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Abstract

Micronutrients play an important role in metabolic reactions in animals. To correct for potential micronutrient deficiencies in forages, the dominant feed source for ruminants, farmers routinely administer supplements to animals prophylactically. Micronutrients that are not absorbed from supplements are excreted, providing a potential flux of micronutrients to soil for uptake by forages. However, it is unclear if the form of supplemental minerals given to animals significantly affects the flux of micronutrients in pasture systems. This study investigated the impacts of supplement form (organic or inorganic), dose, excreta type (urine and/or faeces), and soil organic matter (OM) on the flux of micronutrients in a grazing pasture system. Over two weeks, 24 sheep were supplemented with organic or inorganic forms of Zn, Cu, Mn, and Se at two industrial doses. The excreta were applied to potted soils and seeded with perennial ryegrass (*Lolium perenne* L.). The redistribution of micronutrients was followed from feed to excreta to soil to grass.

Over 90% of the Cu, Zn and Mn, and over 80% of the Se was excreted through faeces, regardless of supplement form or dose. The concentrations of bioavailable Zn, Cu, Mn and Se in faeces were not significantly affected by the supplement form, but recalcitrant fractions of Zn, Cu and Se were, implying a potential long-term effect of supplement form on micronutrient bioavailability in soils. Uptake of Zn, Cu and Mn by ryegrass was most affected by excreta type. Although faeces contributed 20-1200 times more Zn, Cu and Mn than urine, micronutrient uptake by ryegrass was significantly higher in soils applied with urine because N, K and S in urine increased grass growth. Uptake of Se by ryegrass was limited in high OM soils regardless of excreta type, revealing antagonism between the benefits of OM to plant growth and the bioavailability of Se.

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Pei-Tzu Kao

April, 2022

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Besides my supervisors, I would also like to say thanks to my working fellows and student supports at Rothamsted Research and University of Bristol: **Sarah Dunham**, **Mark Durenkamp**, **Joanna Carter**, **Hannah Fleming**, **Lizzy Webster**, **Liz Dixