**	2968^{ns}	2344^{ns}	36.36^{ns}	16288^{ns}	4369*	0.05651^{ns}
Phosphorus	2	100391***	32915***	22102***	300.17***	161151***	41731***	0.40081***
Soil	1	197062***	58136***	38771***	0.94^{ns}	212761***	81483***	0.13948**
Nitrogen x Phosphorus	8	7393**	3112**	2454**	62.35**	17384*	5762***	0.10528***
Nitrogen x Soil	4	16084***	5641**	4863**	49.00*	33500**	6827**	0.05519^{ns}
Phosphorus x Soil	2	6161^{ns}	3753^{ns}	2381^{ns}	39.65^{ns}	16577^{ns}	2499^{ns}	0.05023^{ns}
Nitrogen x Phosphorus x Soil	8	716^{ns}	399^{ns}	343^{ns}	21.66^{ns}	2366^{ns}	1177^{ns}	0.04638^{ns}
Residual	87	2754	1361	1106	18.31	7868	1521	0.02923

df: degrees of freedom, **BM:** biomass, **CB:** cob mass, **GY:** grain yield, **RM:** root mass, **KN:** kernel number, **TSW:** thousand seed weight, **HI:** Harvest Index.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns non-significant

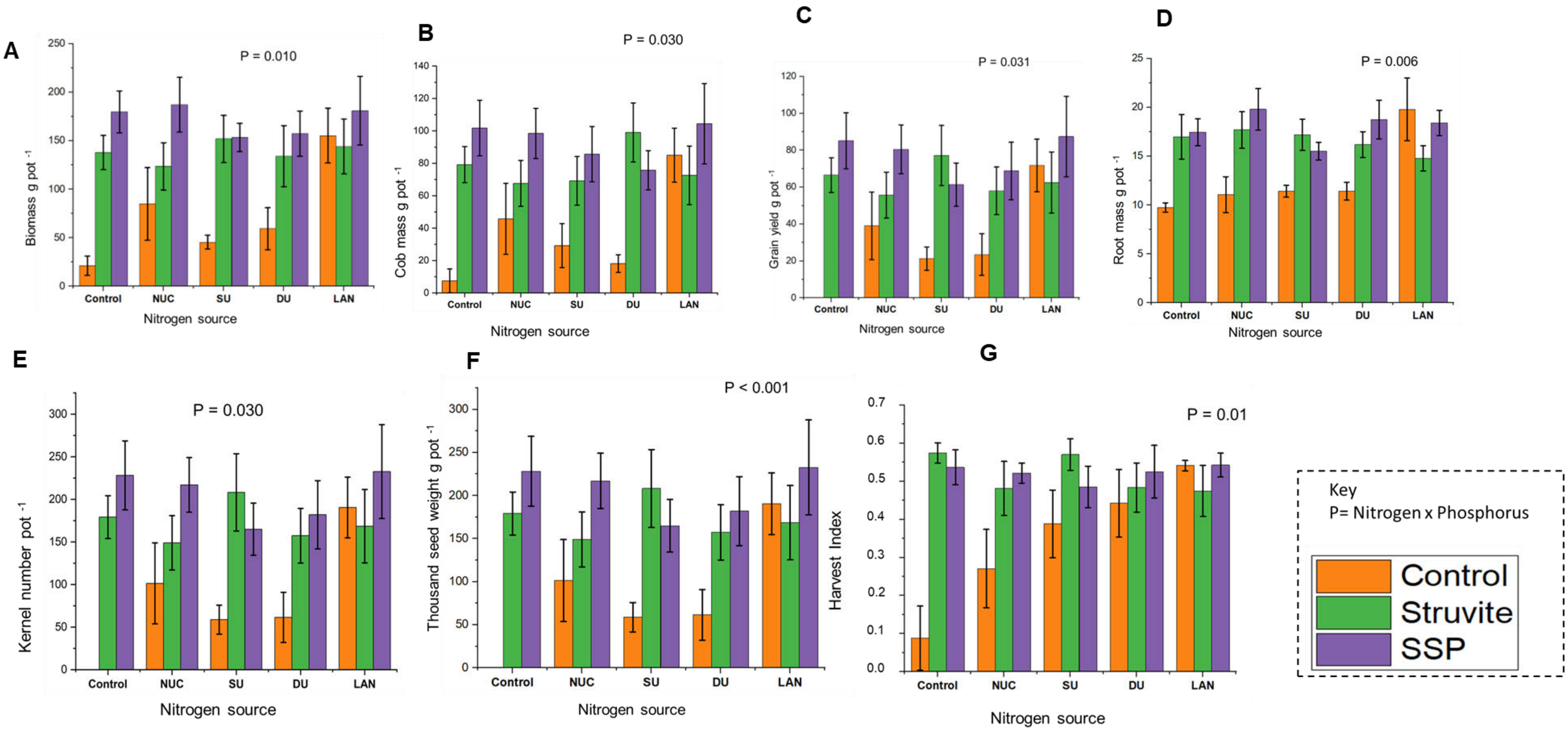

Figure 3. 3 : The effect of nitrogen and phosphorus in human urine-derived and mineral fertilizers on maize yield components: (A) biomass, (B) cob mass, (C) grain yield, (D) root mass, (E) kernel number, (F) thousand seed weight, (G) harvest index. NUC: nitrified urine concentrate; SU: stored urine; DU: dried urine; LAN: Limestone ammonium nitrate; SSP: single superphosphate

The response of maize yield components to the applied combinations of N and P is presented in Figure 3.3 A-G. Maize biomass was lowest in all the zero P treatments except in the LAN treatment where the lowest biomass was observed in the LAN x struvite treatment. Maximum biomass (187±28 g) was observed in the NUC x SSP treatment. Significant differences (P=0.030) were observed in cob mass with regards to N and P interactions. With the exception of the LAN x struvite treatment which had the lowest cob mass, maize cob mass was lowest in all N source x control (no P) treatments (Figure 3.3B). Maize fertilized with N (NUC, stored urine and LAN) and SSP exhibited the highest cob mass. However, the dried urine treatment had the highest cob mass when applied with struvite. Grain yield significantly (p= 0.031) varied between N and P combinations. The N0P0 treatment (control) did not result in grain production (Figure 3.3C). The same trend was observed for grain kernel number and TSW (Figure 3.3E and F, respectively). Root mass ranged from 9.8 g pot^{-1} to 19.8 g pot^{-1} (Figure 3.3D). The maximum root mass was observed in the NUC x SSP treatment. Harvest index exhibited significant differences (P=0.001) between N x P interactions. Treatments lacking P exhibited the lowest HI except plants treated with LAN, where the lowest HI was observed in the LAN x struvite treatment (Figure 3G.)

3.3.4 Effect of nitrogen and soil interactions on yield components

Maize yield components showed a significant N source x soil interaction, (Figure 3.4 A-G). Regarding N sources, biomass was significantly (P<0.001) higher in the Cf than the Ia, with the exception of the SU treatment, where there was no significant difference between the two soils. Maximum biomass was observed in the stored urine (106 g pot^{-1}) and LAN (221 g pot^{-1}) treatments while dried urine and the control treatments exhibited the lowest biomass on the Ia and Cf soils, respectively (Figure 3.4A). Cob mass, grain yield, kernel number and TSW were higher in Cf than Ia. However, cobmass, grain yield, kernel number and TSW observed in the dried urine treatment did not differ significantly between Cf and Ia soils (Figure 3.4B, C, E, F). Significant differences (P=0.04) were observed in maize root mass with regards to N x soil interactions (Figure 3.3D). The dried urine and LAN treatments exhibited higher root mass in the Ia than the Cf soil. Harvest index did not show a significant difference between the two soils, however, the NUC treatment exhibited significantly higher HI in the Cf soil than Ia.

3.3.5 Principal component analysis (PCA) for assessed yield components

The PCA was conducted to visualize the relationship between the N and P combinations and the evaluated yield parameters on the Ia (Figure 3.5a) and Cf soils (Figure 3.5b). The red dot markers are information pertaining to the N x P treatments and can be related to the original variables by means of the red lines. The position of each yield parameter is defined by its eigenvector and contributes to the scores of PCA components 1 and 2.

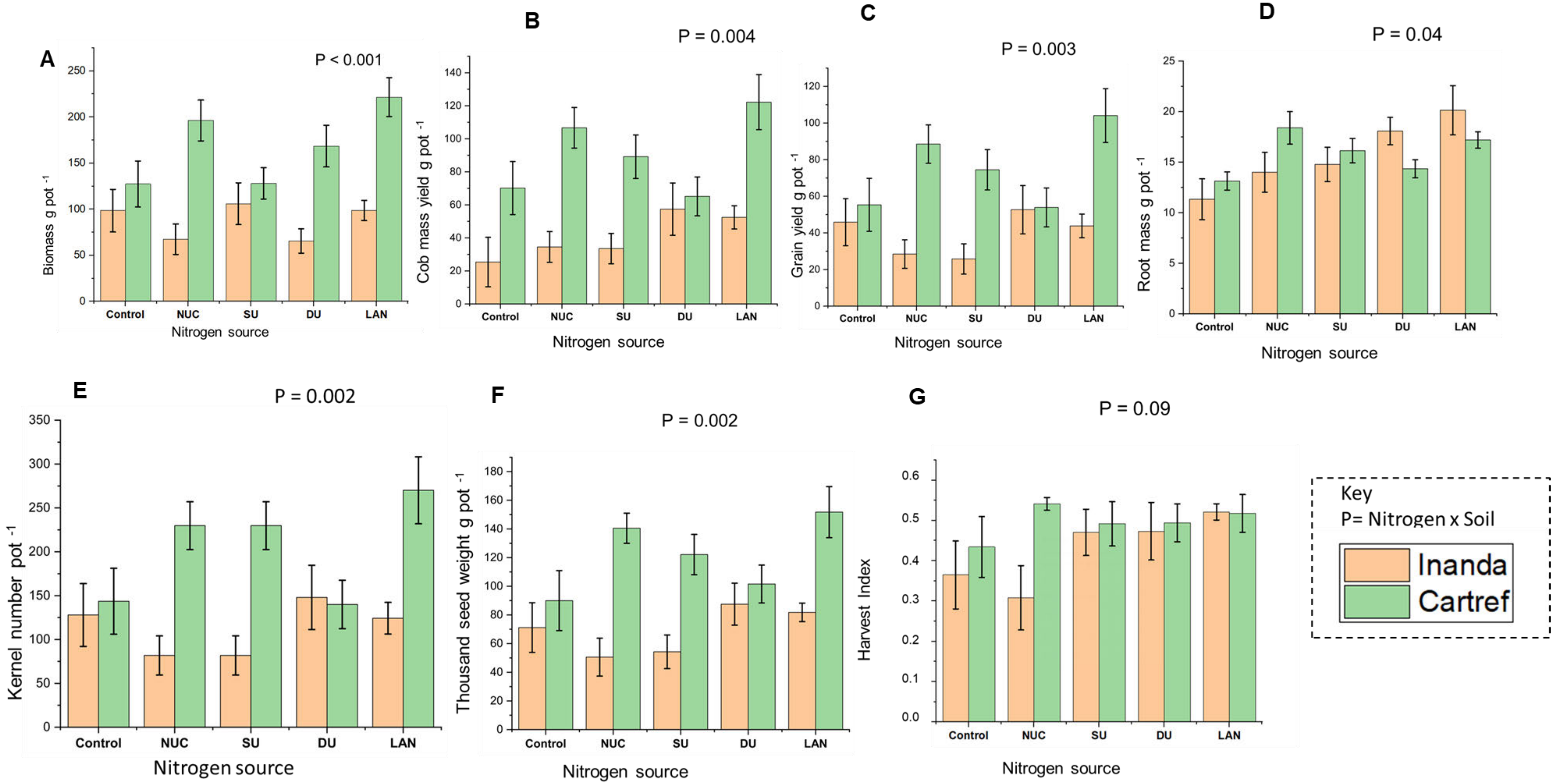

Figure 3. 4: Interaction effect of nitrogen fertilizers and soil on maize yield components: (A) biomass, (B) cob mass, (C) grain yield, (D) root mass, (E) kernel number, (F) thousand seed weight, (G) harvest index. NUC: nitrified urine concentrate; SU: stored urine; DU: dried urine; LAN: Limestone ammonium nitrate

In the Ia, treatments N0P1, N1P1, N3P1, N3P2, N4P0, and N4P1 were grouped based on high root mass, HI and TSW. Treatments N0P2, N1P2, N2P1, and N2P2 were grouped based on biomass, grain yield, cob mass, and kernel number.

In the Cf, treatments N0P1, N0P2, N2P1, N2P2, and N3P1 were grouped based on HI, TSW and kernel number. Treatments N1P1, N1P2, N3P2, N4P0, N4P1, and N4P2 were grouped according to grain yield, cob mass, root mass and biomass. Treatments N0P0, N1P0, N2P0 and N3P0, were negatively associated with the evaluated yield parameters in both soils.

3.3.6 Agronomic efficiency

Significantly lower N-AE values were obtained on the Ia than the Cf except the DU treatment where significantly lower N-AE values were obtained on the Cf soil. (Table 3.4). The NUC and stored urine exhibited negative N-AE values of -26.9 and -1.61 kg (kg N)$^{-1}$, respectively, while dried urine gave the highest N-AE on the Ia soil. In contrast, dried urine gave the lowest and LAN the highest N-AE in the Cf soil. Significantly lower P-AE was observed in struvite than SSP on the Cf while there was no significant difference between them on the Ia.

Table 3. 4. Agronomic efficiency values of different nitrogen and phosphorus fertilizers applied to the Inanda and Catref soils.

Fertilizer	Inanda	Catref
NUC kg (kg N)$^{-1}$	-26.9	35.9
DU kg (kg N)$^{-1}$	6.5	0.3
SU kg (kg N)$^{-1}$	-1.6	21.3
LAN kg (kg N)$^{-1}$	0.2	49.1
Struvite kg (kg P)$^{-1}$	52.7	43.9
SSP kg (kg P)$^{-1}$	49.1	92.6

NUC: nitrified urine concentrate; SU: stored urine; DU: dried urine; LAN: Limestone ammonium nitrate, SSP: single superphosphate

3.4 DISCUSSION

Phosphorus is crucial for cell division, enzyme activation/inactivation and carbohydrate metabolism which are all necessary for tissue development. Additionally, it promotes root growth and winter hardiness, stimulates tillering, and accelerates maturity (Razaq *et al.*, 2017). The present study demonstrated that lack of P limits plant growth even with sufficient applied N, which is supported by previous findings that P application increases plant height and root collar diameter (Huda *et al.*, 2007).

Chlorophyll content is used as a physiological indicator of a plant's N status (Bojović and Marković, 2009). The application of N promotes the formation of active photosynthetic pigments by increasing the amounts of stromal and thylakoid proteins in leaves. However, P is required for the facilitation of metabolic processes and the manufacture of pigment molecules. The highest CCI were found in maize treated with NUC and struvite (N1P1) on the Ia. Struvite contains 9.9% magnesium which is a crucial component of chlorophyll a/b, which, in turn, is part of light-harvesting complexes (Gerendás and Führs, 2013). Additionally, it is a major cofactor in a series of enzymes involved in photosynthetic carbon fixation and metabolism. Therefore, struvite application significantly affected the chlorophyll content in the maize plants.

Yield responses are among the most crucial factors for assessing fertilizer effectiveness as they show the differences between yields that are attainable and those that are limited by nutrient availability in agroecosystems (Liu *et al.*, 2017). The study has revealed varied responses for the evaluated yield components among the assessed N x P treatments. Nitrogen fertilizer application alone did not increase the maize yield components. However, when N was applied in combination with P fertilizer, yield was significantly higher in the SSP than the struvite treatments. These findings are not consistent with results from a study by Antonini *et al.* (2012) which concluded that urine-derived struvite resulted in substantially higher biomass than mineral fertilizer. Struvite-P uptake by many crops is slow due to its low solubility, therefore, crop yield can be lower than it would be with mineral fertilization (Nagy *et al.*, 2019b). Results from a study by Talboys *et al.* (2016) suggest that the potential benefit of struvite is its use on crops whose root systems exude organic acids in large quantities to increase solubility. Field lupin (*Lupinus albus)*, oil seed rape *(Brassica napus*), and chickpea *(Cicer arietinum)* are a few examples of the crops that produce substantial amounts of malate and citrate into the rhizosphere.

An essential yield component for farmers in maize production is grain yield, which translates to economic yield. The ack of grain production in the control treatment (no added N or P) is often the reality for many smallholder farmers in Sub-Saharan Africa. Little to no fertilizer application and erratic rainfall patterns are major constraints for South African smallholder maize farmers (Agbugba *et al.*, 2020). On the other hand, yields from the NUC x SSP and LAN x SSP treatments reflected those from commercial farmers (120-150 g cob^{-1}) (Huetsch and Schubert, 2017). The TSW is a measure of yield quality that is not only correlated with grain yield and milling quality but also with seedling vigour and growth (Darfour *et al.*, 2022). The NUC x SSP, LAN x SSP and dried urine x struvite treatments exhibited the highest TSW demonstrating the ability of plants grown in these treatments to translocate carbohydrates during grain filling stage. Harvest indices observed in the evaluated N x P treatments varied between 0.09 and 0.57. The HI has been used as an indicator of success in partitioning assimilated photosynthate to harvestable product and thus the effective utilization of resources (Metho and Hammes, 2000). Selection of the most efficient fertilizer treatments and improvement in yield prediction systems can both benefit from an understanding of HI variations among N x P treatments. Based on the current results, it is not agronomically feasible to completely replace mineral fertilizers with HUDF due to the lower grain yield observed when the latter are applied alone compared to mineral fertilizers.

The response of maize yield components was influenced by the applied N sources and soil type. Almost all the N sources exhibited higher yield components on the Cf than the Ia soil (Figure 3.4). Inanda is an acidic soil characterized by 30% acid saturation and high exchangeable acidity (Table 3.1). According to Kamprath and Foy (1985) when acid saturation exceeds 22% the detrimental effects of aluminium toxicity become limiting. In such conditions, aluminium fixes P decreasing its availability for plant uptake, and the bioavailability of iron or manganese can be very high and may reach toxic levels. Furthermore, the rate of mineralization and nitrification is reduced because microorganisms responsible for these biogeochemical processes are inactive in acidic conditions (Li *et al.*, 2018). The high root mass observed in LAN and dried urine treatments in the Ia can be attributed to the liming effect of these N sources. In addition to 28% N, LAN contains 20% dolomitic lime resulting in about 4.1% Ca and 2.1% Mg in the product (Rasulov *et al.*, 2021). The method for producing dried urine established by Senecal and Vinnerås (2017) and Simha *et al.* (2018) involves alkalizing the urine to prevent urea hydrolysis and to maximise N recovery. Alkaline products such as lime (Randall *et al.*, 2016), and wood ash (Senecal and Vinnerås, 2017) can be used to achieve the alkalization, thereby contributing to the liming effect of dried urine.

The AE reflects the direct production impact of an applied fertilizer and relates directly to economic return. The low N-AE values obtained on the Ia suggest that it is a less responsive soil as defined by Vanlauwe *et al.* (2010), where responses to N fertilizer are constrained by physical, biological or chemical degradation. According to Vanlauwe *et al.* (2011) it is challenging to explain negative N-AE values unless there are processes by which fertilizer application decreases the relative availability of native soil N in comparison to the no-input control soil. The Ia contains a very high amount of OM which is the source of its natural N. If the applied fertilizers prevented or slowed its decomposition that could result in negative N-AE values. The N-AE values obtained in this study on the Cf for NUC (35.9) and LAN (43.2) are comparable to values reported for sub–Saharan Africa. A meta-analysis conducted on the AE of N fertilizer in maize-based systems in sub-Saharan Africa within the context of integrated soil fertility management revealed that the average N-AE value for researcher-managed plots was 34 kg (kg N) $^{-1}$ based on 73 cases (Vanlauwe *et al.*, 2011).

A P-AE value of 26 kg (kg P) $^{-1}$ was reported by Endris (2019) on maize field study conducted in Ethiopia on a nitisol soil with slight acidity and fertilized with triple superphosphate (TSP), which is much lower than the values found in the present study. In contrast, Kihara and Njoroge (2013) reported average P-AE values of 60 kg (kg P) $^{-1}$ for maize based systems in western Kenya which are not too different from values observed in the current study. The control yields, amount of clay and plant-available soil P and P application rates all have an impact on the agronomic efficiency of P which could explain differences in P-AE (MacDonald *et al.*, 2011). The response to P may also be impacted by other factors, such as nutrient shortages, particularly in the case of micronutrients, which were not tested in the current study.

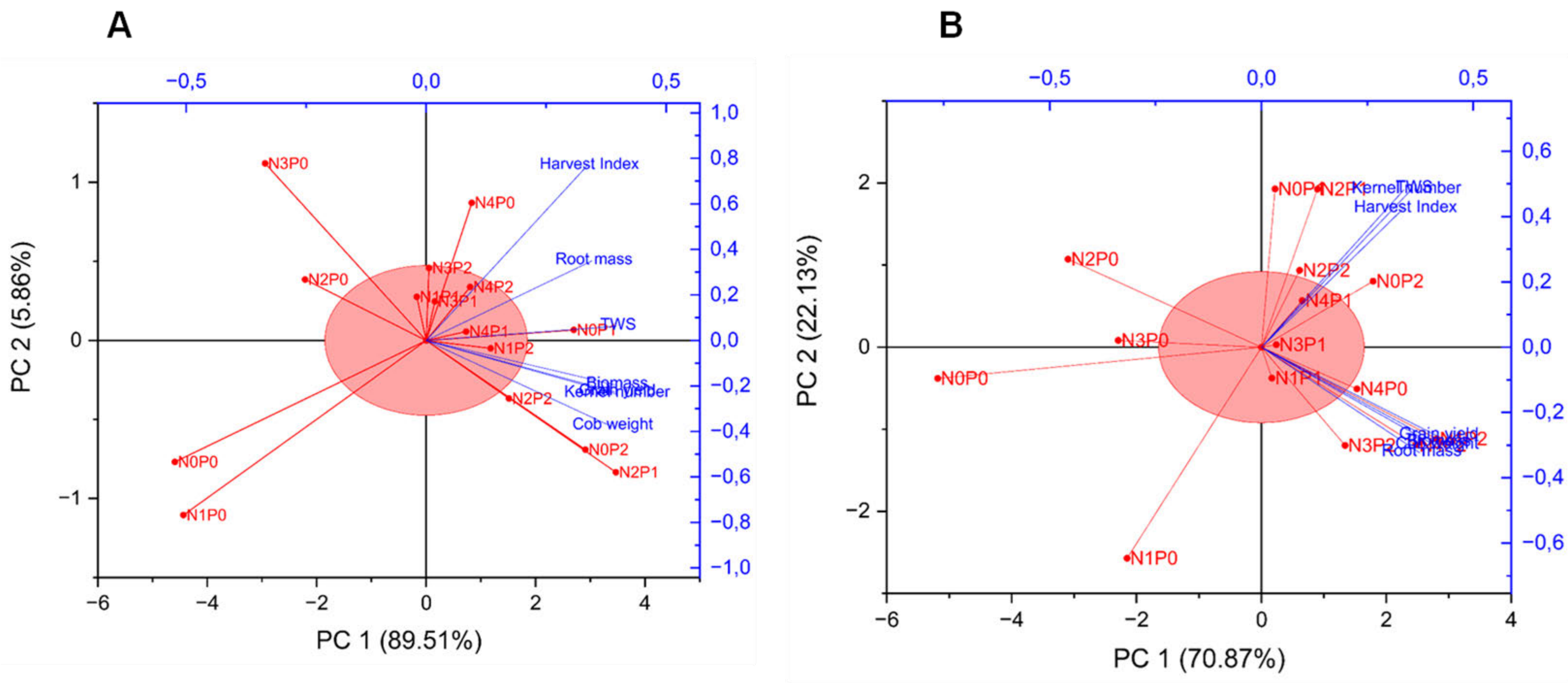

Figure 3. 5 :Principal component (PC) biplot of PC 1 vs PC 2 showing the relationships between yield components and fifteen fertilizer combinations applied to (A) Inanda and (B) Catref *soils*

3.5 CONCLUSION

Maize growth and yield components showed varying responses to soil type and N and P fertilizer combinations. Combination of HUD and mineral fertilizers can result in good yield components but depends on which urine fertilizer is used as a nitrogen source. The best performing N x P treatments on the Ia soil in terms of biomass, grain yield, cob mass, and kernel number were NUC x SSP, dried urine x struvite, dried urine x SSP, LAN x struvite, and LAN x SSP. On the Cf soil the NUC x struvite, dried urine x SSP, LAN x struvite and LAN x SSP treatments showed the best results in terms of yield components. This study demonstrated a new opportunity for combining HUD and mineral fertilizers as N and P sources for crop production. Furthermore, the lower grain yield observed in the stored urine treatments highlights the importance of urine treatment technologies for nutrient recovery. The other HUD fertilizers (NUC, struvite, dried urine) from treatment technologies are odourless, and almost free of heavy metals, factors that play a major role in the acceptance of HUD fertilizers in crop production. Transportation, storage, and application of struvite and dried urine are simpler than those of urine due to their lower weight and volume, and their powdery form. The findings of the present study indicate that dried urine and LAN are the most effective N sources in the Ia and Cf soils, respectively, while struvite and SSP were the most effective P sources in the Ia and Cf soils, respectively. This study has demonstrated the ability of HUDF to improve crop yield, however, their impact on soil quality is unclear and research on their effects on soil properties, especially the biological properties, is required.

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CHAPTER 4: IMPACT OF HUMAN URINE-DERIVED FERTILIZERS ON SOIL QUALITY: A SHORT-TERM STUDY ON MICROBIAL ABUNDANCE, ENZYMATIC ACTIVITY, AND MICROBIAL BIOMASS C AND N

ABSTRACT

Although human urine-derived fertilizers (HUDF) have been used in crop production to maintain soil fertility the extent to which they impact soil quality remains unclear. A laboratory incubation study was conducted to evaluate the effect of stored urine, nitrified urine concentrate, (NUC) and alkalinized dehydrated urine (dried urine) on microbial abundance, extracellular enzymatic activities and microbial biomass carbon (C) and nitrogen (N) in a high organic matter loam soil. Treatments were replicated four times, and the incubation period was for 60 days. Data were collected on day 0, 5, 25, 40 and 60 for analysis of ammonium and nitrate release, arginase, β-glucosaminidase and urease activity, carbon dioxide and nitrous oxide emission, and microbial biomass C and N. The 16S rRNA gene was used to assess the abundance of total bacteria and archaea. Data were subjected to analysis of variance with HUDF treatment and sampling days as main factors. Principal component analysis (PCA) and biplot diagrams Pearson correlation heatmaps were drawn based on mean values. Soil amended with NUC had significantly higher chemical properties. Soil enzyme activities were increased by application of dried urine and stored urine while a reduction in enzyme activity was observed in the NUC treatment. A similar trend was observed with regards to microbial abundance. Overall, the abundance of microbial genes significantly correlated with enzyme activity, while chemical properties were significantly correlated to microbial biomass C and N. The results showed that application of HUDF improved soil quality in short term.

Keywords; human urine, microbial abundance, soil enzymes,

4.1 INTRODUCTION

Human excreta use in agriculture is a centuries-old practice in many countries. In recent years urine treatment technologies have been developed for the recovery of water and nutrients for crop production. Several studies have reported the effects of human urine-derived fertilizers (HUDFs) on crop production (Bonvin *et al.*, 2015; Alemayehu *et al.*, 2020b; Hertzberger *et al.*, 2020; Magwaza *et al.*, 2020). However, one issue that remains elusive is the degree to which they impact soil properties.

The most sensitive parameters to possible toxicity and general soil environmental changes are soil microbial properties. It is important to study these sensitive parameters in order to maintain the sustainability of agroecosystems and ensure the productivity of agricultural lands (Kiani *et al*., 2017). The significance of soil microorganisms in residue breakdown, crop development and regulating biogeochemical processes, particularly the nitrogen (N) cycle, is well established (Macdonald *et al*., 2011, Xu *et al*., 2020). The N cycle, which includes transformation processes such as N-fixation, denitrification, and nitrification, is a crucial biogeochemical cycle for supporting plant growth (Stein and Klotz, 2016; Kuypers *et al.*, 2018). Ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB), particularly *Nitrobacter* and *Nitrospira*, have been reported to play a principal role in nitrification (Könneke *et al.*, 2005) while denitrification is driven by nitrite and nitrous oxide reducers (Zumft, 1997). The abundance and diversity of the microbial communities that are responsible for specific N transformation processes are frequently described using functional gene markers (Ouyang *et al.*, 2018). The genes encoding nitrogenase reductase (*nifH*), ammonia monooxygenase (amoA), nitrite reductase (nirK and nirS) (Braker *et al.*, 1998) and nitrous oxide reductase (*nosZ*) are among the most frequently studied N cycling marker genes (Henry *et al.*, 2006).

Several factors affect microbial abundance, composition and activity in agricultural soils. Soil properties such as texture (Naveed *et al.*, 2016), moisture, pH and temperature (Szukics *et al.*, 2010) have an impact on microbial community composition. Microbial communities are also influenced by agricultural practices such as crop rotation, tillage, and use of cover crops and fertilizer (Sommermann *et al.*, 2018). The abundance and structure of soil microbial communities have been shown to be impacted by the application of both organic and mineral fertilizers (El Azhari *et al*., 2012). The chemical composition of the organic matter, particularly the C:N ratio, the quantity of organic material applied and the community present in the soil before amendment application determines the response of microbial community structure and function (Federici *et al.*, 2017). However, short-term experiments may not show significant changes in soil microbial structure. On the other hand, a study by Allison and Martiny (2008) revealed that 84% of the evaluated 38 studies showed a significant sensitivity of soil microbial community composition and structure to application of mineral fertilizers.

Application of phosphorus (P) fertilizers has a less significant impact on soil microbial communities than N fertilizers, therefore research has focused on effects of N fertilization (Li *et al.*, 2020). The changes in ammonium nitrogen (NH_4^+-N) and pH induced by N fertilization are two of the most important factors affecting the structure and diversity of microbial communities (Zeng *et al.*, 2016). A meta-analysis on long-term effects of mineral fertilizers on soil microorganisms reported that repeated application of inorganic N fertilizers increased soil microbial biomass C by 15% (Geisseler and Scow, 2014). Additionally, an increase in AOB and nosZ abundance but no effect on other genes was reported due to application of inorganic N based on 157 observations from 47 peer-reviewed studies (Ouyang *et al.*, 2018).

In addition to soil chemical properties, soil biological properties such as microbial biomass, enzyme activities and microbial abundance are sensitive indicators of soil quality and productivity. (Sadet-Bourgeteau et al., 2018). Therefore, it is important to understand the effects of HUDF application on soil chemical and biological properties. In the present study, an incubation study was conducted focusing on three N-based urine fertilizers (stored urine, dried urine and nitrified urine concentrate). The objective of the study was to explore the impact of HUDF application on microbial abundance, extracellular enzymatic activities and microbial biomass C and N.

4.2 MATERIALS AND METHODS

4.2.1 Soil

The same Ia soil samples were used for this experiment as described in Section 3.2.1.

4.2.2 Urine fertilizers

The stored urine (SU), nitrified urine concentrate (NUC) and dried urine (DU) described in Section 3.2.2 were used.

4.2.3 Incubation

The aerobic incubation experiment was conducted over a 60-day period in a dark room with temperature maintained at 25 °C and and relative humidity at 80%. Ventilated containers were filled with 100 g of Inanda soil. The soil was amended with 20 mL SU, 10 mL NUC, 0.5 g DU to mimic the recommended N application rate for maize (120 kg N ha^{-1}) as recommended by the Fertility Advisory Services, (full details as per Chapter 3) and no fertilizer (the control) and replicated four times. Deionized water was used to adjust the soil moisture content to a range between 40% and 60% of the soil's field capacity based on periodic determination of weight loss throughout the incubation period. Destructive sampling was used to collect soil samples from the treatments at 0, 1, 2, 3, 5, 7, 14, 25, 35, 45, 50, 55, and 60 days of the incubation.

4.2.4 Soil CO_2 and N_2O

The CO_2 and N_2O evolution study ran concurrently with the incubation study. Soils amended with the same HUDF, and the control (soil alone) treatments were incubated in airtight desiccators together with a beaker containing an aqueous solution of 20 mL sodium hydroxide (NaOH) to trap the evolved CO_2 that was replaced on each sampling day. To keep the treatments moist during the incubation period, a beaker with 20 mL of deionized water was put in each desiccator alongside the NaOH solution. The desiccators were sealed and incubated in the same dark room described in Section 4.2.3). Gas samples were collected on day 0, 5, 25, 40 and 60 of incubation. Samples were collected from sampling port using a 20 mL syringe and transferred to a pre-evacuated 12 mL Exetainer (Labco Ltd., United Kingdom). A coupled Varian 3800 gas chromatograph (Varian Palo Alto, CA, USA) was used to analyse the amounts of N_2O and CO_2 in the gas samples.

4.2.5 Ammonium and nitrate N

Ammonium-N (NH_4^+ -N), and nitrate-N (NO_3^- -N) were extracted from 2 g soil with 20 mL 2M KCl solution in 50 mL plastic test tubes (Maynard *et al.*, 1993). The suspensions were shaken on an overhead shaker for 30 min and then filtered through a. Whatman® No. 2 filter paper. The filtrates were transferred to storage bottles before analysis for NH_4^+ -N, and NO_3^- -N using a Thermo-Scientific Discreet Gallery (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

4.2.6 Microbial biomass carbon and nitrogen

Microbial biomass C (MBC) and N (MBN) were estimated using the chloroform fumigation extraction method (Vance *et al.*, 1987). Fresh soil samples were fumigated using alcohol-free $CHCl_3$ in airtight desiccators for a 5-day period at room temperature. Organic C and N were then extracted with 0.5 M K_2SO_4, from both fumigated and non-fumigated soil samples at 1:5 w/v ratio and shaken for 60 min at 200 rpm. The suspension was then centrifuged for 5 min at 2500 rpm followed by filtration through a Whatman No 1 filter paper. The filtrate was diluted 1:9 (v/v) with deionized water ratio before exposure to alkaline persulfate oxidation followed by sulphuric acid digestion. The samples were then analysed on a Dohrmann Phoenix 8000 UV-persulfate oxidation analyzer (Tekmar-Dohrmann, Cincinnati, OH). The difference between extractable organic C (EOC) and N (EON) from fumigated and unfumigated samples was used to calculate MBC and MBN values. The $CHCl_3$ labile pools of C and N that differ between fumigated and non-fumigated samples were converted to microbial biomass by using coefficients. The MBC and MBN coefficients used in this study were 0.45 for C (Beck *et al.*, 1997) and 0.54 for N (Brookes *et al.*, 1985).

4.2.7 Soil enzymes

Arginase (EC 3.5.3.1), β-glucosaminidase (EC 3.21.30), and urease (EC 3.5.1.5) activity were determined as indicators of microbial activity including the availability of substrates for microbial uptake. Samples were collected for analysis on day 0, 5, 25, 40 and 60 of incubation. Arginase activity was determined by incubating soil slurries with1.0 mM L arginine at 37 °C for 1 h, following procedures described by Bonde *et al.* (2001). The NH_4^+ concentration was quantified as described in Section 4.2.5. β -glucosaminidase activity was assayed by mixing 1.0 g of soil, with 4 mL of 0.1 M acetate buffer (pH 5.5) and 1 mL of 10 mM r-nitrophenyl-N-acetyl-b-D-glucosaminide solution in 50 mL centrifuge tubes and placed in an incubator at 37.8 °C for 1 h (Parham and Deng, 2000). Following procedures outlined by Gianfreda *et al.* (1994), urease activity was measured by mixing 1.0 g soil with 0.1 M phosphate buffer (pH 7.2) and 0.2 M urea solution in 50 mL centrifuge tubes. The mixture was then incubated at 37 °C for 2 h, after which 1 mL of 2 M KCl was added. The mixture was kept at 4 °C for 10 min to end the enzymatic reaction and then centrifuged at 5000 rpm for 10 min. The soil ammonium was then determined following procedures described in Section 4.2.5).

4.2.8 DNA extraction

The 16S rRNA gene was used to assess the abundance of total bacteria and archaea. Nucleic acids were extracted from 0.250 g wet soil using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). The DNA quality and quantity for each sample was analyzed by next generation UV/Vis spectrophotometry with the Qiaxpert (Qiagen, Hilden, Germany). PCR amplification of the bacterial and archaeal (16S rRNA gene) markers was performed using primers 341F and 806R for bacteria (Frey *et al*., 2016).

4.2.9 Microbial functional genes

The qPCR reaction was performed in a total volume of 20 µL using the primers given in Table 4.3. All the reactions were performed with a concentration of 1.5 µM for each primer, 1 X SSO Advanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc., California, USA) and 4 ng of DNA samples. The qPCR cycles started with a polymerase activation step at 98 °C for 3 min. The denaturation occurred at 95°C for 15 sec, the annealing temperature was 52°C (Table 4.3) and the elongation was at 72°C for 15 sec. These last three steps were repeated within 30 times. Melting curves were generated by increasing the temperature from 75 to 95 °C by 0.5 °C every 5 sec at the end of the amplification cycles in order to verify the amplification specificity. All the reactions were done with a thermocycler CFX Connect™ Real-Time System (Bio-Rad Laboratories, Inc., California, USA) and the results were recorded and analyzed with the software of the device Bio-Rad CFX Maestro.Ink. The inhibition test was performed using 2 ng of plasmid DNA, and 4 ng of DNA template in each sample.

Potential amplification inhibition by extraction contaminants was tested across all samples using a qPCR assay of pGEM-T plasmid (GenBankR Accession No. X65308; Promega, Madison, WI, United States) spiked into the soil DNA at equimolar concentration in all samples and using the plasmid specific primers SP6 and T7 for PCR. The qPCR reactions were performed using the primers listed in Table 4.1

Table 4. 1: Primers used for the qPCR

Genes	Primer Sequences	References	Annealing temperatures (°C)	Cycle numbers
16S	**515F:** GTGYCAGCMGCCGCGGTAA **804R:** ATTAGADACCCBNGTAGTCC	(Parada *et al.*, 2016)	52	30
Inhibition Test	**SP6:** ATTTAGGTGACACTATAG **T7:** TAATACGACTCACTATAGGG	(Longepierre *et al.*, 2022)	58	35

The DNA standards were prepared from purified PCR products obtained by amplifying the targets from a pool of DNA from all samples. The concentrations used for the standard curves ranged from 10^{-2} to 10^{-7} ng of DNA per reaction. The qPCR efficiencies (E) ranged between 97.4 and 99.0%.

4.2.10 Data analysis

Data were subjected to analysis of variance using Genstat 20th edition (VSN International, Hempstead, United Kingdom), with HUDF types and sampling days as main factors. Means were separated using Fisher's protected least significant difference (LSD) test when treatments showed significant differences on measured parameters at a 5% level of significance. Principal component analysis (PCA) and the biplot diagrams were drawn based on mean values using Origin Pro 2022 (OriginLab Corporation). The PCA was conducted to visualize the association between functional gene abundance, soil chemical and microbiological properties, and the HUDFs.

4.3 RESULTS

ANOVA for evaluated soil properties indicated significant urine fertilizer treatment, sampling time and interaction effects on most biological properties. The response of soil chemical and microbiological properties to human urine fertilizers over a 60-day incubation period is presented in Figure 4.1-4.4. Application of human urine fertilizers significantly increased NO_3^-, EOC, EON, MBC and MBN, N_2O and CO_2 compared to the control treatment.

Table 4. 2: Analysis of variance showing mean squares and significance for soil chemical and microbiological properties with different human urine-derived fertilizers

Source of variance	DF	Ammonium	Nitrate	MBC	MBN	CO_2	N_2O	Arginase	β-glucosaminidase	Urease
Treatment	3	55086***	3685***	39210***	2953***	10616***	27015***	203***	505***	252***
DOI	3	13294***	4867***	18997***	224***	1625***	9629***	23*	329***	508**
Interaction	9	3212***	2731***	6188***	232***	794***	5792***	19^{ns}	127*	36*
Residual	30	625	65	397	102	249	214	76	231	107

D.F; degrees of freedom, DOI: day of incubation, MBC: microbial biomass carbon; MBN, microbial biomass nitrogen; CO_2: carbon dioxide; N_2O nitrous oxide * p < 0.05, ** p< 0.01, *** p < 0.001, ns non-significant

4.3.1 Ammonium and nitrate

Significant HUDF treatment, sampling time and interaction effects on ammonium and nitrate release were observed (Table 4.2). Ammonium significantly increased in all treatments except the control (unamended treatment) after 5 days of incubation. This was followed by a sharp decrease after 25 days and then a steady decrease was observed at 40 and 60 days. Soil amended with NUC showed the highest ammonium release throughout the incubation period (Figure 4.1). Nitrate-N increased over time in all HUDF treatments, gradually up to 25 days and then more rapidly especially in the DU and NUC treatments. The highest amounts were released in the NUC treatment while the nitrate released from SU and control treatments were comparable throughout the incubation period except at 60 days where nitrate release significantly increased in the SU treatment (Figure 4.1).

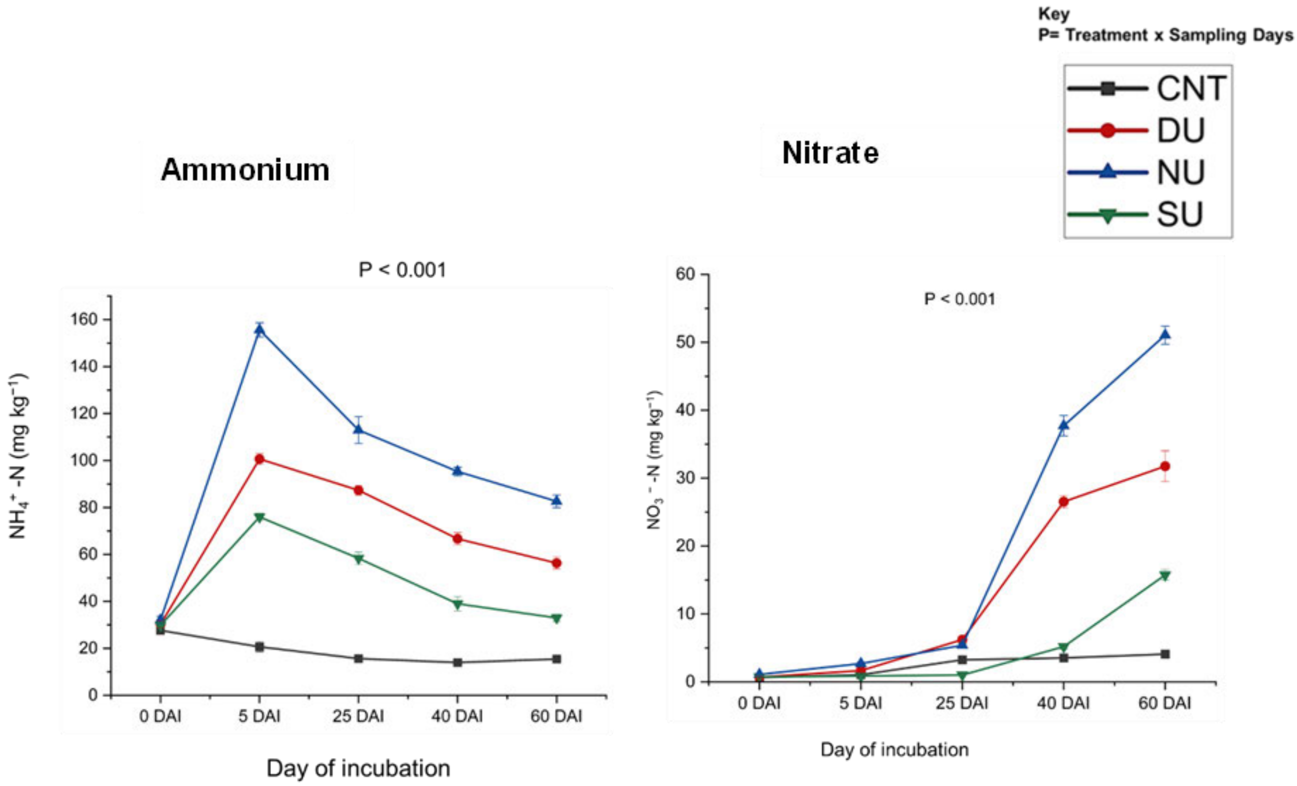

Figure 4. 1: Changes during incubation in ammonium and nitrate in Inanda soil amended with human urine-derived fertilizers. CNT: control; DU: dried urine; NU: nitrified urine concentrate; SU: stored urine.

4.3.2 Carbon dioxide and nitrous oxide emissions

The ANOVA showed significant differences ($p = 0.001$) between treatments with regards to CO_2 and N_2O emissions (Table 4.2). Application of HUDF resulted in a steady decrease in soil CO_2 emissions was observed in the control treatment. Application of HUDF resulted in an increase in CO_2 emmisions and gradual decrease was observed 25 days after incubation, however the SU treatment showed a sharp increase in soil CO_2 emissions at 25 days followed by a sharp decrease at 40 days, which was followed by a gradual decrease (Figure 4.2). Soil N_2O emissions initially increased in all treatments including the unamended soil (control) and later decreased. The highest N_2O emission was observed in the NUC treatment (620 ppm at 40 days).

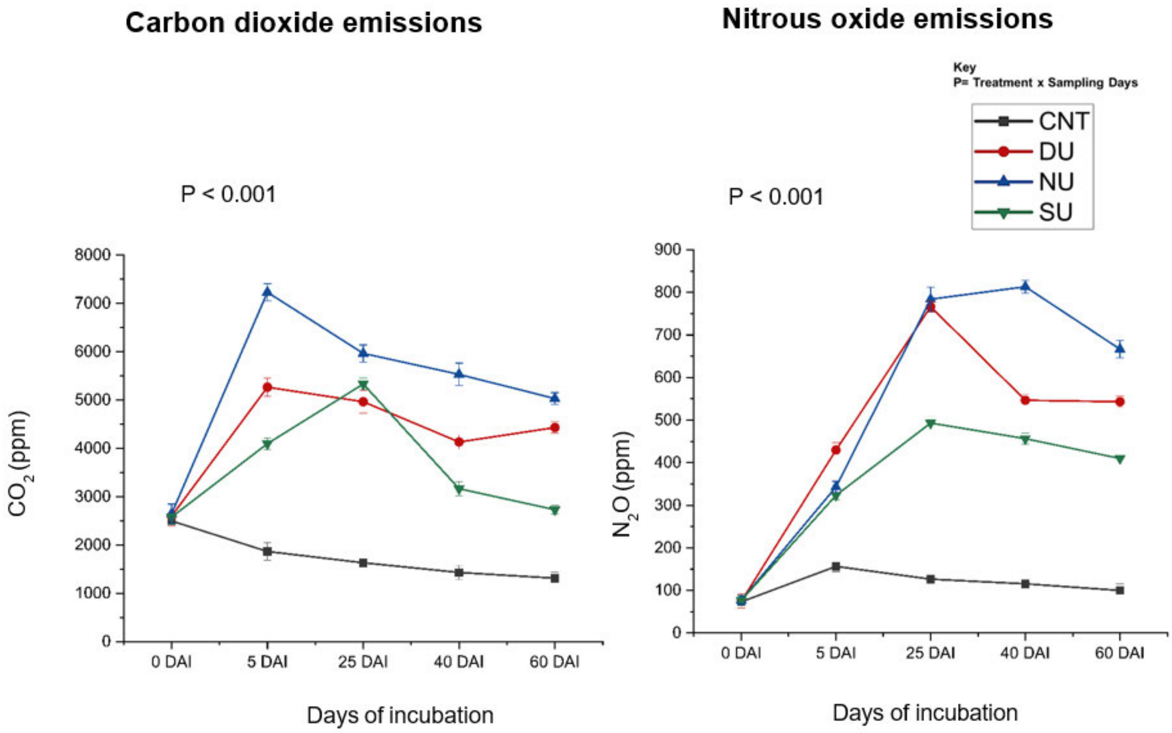

Figure 4. 2: Changes in carbon dioxide and nitrous oxide emissions in Inanda soil amended with human urine-derived fertilizers. CNT: control, DU: dried urine. NU: nitrified urine concentrate; SU: stored urine

4.3.3 Microbial biomass carbon and nitrogen

The application of HUDF significantly influenced MBC and MBN (Table 4.2). Microbial biomass C gradually increased in HUDF amended treatments over the incubation period (Figure 4.3). The NU treatment showed significantly higher MBC in comparison to the DU and SU treatments. This trend was observed throughout the incubation period. In contrast, a sharp increase in MBN was observed in all HUDF treatments at early stage of incubation (0 to 5 days). However, the 25-day samples revealed a decline in MBN in the NUC treatment, which was then followed by an increase at 40 days.

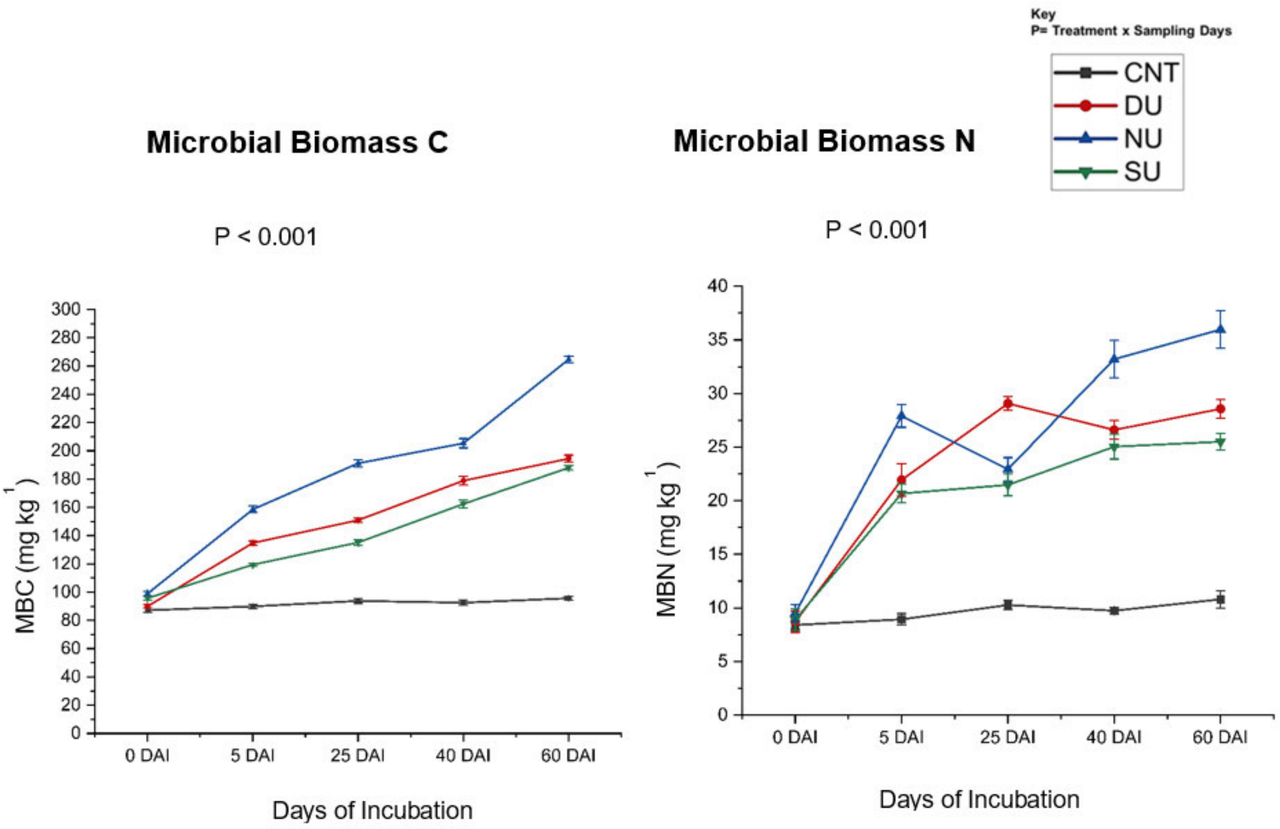

Figure 4. 3: Changes in microbial biomass carbon and nitrogen in an acidic soil amended with human urine-derived fertilizers. CNT: control, NU: nitrified urine concentrate; SU: stored urine; DU: dried urine.

4.3.4 Enzyme activity

The results showed significant HUDF treatment, sampling time and interaction effects on β-glucosaminidase, and urease activity (Table 4.4). The application of all the HUDF treatments had a significant ($p < 0.001$) effect on arginase, β-glucosaminidase, and urease activity (Figure 4.4). Arginase activity initially increased in the DU and NUC treatments at 5 days before gradually declining throughout the course of the incubation period. Arginase activity decreased in the control and SU treatments over the incubation period. In contrast, β-glucosaminidase increased with time of incubation with the DU treatment having the highest enzyme activity and NUC the lowest. Urease exhibited the highest enzyme activity in the SU treatment over the incubation period, while the NUC showed the lowest activity and was not significantly different to the control over the incubation period.

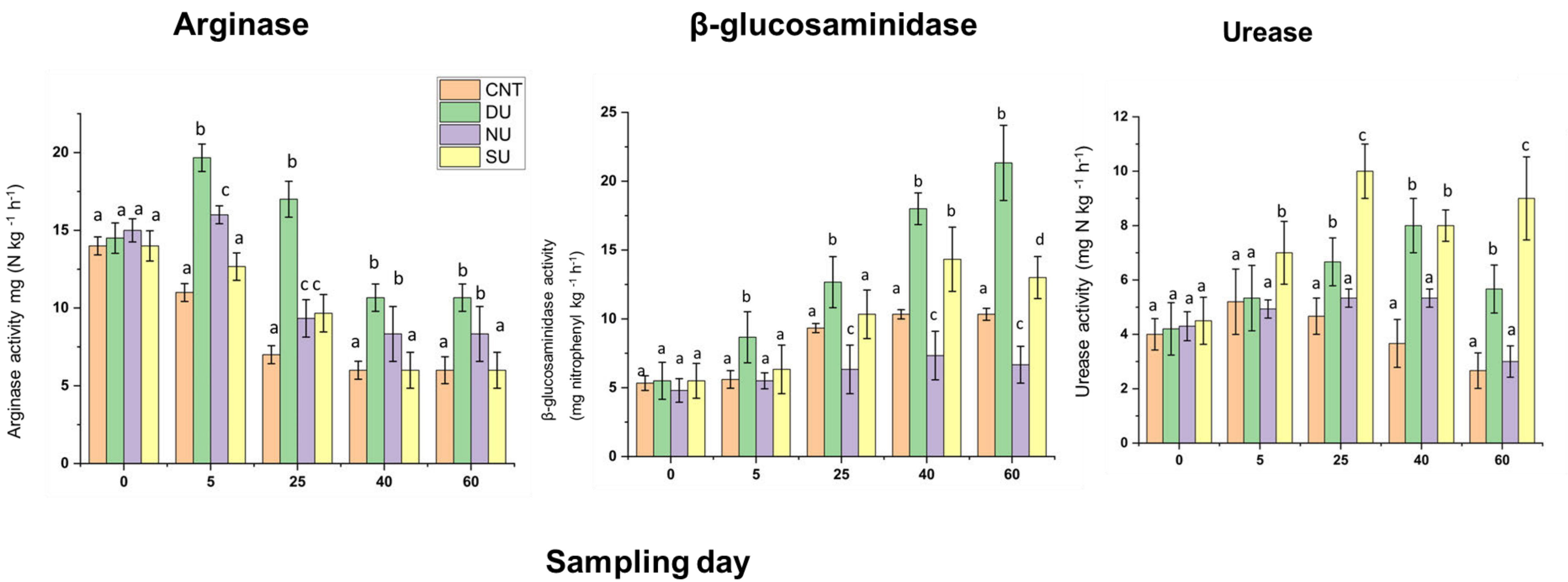

Figure 4. 4: Soil enzyme activity in an acidic soil amended with human urine-derived fertilizers. Error bars represent standard error (n = 4). Different lowercase letters above bars indicate a significant difference among treatments at a specific sampling time. CNT: control; DU: dried urine; NU: nitrified urine; SU: stored urine.

4.3.5 Microbial abundance

Microbial abundance was significantly influenced by HUDF treatments and sampling time. Microbial abundance gradually decreased with time in the control treatment. Application of SU and NUC significantly reduced microbial abundance compared to the control treatment (Figure 4.5). However, soil sampled after 25 days of incubation showed an increase of microbial abundance in the SU treatment compared to the control, and a similar trend was observed to the end of the incubation period. Dried urine significantly increased microbial abundance compared to SU and NUC over the incubation period. The highest average bacterial 16S rDNA copies g^{-1} soil were observed after 25 days of incubation (1.1 ng g^{-1} soil), while the lowest number was recorded after 14 days incubation (0.18 ng g^{-1} soil).

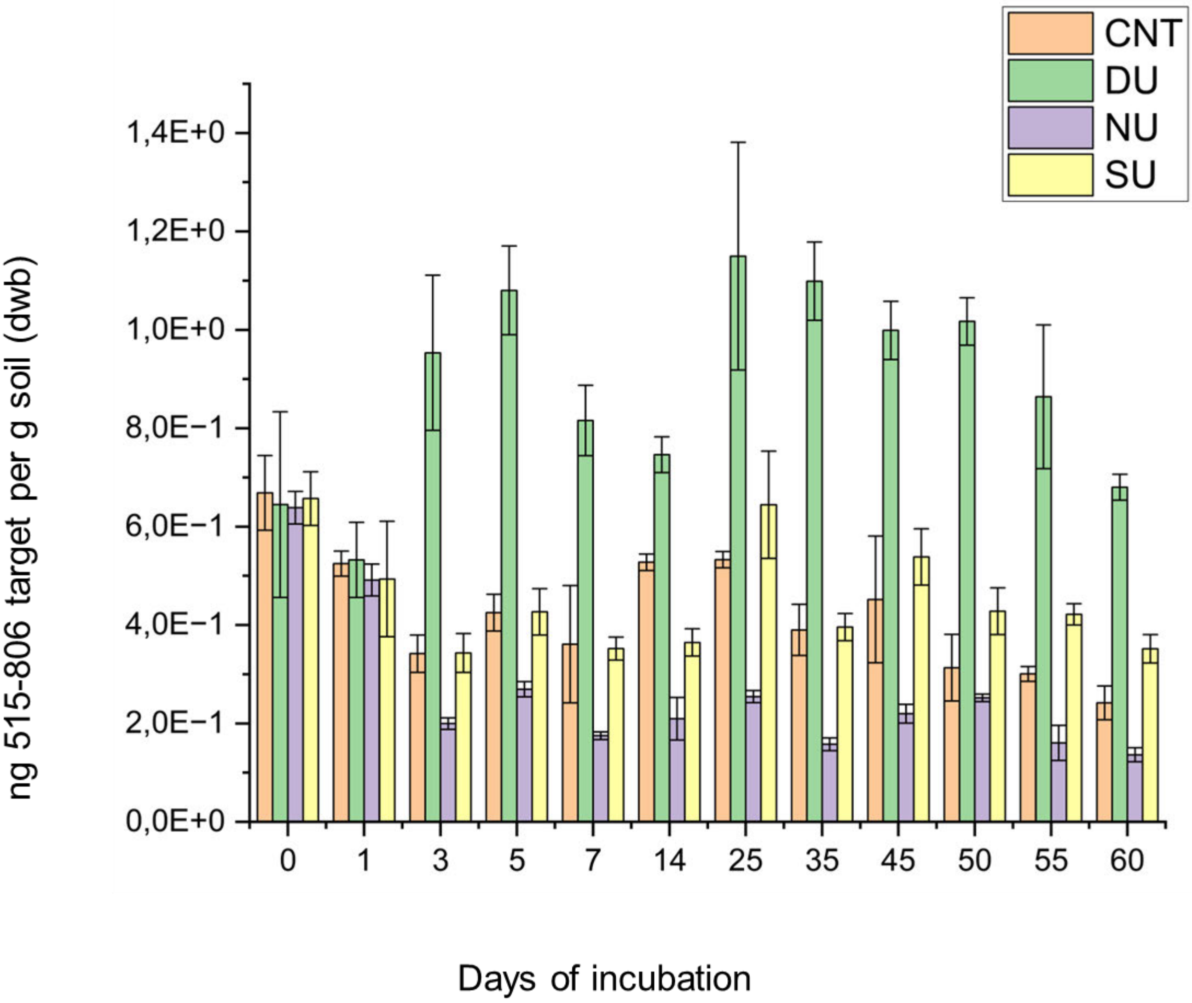

Figure 4. 5: Abundance of bacteria and archaea as indicated by the number of 16S rDNA copies measured using quantitative PCR. Error bars represent standard error (n = 4). CNT: control; DU: dried urine; NU: nitrified urine concentrate; SU: stored urine

4.3.6 Principal component analysis

The first two principal components accounted for 87 % of the total variability (PC1 68%; PC2 19%) between the soil chemical and microbiological parameters (Figure 4.6). Stored urine was characterized by arginase and β-glucosaminidase activity, and DNA concentration. The DU treatment was differentiated by high urease activity, abundance of functional genes, EON, MBN, CO_2 and N_2O emissions while the NUC was characterized by the EOC, and amount of ammonium and nitrate.

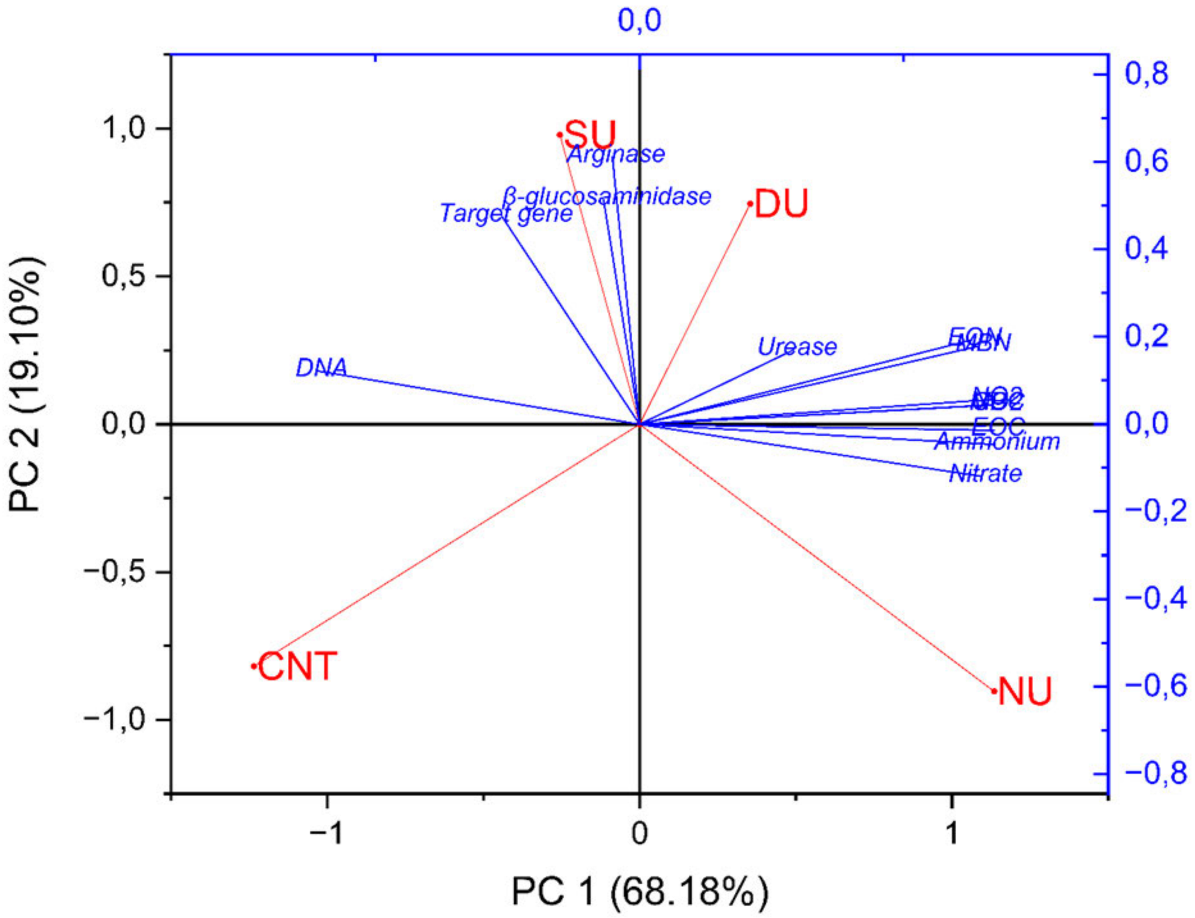

Figure 4. 6: Principal component (PC) biplot of PC 1 vs PC 2 of the relationships between chemical and microbiological components in the Inanda soil amended with human urine-derived fertilizers. CNT: control; DU: dried urine; NU: nitrified urine concentrate; SU: stored urine

4.4 DISCUSSION

Application of SU, NUC and DU influenced soil chemical and microbiological properties, soil enzyme activity and functional gene abundance. The general increase in NH_4^+ -N seen during the early stages of incubation could be explained by the conversion of the high amount of urea in urine. The decline during the later stages could be explained by nitrification, as evidenced by the associated increase in NO_3^- -N. Nitrified urine concentrate exhibited high NH_4^+ -N and NO_3^- -N throughout the incubation period due to their initial high content. Results indicated an increase in MBC and MBN after application of HUDF. In contrast, Lee and Jose (2003) reported a decrease in MBC and MBN after application of inorganic N fertilizers. Li *et al.* (2013) also found reductions in soil MBC (51%) and MBN (42%) in a column study following application of urea and ammonium sulphate. These contrasting results may be the result of variations in soil pH, organic matter, nutrient content and the initial status of the microbial communities. Furthermore, while the evaluated urine fertilizers are N-based sources, they also contain additional nutrients including calcium and magnesium (Table 4.2) which could promote an increase of MBC and MBN (Horn *et al.*, 2021).

The evolution of carbon dioxide is used to predict soil health and measure soil microbial activity (Castro Bustamante and Hartz, 2016), while nitrous oxide emissions are the result of the microbial process of denitrification, in which nitrate (NO_3^-) is converted to N_2 gas. Carbon dioxide production increased during the early stages of incubation (from 5 to 25 days) in the HUDF treated soil suggesting an immediate and significant increase in metabolic activity. The initial high CO_2 production rates from the SU-treated soil will have included some from increased microbial respiration as well as from the hydrolysis of urea. Additionally, solubilization of soil organic C has been reported to occur following urine application (Monaghan and Barraclough, 1993) which could provide sufficient substrate for increased metabolism. Nitrous oxide emissions are primarily produced by nitrification or denitrification depending on soil conditions. High N_2O fluxes in the NUC-treated soil are correlated significantly with denitrification activity when a high content of NO_3^- and NH_4^+ are present in the soil. The peak of N_2O emissions observed in all the HUDF treatments was due to the ammonium oxidation rate increasing and the resulting N_2O pool building up (Clough *et al.*, 2009).

The increase in β-glucosaminidase activity after HUDF application is in line with results reported by (Geisseler and Scow, 2014) in a meta-analysis, which showed a strong increase in the enzyme in N-fertilized soil based on 21 studies. The presence of hippuric acid in urine and the high pH of urine, both of which encourage urea hydrolysis, are likely to have contributed to the rise in urease activity in the DU and SU treatments (Hamamoto *et al.*, 2020). Generally, the highest soil enzyme activity was found in the DU treatment, and this can be attributed to its liming effect. The soil used in this study is acidic characterized by 30% acid saturation and an exchangeable acidity of 1.80 $cmol_c$ kg^{-1} (Table 3.1). Application of DU increases soil pH and may stimulate the microbial population and diversity, resulting in an increase in soil enzyme activity and thus affecting nutrient cycling (Acosta-Martinez and Tabatabai, 2000) .

According to Li *et al.* (2013) microbial activity is reduced and, in some cases, inhibited in acidic conditions as changes in H^+ concentration influence enzymes, substrates, and cofactors by altering their ionization and solubility. However, alkaline products such as lime (Randall *et al.*, 2016), and wood ash (Senecal and Vinnerås, 2017) are used to prevent urea hydrolysis and maximise N recovery during DU production, thereby contributing to the liming effect of DU that promotes microbial activity. Soil enzyme activities were generally reduced by application of NUC. During NUC production, water is extracted from nitrified urine by distillation, resulting in a concentrated nutrient stream. However, the NUC also contains salts, mainly sodium and chloride and the present results suggest that these may have decreased soil microbial activity. Cells become lysed and dried out because of the osmotic stress brought on by sodicity, thus soil microbial abundance is decreased.

Similar to enzyme activity, soil microbial abundance was highest in the DU treatment and lowest in the NUC treatment. The reduction in microbial abundance can be due to the harsh conditions caused by the high ammonium concentration in NUC. Most bacteria and fungi prefer ammonium as a source of N (Merrick and Edwards, 1995). However, due to the toxicity of ammonia, and an increase in ionic strength, ammonium fertilizers can suppress soil microbes when used at high rates (Eno *et al.*, 1955; Omar and Ismail, 1999). In addition, the acidic nature of the NUC could have also contributed to the low microbial abundance.

In the current study, microbial abundance of bacteria genes (16S) was significantly correlated to urease and β-glucosaminidase activity while chemical properties (NH_4, NO_3, EOC, EON, CO_2 and N_2O) were significantly correlated to microbial properties (MBC, MBN). However, according to Yang Ouyang (2018), edaphic properties rather than functional gene abundance drive N transformation processes (N fixation, nitrification, denitrification, anammox, and ammonification). In contrast, Petersen *et al.* (2012) reported that functional gene abundance was more important than soil edaphic properties in predicting N transformation. It is important to note that 16S sequence-based profiling used in this study only detects the bacterial and archea members of the soil community and excludes fungi, which are highly significant in decomposition and nutrient cycling.

4.5 CONCLUSION

This study investigated the impact of dried urine, NUC and stored urine on biological indicators of soil quality which include microbial abundance, extracellular enzymatic activities and microbial biomass C and N. In comparison to NUC and SU application, applying dried urine increased soil enzyme activity (β-glucosaminidase and arginase) and microbial abundance. Application of NUC increased the availability of ammonium and nitrate in the soil at least in the short-term. Overall, the abundance of microbial genes was significantly correlated with enzyme activity, while chemical properties (NH_4, NO_3, EOC, EON, CO_2 and N_2O) were significantly correlated to microbial properties (MBC, MBN). In general, application of HUDFs improved soil quality. However, the presence of pharmaceuticals in HUDF presents a challenge in respect of plant uptake. The following chapter investigates the uptake of some pharmaceuticals from soils fertilized with HUDF and evaluates the risk exposure associated with the consumption of crops grown with HUDF.

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CHAPTER 5: UPTAKE OF SELECTED ANTIRETROVIRALS BY PEPPER (*CAPSICUM ANNUM*), RADISH (*RAPHANUS SATIVUS*), AND RYEGRASS (*LOLIUM PERENNE*) GROWN ON TWO CONTRASTING SOILS AND FERTILIZED WITH HUMAN URINE-DERIVED FERTILIZERS

This chapter was published as

Migeri, S., Lawal, M. A., Hughes, J. C., Badza, T., Abafe, O. A., Martincigh, B. S., & Odindo, A. O. (2023). Uptake of selected antiretrovirals by pepper (*Capsicum annum*), radish (*Raphanus sativus*), and ryegrass (*Lolium perenne*) grown on two contrasting soils and fertilized with human urine-derived fertilizers. *Science of The Total Environment*, https://doi.org/10.1016/j.scitotenv.2023.164551

ABSTRACT

The use of urine-derived fertilizers has several economic and environmental advantages. However, there is concern that pharmaceuticals could enter the food chain after plant uptake and pose potential risks to human and animal health. A pot experiment was conducted to evaluate the uptake of nine target antiretrovirals (ARVs) by pepper (*Capsicum annum*), ryegrass (*Lolium perenne*) and radish (*Raphanus sativus*) grown in two soils of contrasting texture and organic matter content and fertilized with stored urine, nitrified urine concentrate (NUC) and struvite. Nevirapine was the only ARV detected in crops grown with NUC and struvite on both soils, but the concentrations were below the limit of quantification. Plants fertilized with stored urine absorbed lamivudine, ritonavir, stavudine, emtricitabine, nevirapine, and didanosine while abacavir, efavirenz and zidovudine were not detected. The ARVs detected in the soils after harvest were significantly higher in the soil with high organic matter and clay content. The estimated daily dietary intake (DDI) of ARVs by consumption of the pepper and radish fertilized with stored urine was compared to Threshold of Toxicological Concern (TTC) values based on the Cramer classification tree to assess direct human exposure. The calculated DDI for all ARVs were about 300-3000 times lower than the TTC values for class III compounds. Therefore, daily consumption of these crops fertilized with stored urine does not pose a health risk to the consumer. Future research is required to assess the impact of ARV metabolites, which may be more harmful to human health than the parent chemicals.

Keywords: human urine fertilizers, antiretrovirals, plant uptake, human health risk

5.1 INTRODUCTION

The use of source-separated human urine as fertilizer provides a viable option for managing waste, and minimising both environmental pollution and contamination of surface and groundwater. A variety of urine treatment systems have been developed with the goal of nutrient recovery, volume reduction, and pathogen and pharmaceutical elimination. In general, human urine is treated by either concentrating nutrients through eliminating water from urine or selectively extracting nutrients (Patel *et al.*, 2020). Nutrient concentration techniques include membrane distillation, nitrification distillation, and forward osmosis, whereas nutrient extraction is accomplished through adsorption, ion exchange, stripping, and precipitation (Simha *et al.*, 2020). Nitrified urine concentrate (NUC) is processed through biological nitrification and distillation. The nitrification process stabilizes the collected urine. The urine is oxidized into non-volatile nitrate (NO_3^-); and stabilized as non-volatile NH_4^+ (Udert *et al.*, 2016). Struvite ($MgNH_4PO_4.6H_2O$) is produced through urine precipitation, filtration, and drying (Li *et al.*, 2019a). The latest scientific and technological achievements in the safe recovery of nutrients from human urine have been reviewed by Simha and Ganesapillai (2017) and Alemayehu *et al*. (2020). The use of stored urine as a fertilizer has been demonstrated in a variety of crops (Karak and Bhattacharyya, 2011; Pandorf *et al.*, 2019; Alemayehu *et al.*, 2020a), while that of struvite was summarized in a meta-analysis review (Hertzberger *et al.*, 2020). Nitrified urine concentrate was evaluated in hydroponics (Mauerer *et al.*, 2018; Magwaza *et al.*, 2020) and greenhouse studies (Bonvin *et al.*, 2015; Mchunu *et al.*, 2018).The efficiency of urine-derived fertilizers was reported to be comparable to that of mineral fertilizers for both nitrogen (N) and phosphorus (P) (Martin *et al.*, 2020).

Although the use of urine-derived fertilizers has several economic and environmental advantages treatment techniques are unable to completely remove pharmaceuticals and so their widespread agricultural application remains problematic. A meta-analysis on the excretion pathways of 212 pharmaceuticals from the Swiss Pharmaceutical Compendium showed that 64% of each pharmaceutical was excreted via urine while 35% was via faeces (Lienert *et al.*, 2007b). This is of particular concern in fertilizers produced from source-separated urine as these pharmaceuticals could enter the food chain after plant uptake and pose potential risks to human and animal health (Abdel-Shafy and Mohamed-Mansour, 2013).

Plant studies have demonstrated the uptake of pharmaceuticals, however, in most cases plants were exposed to unrealistic concentrations (Carter *et al.*, 2014). The anticonvulsant carbamazepine is the most studied pharmaceutical for uptake studies in various plant species

(Goldstein *et al.*, 2014; Malchi *et al.*, 2014; Marsoni *et al.*, 2014; Mordechay *et al.*, 2018; Li *et al.*, 2019b). Carbamazepine has been reported to be frequently detected in treated wastewater and biosolids (McClellan and Halden, 2010; Jelic *et al.*, 2012) and to be relatively persistent in the environment (Mordechay *et al.*, 2018). However, it is important to note that the use of pharmaceuticals is geographically specific and consequently location determines the type of pharmaceuticals detected at wastewater treatment plants (WWTPs) (Wood *et al.*, 2015). Antiretrovirals (ARVs) are a significant pharmaceutical class to consider for plant uptake studies in South Africa because the country has the highest use of ARV therapy in the world with approximately 5 million people receiving ARV treatment (UNAIDS, 2017). The presence of ARVs has been reported at South African WWTPs (influent and effluent) (Abafe *et al.*, 2018) and in surface waters (Wood *et al.*, 2015). Nevirapine and efavirenz were detected in effluent sampled from WWTPs in Gauteng province (Schoeman *et al.*, 2017). Abafe *et al.* (2018) reported the persistence of atazanavir, efavirenz, lopinavir and nevirapine in effluent from selected WWTPs in KwaZulu-Natal province.

The aim of this study was to determine the uptake of some ARVs after application of urine-derived fertilizers by pepper (*Capsicum annum* var. California Wonder), radish (*Raphanus sativus* var. cherry belle) and ryegrass (*Lolium perenne* var. Matilda) grown in two contrasting soils. Additionally, the risk associated with the consumption of the pepper and radish was evaluated.

5.2 MATERIALS AND METHODS

5.2.1 Human urine-derived fertilizers

Urine used for this study was collected from household urine diversion toilets in KwaMpumuza, Pietermaritzburg, KwaZulu-Natal, South Africa. The urine was stored for approximately 2 months before use at a temperature range of 20 - 25 °C. The struvite and NUC were produced at Newlands Mashu research facility, Durban, South Africa (29°46′3.94″S; 30°58′44.16″ E), following procedures described by Udert *et al*. (2016). The fertilizers were analysed for chemical properties following standard methods for water and wastewater analysis (Rice *et al.*, 2012). Total N was analysed using a NOVA 60 Spectroquant® (Merck Millipore, Germany). Selected properties of the urine-derived fertilizers used are given in Table 3.2.

5.2.2 Soils

Samples were collected from the 0 - 30 cm depth of a clay loam (Inanda (Ia) form; Rhodic Hapludox) and a sand (Catref (Cf) form; Typic Haplaquept) Soil Classification Working Group, 2018; IUSS Working Group WRB, 2015, respectively). The Cf was collected from Kwadinabakubo, South Africa (29° 44' S; 30° 51' E) under natural grassland and the Ia from Worlds View, Pietermaritzburg, South Africa (29° 35' S; 30° 19' E) under a pine plantation. The soils were air-dried and sieved to < 2 mm for analysis. The hydrometer method outlined by Huluka and Miller (2014) was used for the analysis of soil particle size. The bulk density was determined from undisturbed soil cores following methods described by Blake (1965). Organic carbon was estimated by near-infrared reflectance, using the air-dry, milled soil samples. Extractable P was determined using Ambic-2 solution (Hunter 1974) followed by the molybdenum blue procedure (Murphy and Riley, 1962). Total inorganic N (NH_4^+ -N + NO_3^- -N) was extracted from freshly collected soils in a 1:5 soil :2M KCl suspension followed by filtering using Whatman ® No. 2 paper.The filtrates were then analysed using a Nova 60 Spectroquant® (Merck Millipore, Germany) according to standard methods (APHA 2005). Procedures outlined by Okalebo *et al.* (2002) were followed for the other soil analyses listed in Table 5.1.

Table 5. 1 : Selected chemical and physical properties of the Inanda and Catref soils

Property	**Inanda**	**Catref**
pH (KCl)	4.11	4.21
Organic C (%)	6.0	0.5
Total N (%)	0.56	0.05
Extractable P (mg kg^{-1})	12.0	0.7
Acid saturation (%)	30	18
Exch. acidity ($cmol_c$ kg^{-1})	1.80	0.18
Extractable K ($cmol_c$ kg^{-1})	0.07	0.01
Extractable Ca ($cmol_c$ kg^{-1})	3.2	0.4
Exchangeable Mg ($cmol_c$ kg^{-1})	0.0	0.4
Total cations ($cmol_c$ kg^{-1})	5.9	1.2
Bulky density (g cm^{-3})	0.80	1.43
Clay (%)	23	12
Silt (%)	48	15
Sand (%)	29	73

5.2.3 Experimental set up and management

The soils were air-dried, sieved through an 8 mm sieve, and packed into 10 L pots (radish and sweet pepper) or 2 L pots (ryegrass) after fertilizer application. Nitrogen, P and potassium (K) application rates were recommended by the Soil Fertility and Analytical Services Division Department of Agriculture, Cedara, KwaZulu-Natal, South Africa (Appendix 5.1). Stored urine and NUC were applied based on crop N requirements while struvite was applied based on the crop P requirement. Muriate of potash was applied to supplement for K in all treatments. The liquid fertilizers (NUC and stored urine) were diluted with 1 L of water before application, and struvite was applied directly to the soil. For the pepper trial, one seedling was transplanted into each pot in the evening to avoid heat stress and thus minimize the transplanting shock. Two seeds of radish were planted in each pot, while 5 g of ryegrass seeds were sown into each pot and lightly covered with soil. Plants were watered with tap water throughout the experiment by maintaining field capacity at 60–80%. Pepper fruits were harvested approximately 90 days after planting while radish bulbs and ryegrass were harvested 60 days after planting. After harvest, pepper fruits, radish bulbs and ryegrass leaves were freeze dried and ground and soil samples were collected, air dried and sieved (1 mm) for pharmaceutical analysis. The tunnel experiment was carried out under local environmental conditions, so that climate factors such as light, temperature and humidity were not controlled.

5.2.4 Experimental design

The experiment was laid out as a randomized complete block design (RCBD) and designed as a 3 x 3 x 2 treatment structure replicated three times to give 54 experimental units. The factors were three nutrient sources (stored urine, NUC and struvite), three crops (pepper, radish and ryegrass) and two soils (Catref and Inanda).

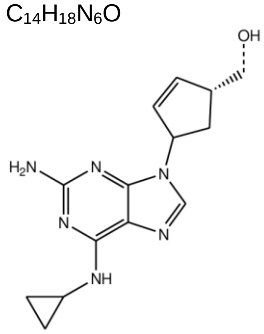
5.2.5 Pharmaceutical analysis

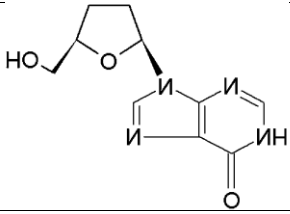
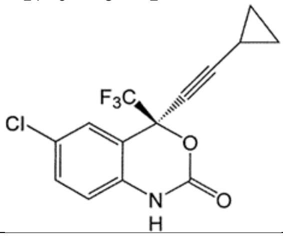
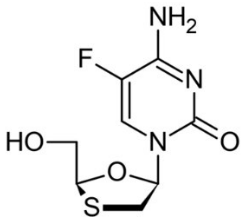
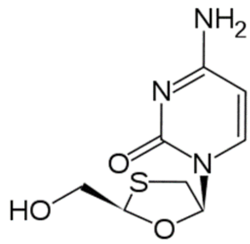
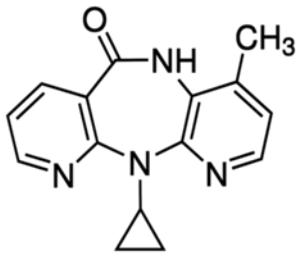
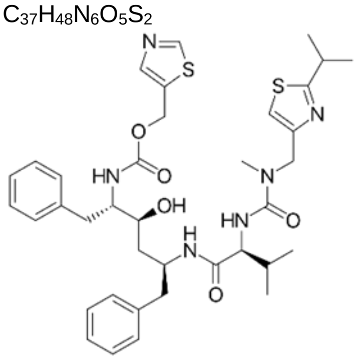
Urine fertilizers (stored urine, NUC and struvite), plant and soil samples were analysed in an ISO17025 accredited laboratory at the Agricultural Research Council, Pretoria. The extraction method of Garcia-Rodríguez *et al.* (2014) was modified for the extraction of ARVs in solid matrices. Briefly, 0.2 g aliquots of each plant and soil sample was spiked with 50 µg L^{-1} internal standard mixture containing enrofloxacin-d5 hydrochloric, flubendazole-d3, sulfamethoxazole-(phenyl- $^{13}C_6$) and nevirapine-d4. The samples were extracted with 12 mL of 1:1 methanol: water mixture in an ultrasonic bath set at 25 °C for 30 mins, followed by centrifugation at 6000 rpm for 10 mins. One millilitre of the supernatant was filtered through 0.22 µm nylon syringe filter prior to injection onto UHPLC-MS/MS.

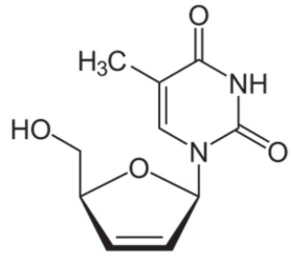
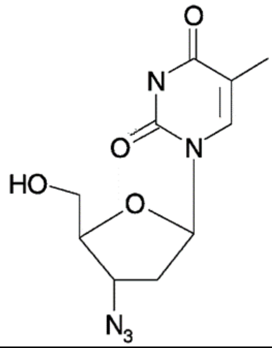
The analysis of ARV drug residues in the samples was achieved by using a Perkin Elmer QSight™ 220 triple quadrupole mass spectrometer coupled with a Perkin Elmer LX50 ultrahigh performance liquid chromatograph. The separation of ARVs was achieved with a Shim-pack GIST C18 column (100 × 2.1 mm, 1.9 µm particle size) with column oven temperature set at 25 °C and a mobile phase comprising of 0.1 % formic acid in (A) water and (B) methanol at constant flow rate of 0.3 mL min^{-1}. The gradient elution program modified from (Abafe *et al.*, 2018) is presented in Appendix 5.2. The injection volume was 10 µL. The retention time of each of the ARV is presented in Appendix 5.3.

The mass spectrometer was equipped with both a positive and negative polarity electrospray ionization (ESI) source which was used for the analysis of multiresidue ARV drugs by fast polarity switching. The electrospray voltage was set at 4000 V. Nitrogen was used as drying and nebulizer gas, set at 150 and 400 arbitrary units, respectively. The optimized hot surface-induced desolvation temperature was set at 320 °C, while the ion source temperature was set at 350 °C. The acquisition of ARVs was achieved by using the time-managed multiple reaction monitoring mode. Data were acquired by using Simplicity™ 3Q software (version 1.4.1806.29651). Selection of the target ARVs (Table 5.2) was based on those recommended for public sector ARV treatment in South Africa. Concentrations of the ARVs in each of the urine-derived fertilizers are given in Appendix 5.4.

Table 5. 2: Selected physico-chemical properties of the target antiretrovirals.

Compound	Pharmaceutical class	Structure and formula	Molecular mass g mol^{-1}	pKa	Log K_{ow}
Abacavir	Antiretroviral-nucleoside reverse transcriptase inhibitors	$C_{14}H_{18}N_6O$ 	86.33	5.01	1.22
Didanosine	Antiretroviral- nucleoside reverse transcriptase inhibitors	$C_{10}H_{12}N_4O_3$	236.2	9.13	-1.24

					
Efavirenz	Antiretroviral- non-nucleoside reverse transcriptase inhibitors	$C_{14}H_9ClF_3NO_2$ 	315.67	10.2	4.7
Emtricitabine	Antiretroviral- nucleoside reverse transcriptase inhibitors	$C_8H_{10}FN_3O_3S$ 	247.24	2.65	-0.43
Lamivudine	Antiretroviral- nucleoside reverse transcriptase inhibitors	$C_8H_{11}N_3O_3S$ 	229.26	4.3	-9.54
Nevirapine	Antiretroviral-non-nucleoside reverse transcriptase inhibitors	$C_{15}H_{14}N_4O$ 	266.30	2.8	3.89
Ritonavir	Antiretroviral-protease inhibitors	$C_{37}H_{48}N_6O_5S_2$ 	720.9 g	2.6	6.27
Stavudine	Antiretroviral-nucleoside reverse transcriptase inhibitors	$C_{10}H_{12}N_2O_4$	224.21 g/mol	9.95	-0.72

					
Zidovudine	Antiretroviral- nucleoside analogue and reverse transcriptase inhibitor	$C_{10}H_{13}N_5O_4$ 	267.24	9.6	0.05

Data from https://pubchem.ncbi.nlm.nih.gov/.

5.2.6 Bioconcentration

Bioconcentration factors were calculated from Equation 5.1:

$$BCF = \frac{Ccrop}{Csoil} \text{ .... Equation 5.1}$$

Where: C_{crop} is the concentration of an antiretroviral in a specific organ (ng g^{-1} dry weight (dw)); C_{soil} is its concentration in the soil (ng g^{-1} dw) at the end of the experiment.

5.2.7 Human exposure and risk assessment

Estimated daily dietary intake (DDI) of ARVs by consumption of the pepper and radish grown using the urine fertilizers was compared to Threshold of Toxicological Concern (TTC) values based on the Cramer classification tree. This method has been used to assess the risks of drinking water that contains 10,11-epoxycarbamazepine (Houeto *et al.*, 2012) and consumption of carrots and sweet potatoes irrigated with treated wastewater (Malchi *et al.*, 2014).

The DDI of each chemical per kilogram bodyweight (ng kg^{-1} bw) was calculated (García *et al.*, 2019) using Equation 2.

$$DDI = \frac{Ccropxconsumption}{bw} \text{ .... Equation 5.2}$$

Where: C_{crop} is the detected ARV concentration (ng g^{-1}); consumption is the South African average consumption of pepper and radish (35 and 40 g d^{-1}, respectively); bw is the South

African average bodyweight (70 kg) (National Department of Health 2019) . The concentrations of ARVs in the two crops were converted to a fresh weight basis using the average water content of each crop (95.3% for pepper, and 91.4% for radish).

Compound classification and TTC values were determined using Toxtree software, which is an open-source software that was commissioned for development by the European Commission Joint Research Centre's European Chemicals Bureau (ECB) solely for the purpose of determining the Cramer classification of chemical compounds (Bhatia *et al.*, 2015). The Cramer decision tree classifies materials into one of three classes (I – low, II – intermediate and III – high). The TTC values are 30, 9 and 1.5 g kg^{-1} bw per day for Cramer Classes I, II, and III, respectively. The likelihood that chemicals might have negative impacts on health is low for exposures below the TTC levels (Committee *et al.*, 2019). A non-TTC technique is necessary to determine any potential negative health consequences if the expected exposure to a chemical is higher than the pertinent TTC value (Blackburn *et al.*, 2020).

Target hazard quotients, expressed as the ratio of exposure to reference dose, are used to present risk for noncarcinogenic effects. The US Environmental Protection Agency (EPA) defines a reference dose as "an estimate of a daily oral exposure to a chemical to the human population including sensitive subpopulations that is likely to be without risk of deleterious non-cancer effects during a lifetime." The consumption of plants grown in human excreta-amended soils represents a single pathway of human exposure. Therefore, a hazard quotient of ≥ 0.1 was identified as a potential hazard to human health. The hazard quotients calculated for each individual antiretroviral were added together as a conservative approach to determining the risk of a mixture of PPCPs in plant tissues to human health.

5.2.8 Data analysis

Data were subjected to analysis of variance (ANOVA) using GenStat□ Version 18 (VSN International, UK). Means of significantly different variables were separated using Least Significant Differences (LSD) at P = 0.05.

5.3 RESULTS AND DISCUSSION

5.3.1 Antiretrovirals in plants

5.3.1.1 Stored urine treatment

Pepper, radish, and ryegrass treated with stored urine absorbed six of the nine target ARVs in detectable amounts. Lamivudine, ritonavir and stavudine were taken up by pepper, radish, and ryegrass grown in both soils (Figure 5.1). Lamivudine and ritonavir were detected at significantly higher concentrations ($P < 0.05$) in pepper than radish and ryegrass grown in both soils (Figure 5.1A, B). Stavudine was taken up in the order of radish > pepper > ryegrass on both soils (Figure 5.1 C). The only plant that absorbed emtricitabine was radish (Figure 5.1 D), with a significantly higher concentration ($P < 0.05$) when grown in Ia soil than Cf soil (2.43 and 1.77 ng g^{-1} dw, respectively). For the Cf soil, nevirapine was absorbed by both pepper and radish, whereas on the Ia soil it was only detected in pepper (Figure 5.1E). Didanosine was taken up to the greatest extent of all the detected ARVs with measured concentrations up to 7.8 ng g^{-1} dw (Figure 5.1 F). However, it was only found in pepper grown on both soils.

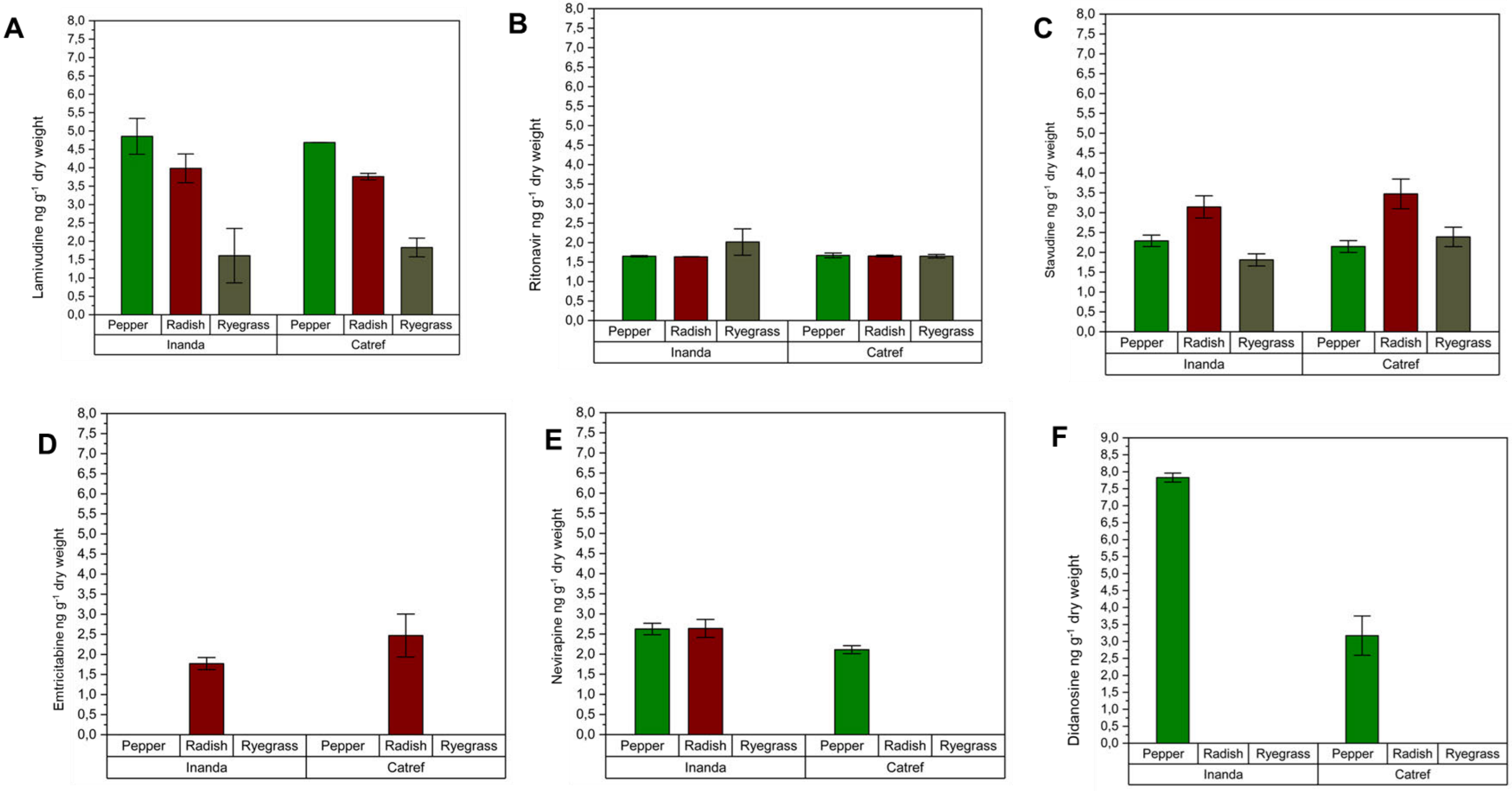


Figure 5. 1: Concentration (ng g^{-1} dry weight) of (A) lamivudine, (B) ritonavir, (C) stavudine, (D) emtricitabine, (E) nevirapine, and (F) didanosine in pepper, radish and ryegrass grown on Catref and Inanda soils fertilized with stored urine.

The uptake of a particular pharmaceutical by a plant depends on its physicochemical properties. Molar mass is an important factor associated with membrane permeability (Li *et al.*, 2019b). Pharmaceuticals are readily absorbed by plants when their molar mass is less than 1000 g mol^{-1} (Zhang *et al.*, 2017). The molar masses of the detected ARVs range from 224.2 - 720.9 g mol^{-1}, with ritonavir having the largest (Table 5.2). The other five detected ARVs with a molar mass < 270 g mol^{-1} (lamivudine, stavudine, emtricitabine, nevirapine and didanosine) were absorbed at significantly higher concentrations than ritonavir. According to Goldstein *et al.* (2014), non-ionic pharmaceuticals can easily cross membranes while moving from the xylem to the phloem. As a result, they are mainly transported in the direction of the transpiration stream and primarily accumulate in the leaves. Ionic pharmaceuticals are repelled by the negatively charged cytosol and accumulate to a greater extent in the fruit. In this study ARVs accumulated more in the pepper fruit and radish bulbs than the ryegrass leaves. The hydrophobicity of a pharmaceutical is typically used for interpretation of uptake of organic compounds into plant roots. A positive linear relationship between the root uptake and chemical hydrophobicity was reported for neutral compounds so that pharmaceuticals with high octanol - water partition coefficient (K_{OW}) values are easily absorbed by plants (Wu *et al.*, 2013). This current study demonstrates contrary results, hydrophobic ARVs (K_{OW} 3.89-6.27) which include efavirenz, nevirapine and ritonavir were absorbed to a lesser extent than hydrophilic ARVs (K_{OW} -0.72-1.22). This suggests that factors other than hydrophobicity might have an impact on how certain ARVs are absorbed.

In addition to the properties of the pharmaceutical compounds, plant uptake is also influenced by the physical and chemical properties of the soil. With the exception of ritonavir and stavudine, ARV concentrations detected in plants grown on Cf soil were significantly higher than found in those grown on Ia soil. This trend was probably due to the higher organic matter content of Ia soil (10.32%) than Cf soil (0.86%). High soil organic matter has been reported to reduce plant uptake of pharmaceuticals because compounds are strongly bound to soil organic matter, hindering availability for plant uptake (Fu *et al.*, 2016; Paz *et al.*, 2016; Mordechay *et al.*, 2018). Concentrations of ARVs available for uptake by plants may also be altered by abiotic and microbial transformation processes in the soil (Miller *et al.*, 2016). Enzymes, such as laccases and peroxidases, are secreted by plants and rhizosphere-associated microbes, and they can transform pharmaceuticals (Martin *et al.*, 2014). A higher microbial activity results from rhizosphere microorganisms utilising carbohydrates in root exudates as a carbon source.

The lipid and carbohydrate content of root cell walls, which impact the permeability of root cell membranes, are the main biological factors in plants that influence pharmaceutical uptake (Keerthanan *et al.*, 2021). Pepper, radish and ryegrass have different root systems, leaf sizes and lipid content. Additionally, the ARV analysis targeted the harvestable and edible components of the crops. Pepper fruits absorbed five of the six detected ARVs. Radish bulbs followed the same trend except that they absorbed emtricitabine instead of didanosine, while ryegrass leaves only absorbed lamivudine, ritonavir and stavudine. The detected ARVs were absorbed at significantly ($P<0.05$) higher concentrations in pepper fruits than radish bulbs and ryegrass, except for stavudine and nevirapine. The differences in ARV uptake between plant species may be attributed to differences in their metabolic systems, which may involve a network of enzymatic reactions, and growth and transpiration rates (Coleman *et al.*, 1997; Wu *et al.*, 2013). Ryegrass has been reported to have a high root lipid content (Huang *et al.*, 2010), which may explain the limited uptake of ARVs by ryegrass in comparison with pepper fruits and radish bulbs.

5.3.1.2 Nitrified urine concentrate and struvite treatments

Nevirapine was the only ARV absorbed by the three crops fertilized with NUC and struvite in both soils although all the concentrations were below the limit of quantification (< LOQ). Duygan *et al.* (2021) reported significant elimination of ARVs including atazanavir, darunavir and ritonavir during the nitrification process of NUC production and this may account for the absence of the target ARVs. Nevertheless, the current study's use of nitrification of urine alone does not guarantee complete elimination of pharmaceuticals (Etter *et al.*, 2015). Several post nitrification processes for pharmaceutical removal have been developed such as microfiltration and electrodialysis in combination with nanofiltration (Pronk *et al.*, 2006), and ozonation combined with ultraviolet light and hydrogen peroxide (Dodd *et al.*, 2008). However, the reactivity of the oxidants with other compounds in the urine matrix limits the efficacy of both procedures (Zhang *et al.*, 2015). Addition of powdered activated carbon as a post nitrification process has been successfully tested to remove approximately 90% of pharmaceuticals (Köpping *et al.*, 2020). In contrast, struvite production has been reported to be inefficient in terms of pharmaceutical elimination. According to a study by Ronteltap *et al.* (2007), 98% of each of the seven drugs that were examined during the synthesis of struvite were still present in the liquid phase of struvite following precipitation. Also, the filtration and drying processes do not remove pharmaceuticals that remain after precipitation of struvite (Bischel *et al.*, 2015b). However, pharmaceutical plant uptake studies have demonstrated limited/no uptake from struvite. de Boer *et al.* (2018) determined bioaccumulation of five common pharmaceuticals (propranolol, diclofenac, sulfamethoxazole, ibuprofen and carbamazepine) by tomato fruits fertilized with spiked struvite and detected no pharmaceuticals. Results from the present study indicate that the uptake of ARVs into pepper, radish and ryegrass grown fertilized with struvite and NUC under controlled conditions is limited.

5.3.2 Antiretrovirals in soils

The concentrations of ARVs in the soils fertilized with stored urine at the end of the experiment showed a wide range from < LOQ to 3.138 ng g^{-1} dw (Table 5.3). Generally, ARV concentrations detected in soil were significantly lower than those detected in plants. Lamivudine was only detected in quantifiable concentrations in the Cf planted to radish while it was not detected or was < LOQ in the remaining treatments. Ritonavir was detected in all treatments except in the Ia under pepper production. Ritonavir concentrations detected in the Ia were significantly ($P < 0.05$) higher than in the Cf. Stavudine was not detected in the Ia and was < LOQ in the Cf planted to radish. In the remaining treatments the lowest and highest concentrations of stavudine were detected in the soils planted to pepper at 0.712 ng g^{-1} (Cf) and 1.461 ng g^{-1} (Ia). Except in the Cf under ryegrass, where concentrations were < LOQ, emtricitabine was not detected in all treatments. In addition to the ARVs identified in plant samples, efavirenz was detected in soil, although it was only quantifiable in the Ia planted to radish and ryegrass.

The main factors that influence pharmaceutical persistence in soil are its photostability, binding and adsorption potential, rate of breakdown, and leaching (Wu *et al.*, 2015b). Pharmaceuticals undergo sorption/desorption and transformations after being introduced to soil. The production of non-exchangeable or bound residue with much lower bioavailability can be the outcome of the pharmaceutical's sorption and transformation (Wu *et al.*, 2015b). The most significant pharmaceutical sorption mechanisms include sorption to organic matter, surface adsorption to mineral components, ion exchange, complex formation with metal ions such as Ca^{2+}, Mg^{2+}, Fe^{3+} or Al^{3+}, and H-bonding (Tolls, 2001). All ARVs detected in soil are neutral compounds which have been reported to be more recalcitrant and persistent while weakly acidic pharmaceuticals exhibit rapid degradation because they have carboxylic groups that are more susceptible to microbial transformations (Grossberger *et al.*, 2014).

These results demonstrate the ability of the two soils to adsorb ARVs from stored urine, potentially endangering environmental health especially of soil microbes. Antiretrovirals are created to be active at low concentrations, therefore they may pose an ecotoxicological risk to the environment. Soil microorganisms would be of major concern especially those involved in key ecosystem services such as nutrient biogeochemical cycling (Caracciolo *et al.*, 2015). A significant concern with regard to antimicrobial (antiviral, antibacterial, and antifungal) pharmaceuticals is the potential for resistance to be strengthened and propagated in a way that endangers crucial medications for human and animal treatment (Patel *et al.*, 2019).

Table 5. 3: Concentration (ng g^{-1} dry weight) of antiretrovirals in Catref and Inanda soils planted to pepper, radish and ryegrass and fertilized with stored urine, nitrified urine concentrate (NUC) and struvite.

	Catref			Inanda		
Antiretroviral	Pepper	Radish	Ryegrass	Pepper	Radish	Ryegrass
Stored urine						
Lamivudine	ND	1.431	ND	ND	< LOQ	ND
Ritonavir	1.128	1.118	1.184	ND	1.151	1.264
Stavudine	0.712	< LOQ	1.344	1.461	ND	1.382
Emtricitabine	ND	ND	< LOQ	ND	ND	ND
Efavirenz	< LOQ	< LOQ	ND	< LOQ	0.967	1.348
Nevirapine	1.260	ND	1.248	ND	1.473	1.378
Didanosine	3.040	3.138	ND	ND	2.353	ND
NUC						
Nevirapine	<LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Struvite						
Nevirapine	0.671	0.659	< LOQ	0.710	0.795	< LOQ

< LOQ: below limit of quantification, ND: not detected

Nevirapine was detected and quantified in struvite treated soils at the end of the experiment with concentrations ranging from 0.659-0.795 ng g^{-1} dw while NUC-treated soils exhibited concentrations <LOQ (Table 5.3). There was no significant difference of nevirapine concentrations in either soil between those planted to pepper and radish. However, nevirapine concentrations were higher in the high organic matter, clay soil (Ia). Nevirapine has planar aromatic structures (Table 5.2) that are ideal for intercalation in various clay minerals (Lambert, 2018) creating a possibility for adsorption in the interlayer spaces of the minerals of soils with higher clay content, that would render it less available for plant uptake .

5.3.3 Bioavailability of antiretrovirals in stored urine-treated plants

Bioconcentration factor (BCF) values were computed based on the pharmaceutical concentration in bulk soil in order to better understand the accumulation potential of lamivudine, ritonavir and stavudine in pepper, radish and ryegrass (Figure 5.2). The BCF was not calculated for NUC and struvite treatments as ARVs were not detected or were < LOQ in both plants and soil. Bioconcentration factors for all three ARVs varied significantly in pepper. The stavudine BCF was significantly higher in peppers grown on Cf soil than those on Ia soil. Generally, the BCF for lamivudine was about twice that for ritonavir in radish on the Cf soil. Although both lamivudine and ritonavir are weak bases, ritonavir is hydrophobic, thus, its bioavailability for plant uptake is limited (Fu *et al.*, 2016). Ryegrass showed a higher BCF for stavudine than ritonavir catref soil. However, the BCF for ritonavir was < 1, indicating its limited availability and accumulation in ryegrass. This is contrary to the proposed accumulation of non-ionic pharmaceuticals in transpiring organs such as leaves (Wu *et al.*, 2013; Goldstein *et* *al.*, 2014; Mordechay *et al.*, 2018). The limited uptake of ritonavir compared with that of stavudine is probably a result of their contrasting log K_{ow} and molar mass (6.02 and 720.2 g mol^{-1}; -0.72 and 224.1 g mol^{-1}, respectively).

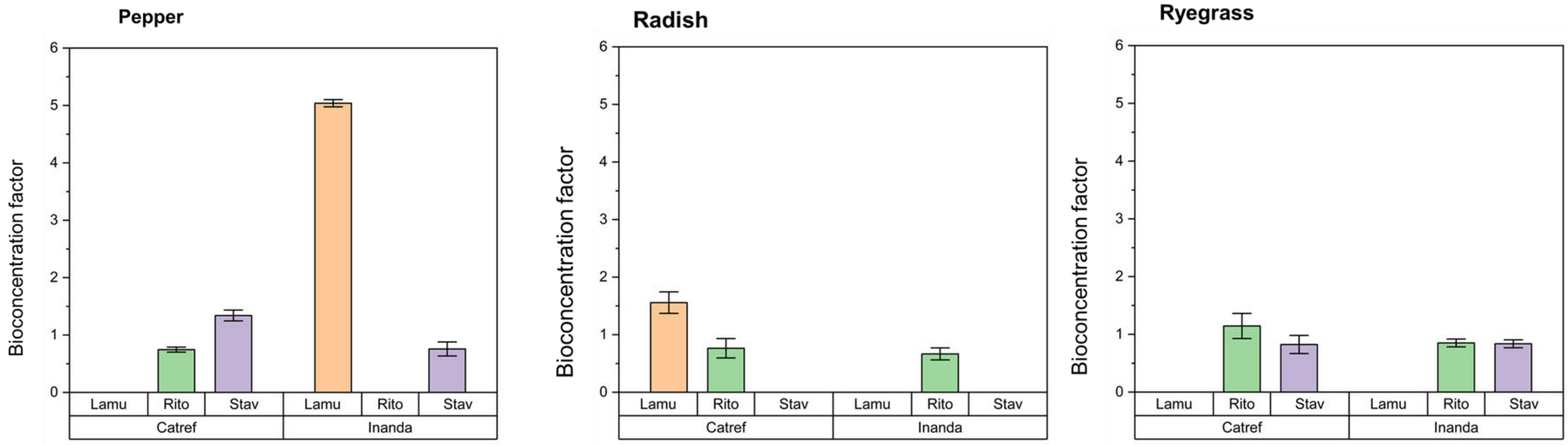


Figure 5. 2: Bioconcentration factors (mean ± SE) for lamivudine (Lamu), ritonavir (Rito) and stavudine (Stav) in pepper, radish and ryegrass grown in Catref and Inanda soils and fertilized with stored urine. n = 3.

5.3.4 Human exposure and risk assessment

The assessment of human exposure through dietary intake of pepper and radish grown in soils fertilized with stored urine is presented in Table 5.4. Ryegrass was excluded from this assessment because its primarily cultivated for livestock feed. The NUC and struvite treatments were also excluded from the evaluation due to the non-quantifiable ARV concentrations in these treatments. All six assessed ARVs were classified as Class III compounds based on the Cramer classification tree (Appendix 5.5-5.10). The DDI of ARVs through consumption of pepper grown on both soils was highest for didanosine and lowest for ritonavir (Table 5.4). In radish, lamivudine exhibited higher DDI for both soils. The calculated DDI for all ARVs were about 300-3000 times lower than the TTC value for class III compounds. According to Prosser and Sibley (2015) for consumption to be considered safe, there should be a considerable gap (> factor 10 to factor 1000) between the DDI and the lowest therapeutic dose, in this study the TTC value. This is the case for all assessed ARVs.

Table 5. 4: Estimated daily dietary intake (DDI) of antiretrovirals by consumption of pepper and radish grown on the Catref and Inanda soils and fertilized with stored urine. In all cases the Threshold of Toxicological Concern (TTC) value was 1500 ng kg^{-1} bw day^{-1} and all antiretrovirals were in Class III (Cramer classification)

Soil	Antiretroviral	DDI (ng kg^{-1} bw day^{-1})		Adult intake to exceed TTC (kg day^{-1})	
		Pepper	Radish	Pepper	Radish
Catref	Lamivudine	1.9	1.8	27.6	33.3
	Ritonavir	0.5	0.5	105.0	120.0
	Stavudine	0.7	1.2	75.0	50.0
	Emtricabine	nd	0.7	-	89.6
	Nevirapine	1.6	0.8	32.0	75.0
	Didanosine	4.5	nd	11.7	-
Inanda	Lamivudine	1.8	1.6	29.2	37.5
	Ritonavir	0.5	0.6	105.0	100.0
	Stavudine	0.6	1.2	87.5	50.0
	Emtricabine	nd	0.9	-	66.7
	Nevirapine	1.4	nd	37.5	-
	Didanosine	1.9	nd	27.6	-

nd: not detected

The amount of pepper required for daily consumption to reach the TTC value ranges from 11.7 to 105 kg in adults. The evaluated ARVs would require an adult to consume approximately 30-120 kg of radish daily to reach the TTC value. Clearly this is an unrealistic consumption, and therefore these results demonstrate that the daily consumption of pepper and radish fertilized with stored urine does not pose a health threat to the consumer.

5.4 CONCLUSION

This study investigated the plant uptake of a range of ARVs from two contrasting soils fertilized with human urine-derived fertilizers. Plant uptake of these compounds from NUC and struvite was very limited. Soil application of stored urine resulted in uptake of some of the ARVs into pepper fruits, radish bulbs, and ryegrass leaves. Antiretroviral drugs were more available for uptake in the plants grown on the soil with low organic matter and clay content. Nevirapine was the only ARV quantified in struvite fertilized soils. The daily consumption of pepper and radish fertilized with stored urine does not present a health risk to the consumer. However, more field work and research are necessary to confirm these findings. Additionally, the eco-toxicological potential effects of human urine-derived fertilizers on soil microbiota remain a potential concern. Further studies are also necessary to evaluate the effects of ARV metabolites that could have a greater impact on human health than the parent compounds.

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CHAPTER 6: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 INTRODUCTION

The use of human excreta for crop production has been a common practice in certain countries for centuries. However, it is only recently that urine treatment technologies have been developed for the recovery of water and nutrients necessary for crop production. Several studies have demonstrated the effect of human urine-derived fertilizers (HUDF) on crop production. However, mineral and urine fertilizers' interactive effects have not been explored. Additionally, uncertainties regarding the impact of HUDF on soil microbial communities, potential plant uptake of pharmaceuticals and risk to human health remain a major challenge in adopting these fertilizers. Therefore, this study was to assist in understanding the effects of combining mineral and urine fertilizers, the impacts of urine fertilizers on soil microbial communities, the uptake of selected antiretrovirals and the assessment of human health risks associated with consumption of crops fertilized with HUDF.

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Write a discussion paragraph focusing on how these three distinct studies are linked in terms of the rationale and the results obtained. The objectives are to assess the combination effect of nitrogen and phosphorus in mineral and human urine-derived fertilizers on maize production in two contrasting soils, to investigate the effect of urine fertilizer application on soil biological properties as indicators of soil quality, and To determine the uptake of selected ARVs after application of human urine-derived fertilizers by pepper, radish and ryegrass grown in two contrasting soils and assess the risk associated with consumption of crops fertilized with human urine-derived fertilizers.Three distinct studies converge on a shared theme of exploring the multifaceted impacts of urine-derived fertilizers on agricultural productivity and soil health, with implications for human health. Firstly, the investigation into the combined effect of nitrogen and phosphorus from both mineral and human urine-derived fertilizers on maize production elucidates the potential of urine-based fertilization as a sustainable alternative to conventional mineral fertilizers. Secondly, the assessment of soil biological properties subsequent to urine fertilizer application serves as a vital component in evaluating soil quality, reflecting the broader ecological ramifications of agricultural practices. Lastly, the study on the uptake of antiretroviral drugs (ARVs) by crops fertilized with human urine-derived fertilizers underscores the intricate linkages between agricultural inputs and food safety, highlighting the necessity of scrutinizing potential risks associated with unconventional fertilization methods. Collectively, these studies underscore the interconnectedness between agricultural practices, soil health, and food safety, emphasizing the imperative for holistic approaches in sustainable agriculture that consider both ecological and human health dimensions. The following sections provide concise conclusions and key recommendations for the specific research work carried out.

To assess the combination effect of nitrogen and phosphorus in mineral and human urine-derived fertilizers on maize production in two contrasting soils

This study presented a new opportunity for combining HUDF and mineral fertilizers as nitrogen (N) and phosphorus (P) sources for crop production. Maize growth and yield components showed varying responses to soil type and HUDF and mineral fertilizer combinations. NUC and dried urine as N sources combined with single superphosphate as a P source showed the best performance in both soils concerning biomass, grain yield, and yield-related variables. The significance of urine treatment technologies for nutrient recovery was demonstrated by the constantly lower yield observed in the stored urine treatment. The NUC, struvite, and dried urine from treatment technologies are odourless, and almost free of hormones, pharmaceuticals, and heavy metals. These factors play a major role in accepting HUDF in crop production. Transportation, storage, and application of struvite and dried urine are simpler than those of urine due to their lower weight, volume, and dry nature. This study showed that LAN and dried urine are the most efficient sources of N in the Inanda and Catref soils, respectively. In contrast, struvite and SSP are the most efficient P sources in the Inanda and Catref soils, respectively. Future studies should focus on field trials and crops beyond short-season crops, including fruit trees.

To investigate the effect of urine fertilizer application on soil biological properties as indicators of soil quality.

The application of HUDF increased the availability of nutrients in the soil. Nitrified urine concentrate-treated soils had significantly higher ammonium and nitrate-N, MBC and MBN, and CO_2 and N_2O emissions. Dried urine had a stronger effect than other urine fertilizers on soil enzyme activity and abundance of total bacteria and archaea. Microbial abundance was less significant than the soil chemical and microbiological properties in explaining the variation in corresponding enzyme activity. Overall, microbial abundance was significantly correlated with enzyme activity, while the CO_2 and N_2O emissions were significantly correlated to MBC, and MBN. Long-term and field-based studies are recommended to understand the realistic effects of HUDF on soil microbial communities. Additionally, microbial diversity and community structure in soil fertilized with HUDF should be explored.

To determine the uptake of selected ARVs after application of human urine-derived fertilizers by pepper, radish and ryegrass grown in two contrasting soils and assess the risk associated with consumption of crops fertilized with human urine-derived fertilizers.

This study investigated the plant uptake of nine target ARVs from two contrasting soils fertilized with HUDF. Plant uptake from NUC and struvite-treated soils was limited. Soil application of stored urine resulted in six of the target ARVs' uptake into pepper fruits, radish bulbs and ryegrass leaves. Antiretrovirals were more available for uptake in the plants grown on the soil with low organic matter and clay content. Nevirapine was the only ARV quantified in struvite-fertilized soils. The daily consumption of pepper and radish fertilized with stored urine does not present a health risk to the consumer. Although various pharmaceuticals have been studied regarding their uptake and metabolization in plants, there is still a lot of work to be done in the future, with significant groups of pharmaceuticals still absent entirely. It would be beneficial to create a priority list of pharmaceuticals with the largest plant uptake potential under realistic field settings so that research and assessment may be more focused. This prioritization would maximize investment in research and generate information that may be used to develop guidelines and mitigation options to minimize the potential risks associated with using human excreta-derived fertilizers. Furthermore, this information could be used in developing predictive models that do not overpredict pharmaceutical plant uptake.

6.2 Future perspective on resource recovery sanitation innovations

Implementing resource recovery sanitation innovations goes beyond technical knowledge, as social norms and local policy frameworks influence it. In most cases, existing policies inhibit resource recovery sanitation technologies because innovation may result in the development of new technology systems that are not covered by current policies and regulations (Kvarnström *et al.*, 2011). A study conducted by McConville *et al.* (2022) in Uganda on the possibilities for changing resource recovery in Kampala's on-site sanitation regime revealed that approximately 99% of Greater Kampala's population is connected to on-site sanitation or faecal sludge management regimes. However, the technology policy on building regulations and prohibiting human waste in organic farming negatively affects the on-site innovations. Furthermore, policies on the circular economy and locally produced fertilizers are too general to make a difference in using human waste in crop production. Regular reviewing, reframing and updating existing policies and legal frameworks to allow an enabling environment for resource recovery is recommended.

The South African policy and legal environment, on the other hand, encourages the scaling up of resource recovery technologies (Musazura and Odindo, 2022). However, several blockages remain, including a lack of collaboration and coordination between government departments concerning resource recovery, lack of financial support from the government to promote resource recovery, absence of established markets and unwillingness to pay for the end product (Gwara *et al.*, 2020). McConville *et al.* (2022) defined sanitation solutions as socio-technical systems; therefore, such systems need to be built on transdisciplinary competencies, including every stakeholder within the resource recovery value chain, such as end users, technocrats and policymakers.

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APPENDICES

APPENDIX 5. 1: Recommended nutrient application rates (kg ha^{-1}) for the crops and soils used

Crop	Catref			Inanda		
	N	P	K	N	P	K
Radish	120	80	80	100	80	50
Ryegrass	200	60	50	140	50	50
Pepper	160	80	50	120	80	50

APPENDIX 5. 2: Linear gradient used to achieve separation of the antiretrovirals

Time (min)	% A	% B
0.00	85	15
1.50	85	15
6.00	0	100
8.00	0	100
8.01	85	15
10.50	85	15

APPENDIX 5. 3 : Mean retention times (RT) for the antiretrovirals

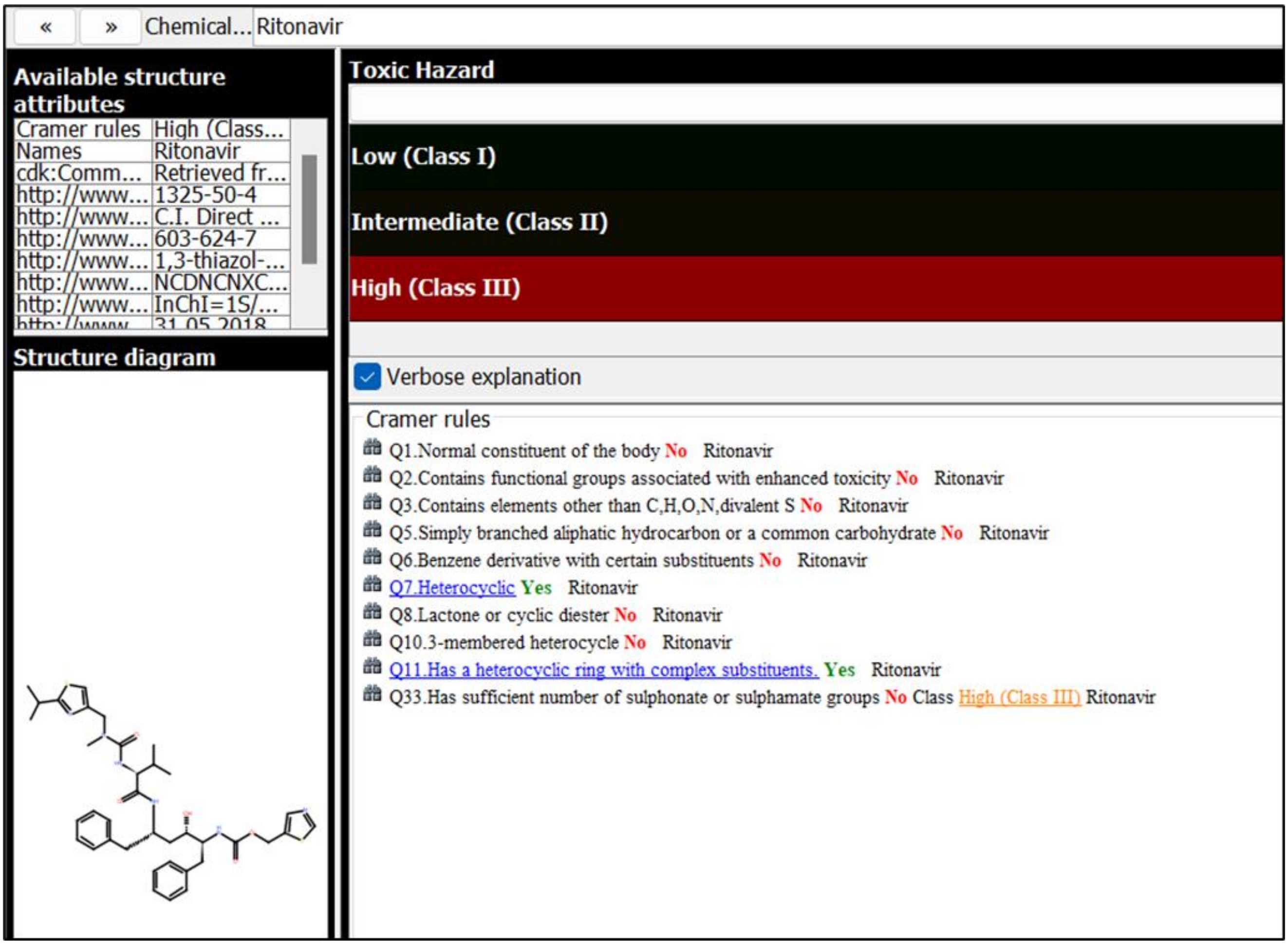
ARV	Mean RT (SD)/min
Abacavir	4.80 (0.01)
Efavirenz	7.44 (0.02)
Emtricitabine	3.94 (0.01)
Lamivudine	2.03 (0.02)
Lopinavir	7.56 (0.02)
Nevirapine	6.06 (0.01)
Ritonavir	7.38 (0.01)
Tenofovir	6.19 (0.01)
Didanosine	4.01 (0.02)

Stavudine	4.24 (0.02)
Zidovudine	5.30 (0.01)

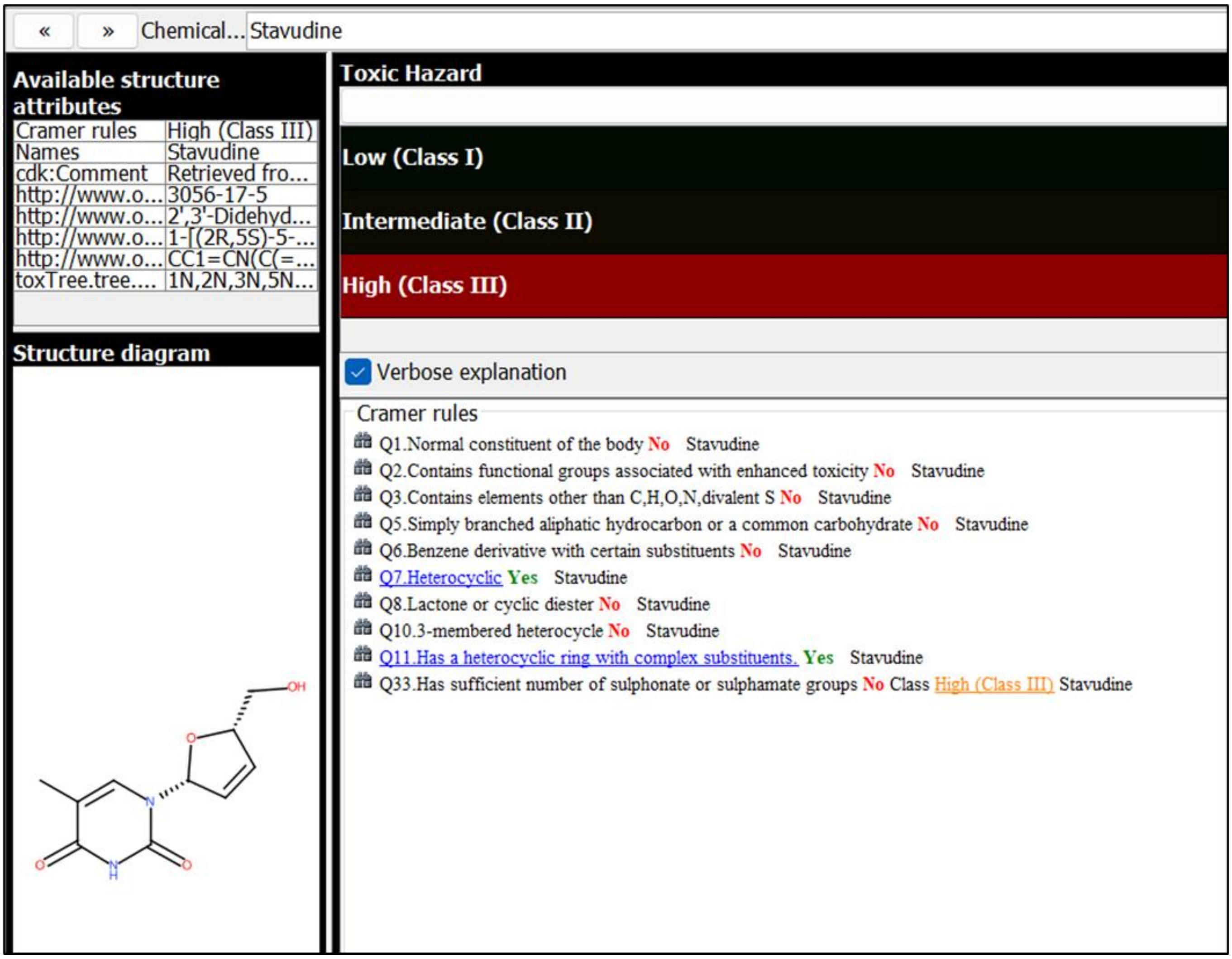
APPENDIX 5. 4 : Antiretroviral concentrations in stored urine, nitrified urine concentrate (NUC) and struvite

Antiretroviral	Stored urine (ng L^{-1})	NUC (ng L^{-1})	Struvite (ng g^{-1})
Lamivudine	2300	nd	nd
Nevirapine	2000	347	45.3
Efavirenz	1420	nd	nd
Zidovudine	590	nd	nd
Abacavir	387	nd	< LOQ
Efavirenz	240	nd	nd
Ritonavir	170	nd	nd
Stavudine	778	nd	nd
Didanosine	1366	nd	< LOQ
Emtricitabine	243	nd	nd

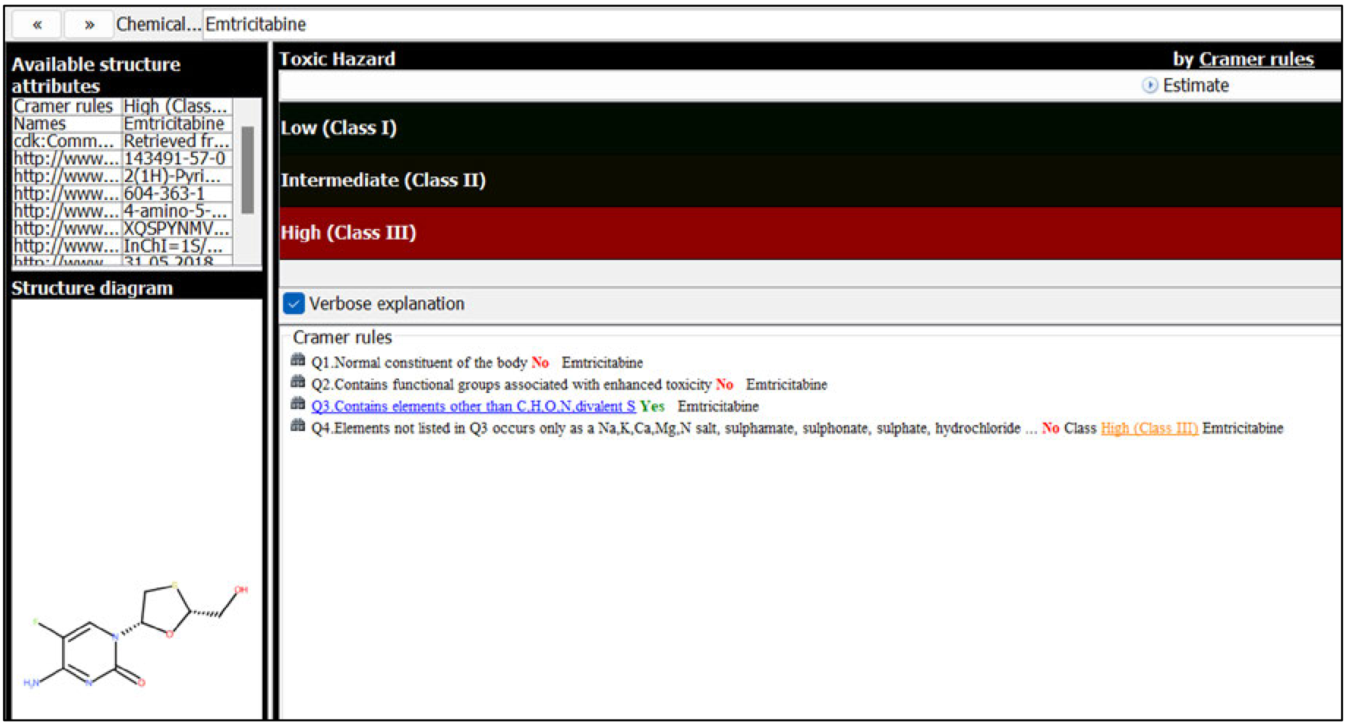
APPENDIX 5. 5. Cramer classification of ritonavir. Results are based on the toxic hazard estimation of Toxtree software



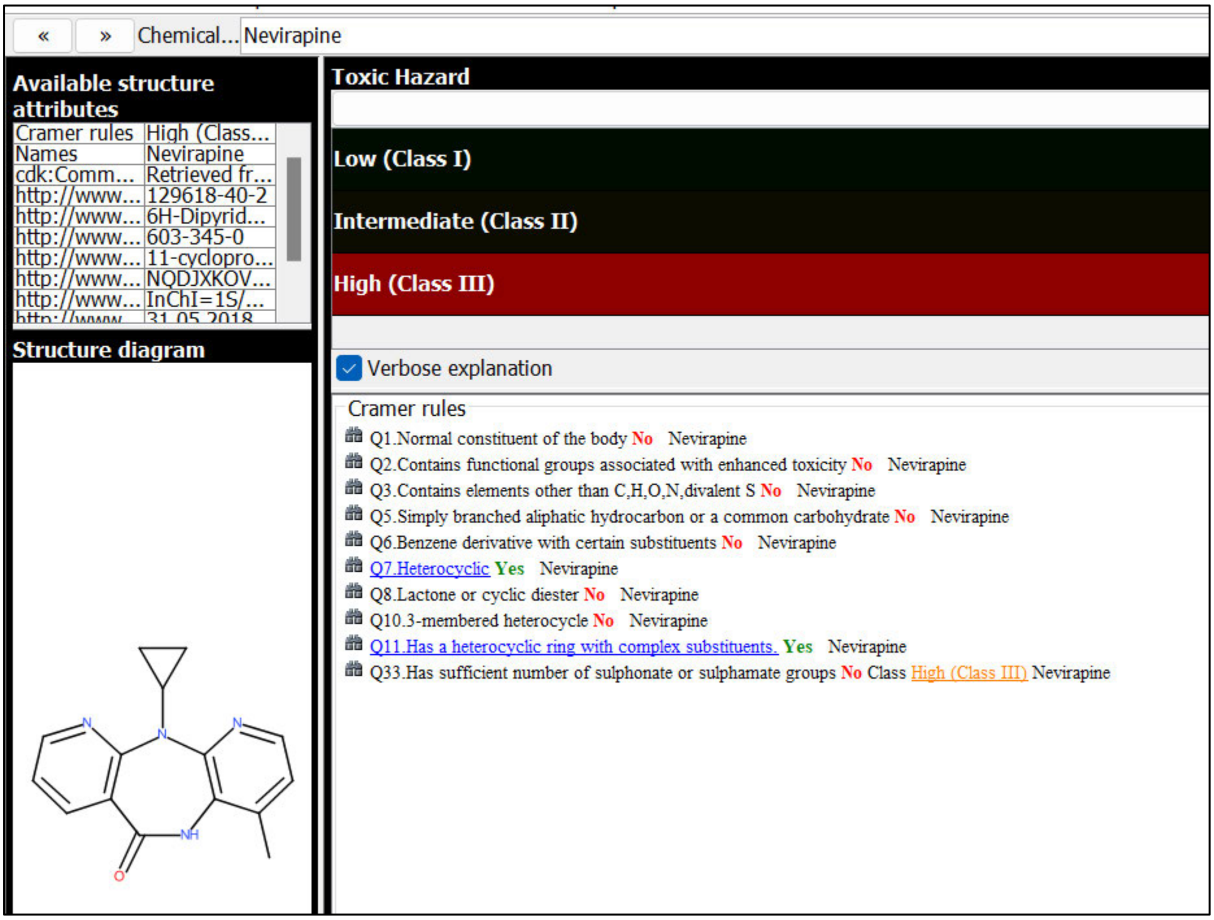
APPENDIX 5. 6: Cramer classification of stavudine. Results are based on the toxic hazard estimation of Toxtree software.



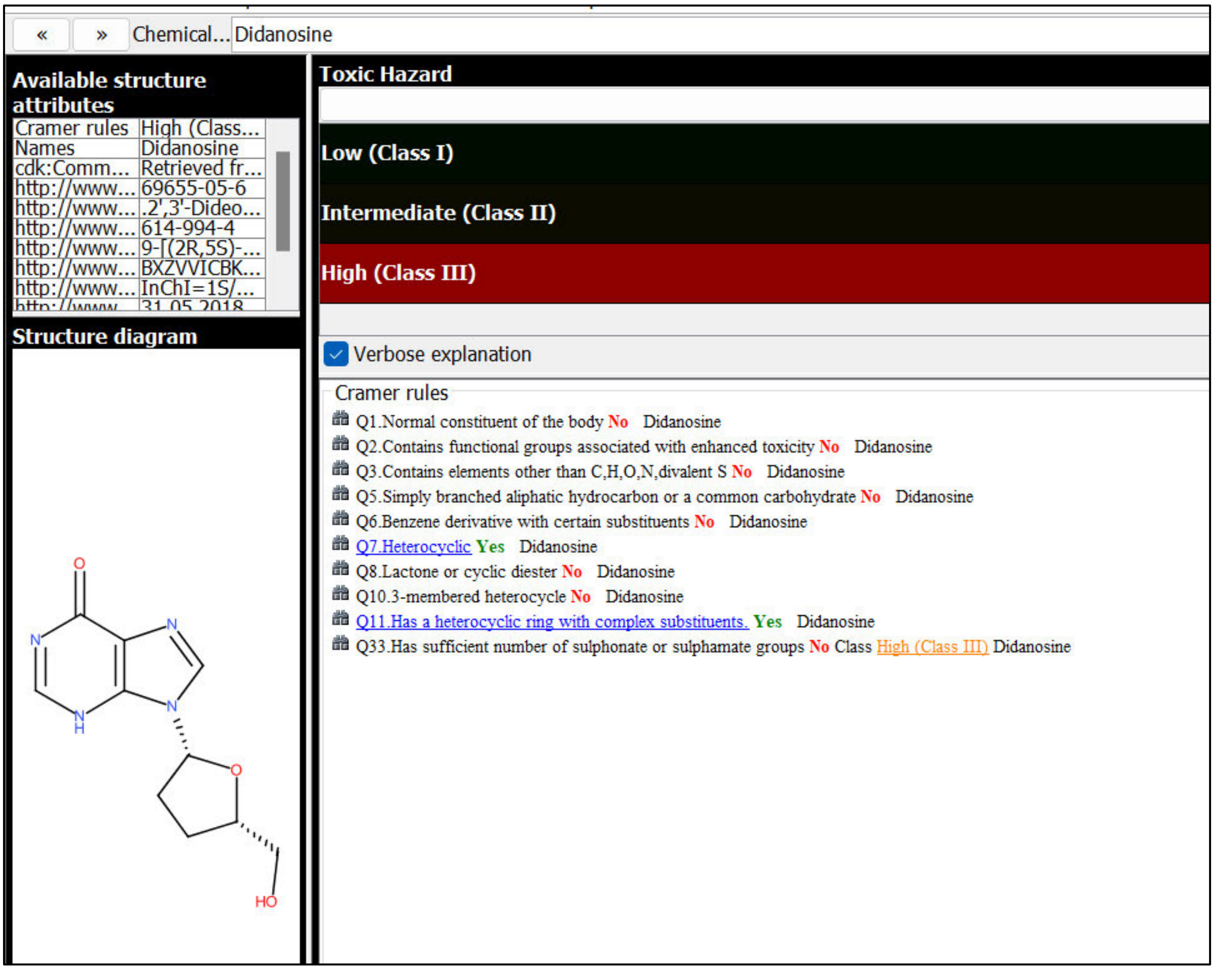
APPENDIX 5. 7: Cramer classification of emtricitabine. Results are based on the toxic hazard estimation of Toxtree software.



APPENDIX 5. 8: Cramer classification of nevirapine. Results are based on the toxic hazard estimation of Toxtree software.



APPENDIX 5. 9: Cramer classification of didanosine. Results are based on the toxic hazard estimation of Toxtree software.



APPENDIX 6. 1:SANITATION SAFETY PLAN

The World Health Organisation (WHO) developed a Sanitation Safety Planning (SSP) manual to minimise health risks and maximise the benefits of human excreta use (World Health Organisation, 2015). Sanitation Safety Planning (SSP) is a risk-based management tool for sanitation systems. It assists users to systematically identify and manage health risk along the sanitation chain; guide investment based on actual risks, promote health benefits and minimize adverse health impacts and provide assurance to authorities and the public on the safety of sanitation-related products and services. The SSP was developed to assist the implementation of the WHO Guidelines for Safe Use of Wastewater, Excreta and Greywater (2006). It is a site-specific tool targeted at a variety of operators and different levels.

Therefore, the objective of this study was to evaluate the risk associated with human urine use in agriculture based on the World Health Organisation's Sanitation Safety Planning (SSP) manual.

The target audience for this sanitation safety plan includes municipal authorities, regulators, town planners and government officials in relevant departments. It can be adopted at a city, regional or national level to influence policy on sustainable sanitation solutions in South Africa.

MODULE 1: DESCRIBE THE SANITATION SYSTEM

The sanitation systems described in this SSP includes collection, transportation, treatment of human urine and use of human urine-derived fertilizers (HUDF) for crop production as illustrated in Figure A 6.1.1

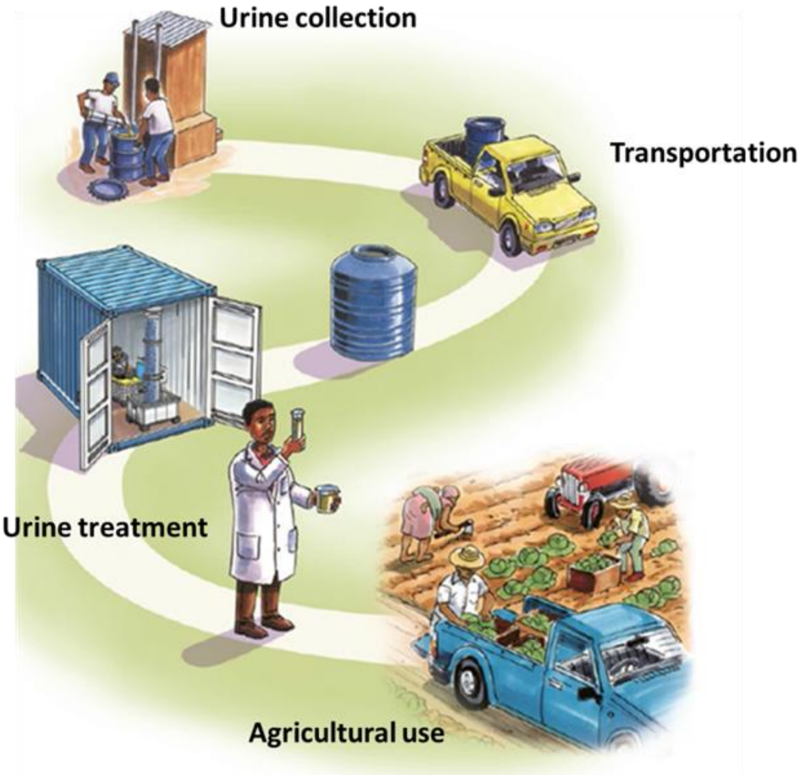


Figure A 6.1.1 Sanitation system for urine collection, transportation Adapted from VUNA report (Etter *et al.* (2015))

The urine diverting toilets (UDTs) and urine treatment technologies for the production of struvite and NUC are part of the sanitation systems included in this SSP.

Stored Urine

Urine is an excreta stream recovered from the UDTs. The urine is high in mineral nutrients compared to faecal matter, thereby making it an important resource for agricultural use (Nagy *et al.*, 2019a). According to Rose *et al.* (2015), an adult person excretes about 1.47 L of urine per day (59 g capita^{-1} day^{-1}), of which 10.56 g capita^{-1} day^{-1} is in the form of N. According to Nagy *et al.* (2019b), the urine contributes about 80% N, 50% phosphorous (P) and 60% potassium (K) of the total domestic wastewater composition. The pathogens in urine are generally low unless it is accidentally contaminated with faecal matter (Bischel *et al.*, 2015b). The pathogens in urine are inactivated during storage for about 2 months due to high pH (Krause and Rotter, 2018). However, the challenge faced with stored urine is the high concentrations of pharmaceuticals, which do not degrade with storage. Bischel *et al.* (2015a) reported the presence of human pathogens, pharmaceuticals and antibiotic resistance genes in urine and suggested the need for its treatment before agricultural use. Stored urine can be processed to produce struvite and nitrified urine concentrate (NUC) fertilizers which can be applied to agricultural land for crop production.

Struvite is a magnesium, ammonium phosphate ($MgNH_4PO4.6H_2O$) produced through urine precipitation, filtration and drying (Figure A6.2). Struvite crystals form after the addition of a magnesium source, followed by filtration to separate the crystals from the liquid and finally drying of the crystals removes all the liquid. Further pathogen die off occurs during the drying process. However, according to (Bischel *et al.*, 2015b) viruses and pharmaceuticals were contained in the residual urine before struvite drying, these are not treated during the procedure and remain a potential human and environmental health risk.

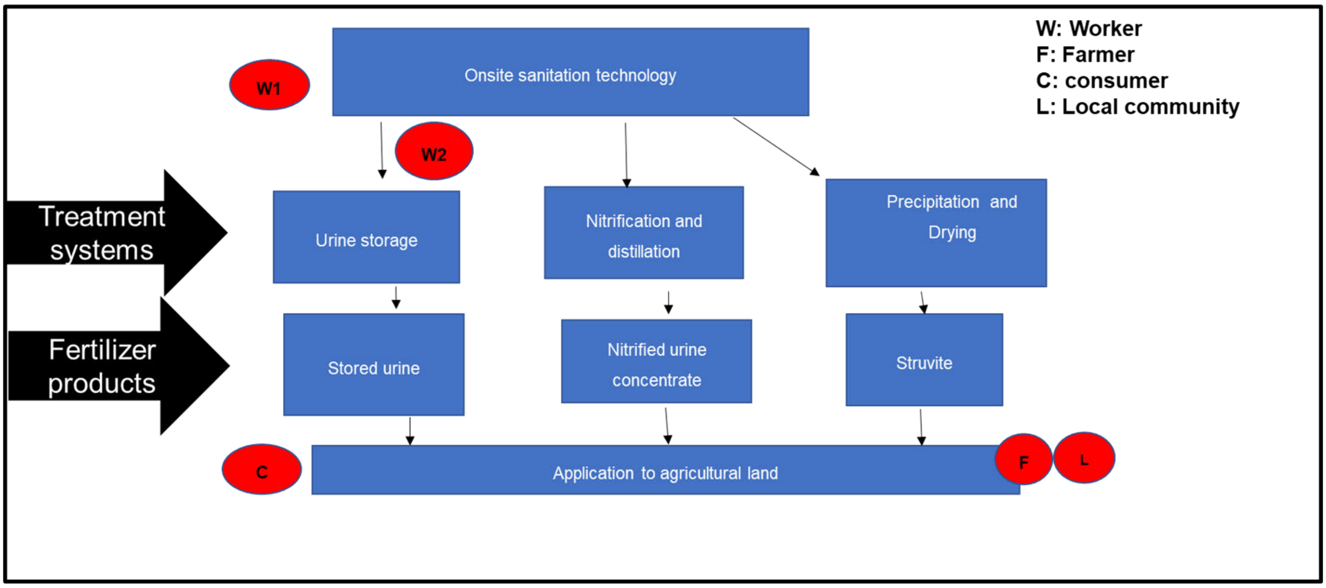


Figure A.6.1.2: urine treatment process flow diagram and exposure groups. W1: those who collect urine from UDDTS; W2: those who treat urine to fertilizers; F: those who use human urine-derived fertilizers for crop production, C: those who consume crops grown with human urine-derived fertilizers; L: those who live adjacent to fields using human urine-derived fertilizers for crop production.

Nitrified urine concentrate is processed through biological nitrification and distillation (Figure A 6.1.2) The nitrification process stabilises the collected urine. Half of the urine being processed is oxidised into non-volatile nitrate; the other half is stabilised as non-volatile ammonium. Nitrification is insufficient for complete pathogen inactivation and pharmaceutical degradation. Distillation at 80 °C for several hours follows nitrification to produce a pathogen free product (Udert *et al.*, 2015). However, pharmaceuticals remain a cause of concern in the use of NUC as a fertilizer.

Compliance and contexual information

This involves national and international standards for the safe use of human excreta in agriculture. Human urine derived fertilizers are intended to improve and maintain plant and soil productivity therefore by definition they are fertilizers according to the South African Fertilizers, Farm Feeds Stock Remedies Act, 1947 (Act No. 36 of 1947). The Department of Water Affairs and Forestry (DWAF), Department of Environmental Affairs and Tourism (DEAT), Department of Health (DoH) and Department of Agriculture (DoA) have a regulatory role in the authorisation of urine for agricultural use. The existing fertilizer legislation in South Africa does not specifically exclude the use of urine fertilizers for crop production. However, due to its potentially harmful components and typical management by sanitation systems, human urine is not regarded as regular fertilizer.

Several regional and international recommendations have been developed to manage the risk associated with the use of human excreta in agriculture. Guidelines for safe use of wastewater, excreta and greywater developed by the WHO provide preventive measures for exposure associated with the use of excreta. The WHO guideline outlines acceptable pathogen limits and approaches taken to achieve limits for the use of urine in crop production. National approaches in line with environmental, socio-cultural and economic circumstances need to be drafted. Guidelines for the utilisation of wastewater sludge in agriculture developed by the Water Research Commission outlines requirements and restrictions associated with sludge in a South African context. Although the guideline focuses on sludge, it gives a baseline for the beneficial use of HUDF in crop production. Therefore, herein the regulatory procedure required for wastewater sludge use is adapted for the use of urine and its products in agriculture. A summary of the main issues noted is listed in Table A 6.1.1.

Table A.6.1.1: Compliance and contexual information for the safe use of

Standard and regulations	
South African, Water Act, 2013. Wastewater limit values applicable for irrigation	Total suspended solids < 25 mg/L Faecal coliforms < 1000/100 mL Chemical Oxygen Demand < 75 mg/L Electrical Conductivity maximum of 150 mS/m
WHO,Guidelines for Safe Use of Wastewater, Excreta and Greywater (2006)	Faecal coliform < 1000 CFU/100ml Intestinal nemetodes ≤1 egg/l

Types of hazards

Biological hazards

Pathogens (bacteria, viruses and protozoa) are a major cause of concern for the use of HUDF in crop production. Soil transmitted helminth, *Taenia spp and Ascaris* eggs are a major health concern due to their long period of persistence in the environment (WHO, 2006).

Physical hazards

Malodours especially during urine collection from the UDDTs is the most prevalent physical hazard.

Chemical hazards

Pharmaceuticals are a potential cause of concern in both wastewater and HUDFs. Heavy metals are not a major concern in urine products as the concentration of these metals are below the permissible concentrations.

MODULE 2: IDENTIFY HAZARDOUS EVENTS, ASSESS EXISTING CONTROL MEASURES AND EXPOSURE RISKS

Control measures

These are actions taken to reduce the potential hazards to exposure groups described above. The control measures are meant to reduce the likelihood of the risk of exposure. Control measures within each sanitation step are listed in Table A.6.1.2.

Table A.6.1.2: Control measures to reduce the potential hazards to exposure groups

Sanitation process	Type of control measure	Control measures currently in place
Urine collection	Non-technical	Vaccination of workers against Hepatitis A and B, cholera and typhoid
	Non-technical	Personal Protective Equipment (boots, overalls, gloves and face masks) issued to workers, and it is worn all the time
Treatment or processing	Treatment	Urine storage, nitrification and distillation of urine, Urine precipitation, filtration and drying.
	Non-technical	Vaccination of treatment workers against Hepatitis A and B, cholera and typhoid
	Non-technical	Laboratory induction for people involved in the treatment process and proper use of standard operating procedures (SOP). PPE (laboratory coats, googles and gloves) provided.
	Non-technical	Treatment site fenced and restricted access to the site
Farmers		
	Non-technical	Personal Protective Equipment
Consumption of produce	Non-treatment	Avoid eating uncooked crops and root crops

Assess and prioritize the exposure risk

Risk assessment involves, identifying and analysing hazardous events. Table 3 outlines a risk assessment sheet related to the use of HUDF for crop production. The risk assessment covers all sanitation steps, from the collection of human urine to the consumption of crops produced from these products. The scoring method is adapted from the semi quantitative risk assessment matrix (World Health Organisation, 2015). The South African Occupational Health and Safety Act, 1993 requires that a safe environment, without health risks is provided to the workers. Therefore, a risk assessment sheet is a good tool to analyse sanitation related risks.

Table A.6.1.3. Risk assessment of use of human urine-derived fertilizers in crop production

Sanitation step	Hazard identification				Existing control	Risk assessment L: Likelihood S: Severity R: Risk level Allowing for existing control				Comments
	Hazard event	Hazard	Exposure route	Exposure groups		L	S	Score	R	
Urine collection	Exposure to untreated urine	Microbial	Dermal	W1	PPE barrier to dermal exposure and Vaccination	2	2	4	L	Contamination might occur with accidental contact PPE
	Foul smell	Malodour	Inhalation	W1	Face masks	3	2	6	L	Wearing of face masks is sometimes not observed during urine collection
Treatment	Exposure to untreated urine	Microbial	Dermal	W2	PPE, Vaccination, laboratory induction, Follow SOP	1	1	1	L	The treatment process involves sophisticated machinery, therefore highly skilled people are involved. Therefore, they pay attention to the control measures.
Application of urine fertilizers to agricultural land	Exposure to pathogens	Microbial	Ingestion and dermal	F1	Hand washing after fertilizer handling, PPE	4	4	16	M	Struvite application poses a higher risk than NUC application because its production does not completely remove *Ascaris* eggs.
Consumption of crops grown with urine fertilizers	Micropollutants	Pharmaceuticals and personal care products	Ingestion	C1	N/A	2	2	4	L	There is a knowledge gap for the uptake of micropollutants and its associated risk on human health

KEY

Semi-quantitative risk assessment matrix

			SEVERITY (S)				
			Insignificant	Minor	Moderate	Major	Catastrophic
			1	2	4	8	16
LIKELIHOOD (L)	Very unlikely	1	1	2	4	8	16
	Unlikely	2	2	4	8	16	32
	Possible	3	3	6	12	24	48
	Likely	4	4	8	16	32	64
	Almost Certain	5	5	10	20	40	80
Risk Score R = (L) x (S)			<6	7–12		13–32	>32
Risk level			Low Risk	Medium Risk		High Risk	Very High Risk

Pathogens

The risk assessment of the developed sanitation safety plan revealed that there is a low pathogen risk to the farmers using urine products. The source of risk was identified as accidental ingestion and dermal exposure which can both be avoided through proper use of personal protective equipment. Consumers are potentially exposed to pathogen risk through consumption, cooking the product reduces the pathogen risk.

The pathogen limits for the use of wastewater in agriculture will be used as a guideline for the liquid products (NUC and stored urine) while limits for use of wastewater sludge will be used for struvite. According to the South African National Water Act 36 of 1998 the limit for faecal coliforms in wastewater use in agriculture is 1000 CFU/mL. Production of NUC involves distillation which allows inactivation of all pathogens. Stored urine is considered sterile unless contaminated with faecal matter or menstrual blood. Therefore, both NUC and stored urine meet the pathogen limits for use in agriculture. Pathogen limits for wastewater sludge are 1000 CFU/g for faecal coliforms and 1 ova/4g for helminth ova (Snyman *et al*., 2006) struvite meets the requirements for use in crop production. Most pathogens are inactivated during the drying process of struvite production; however, it has been reported that the drying process does not allow for helminth ova die off. Therefore, the microbial quality of the produced struvite greatly depends on the quality of source-separated urine collected.

Pharmaceuticals

Pharmaceuticals are a major concern when considering urine fertilizers for agricultural use. According to a metanalysis compiled by (Lienert *et al.*, 2007b) on pharmaceutical excretion pathways, 64% of each of the 212 analysed pharmaceuticals was excreted through urine. Urine storage has minimum benefits for pharmaceutical degradation, while 98% of pharmaceuticals have been reported to remain in struvite after precipitation (Ronteltap *et al.*, 2007). Production of NUC is coupled with post nitrification processes such as the addition of powdered activated carbon and electrolysis to achieve 90% of pharmaceutical elimination. The study on the uptake of selected antiretrovirals (ARV) by pepper, radish and ryegrass grown on human urine-derived fertilized soils (Chapter 5), revealed that the daily consumption of these crops fertilized with stored urine does not pose a health risk to the consumer. However, currently, there are no regulations with regards to pharmaceuticals and concentrations that pose a threat to human health. The wide range of pharmaceutical classes, mode of action and chemical structures makes it difficult to come up with such regulations.

MODULE 3: DEVELOP SUPPORTING PROGRAMMES AND REVIEW PLANS

This module supports the development of people's skills and knowledge, and an organisation's ability to meet SSP commitments. The supporting programmes listed below may be considered to support meeting the objectives of the SSP.

Supporting programmes

- Public awareness programmes and training for the proper use of UDDTs to avoid contamination from faecal matter.
- Training programmes for the operations and maintenance teams including urine collectors.
- Site and laboratory inductions for people with access to the treatment sites
- During the implementation stage, engage the commercial farmers so that the local communities can learn from them.
- Raising awareness on hygienic practises and post-harvest handling of crops produced with HUDF.
- Knowledge dissemination must be simplified in a way that can be understood by anyone in different fields and with any level of education.

Summary and conclusion

The current sanitation plan was developed with focus on urine collection from UDTs, urine treatment, application of HUDFs for crop production and consumption of the produced crops.

The potential exposure groups include people collecting and treating urine, farmers using urine and its derived products for crop production and consumers of the produced crops. Pathogens and organic pollutants specifically pharmaceuticals are the major concerns to human health associated with the use of urine and its products. The identified exposure routes are accidental ingestion, dermal and inhalation. Recommended control measures include vaccination of people involved in urine collection and treatment, use of PPE by farmers during application of HUDFs. To reduce the possibility of consumers being exposed to pathogens, cultivation of food crops that are eaten raw, mostly vegetables, should be avoided. In cases where vegetables are grown, post-harvest handling including washing and use of homemade disinfectant (e.g. vinegar solution) after harvesting is recommended.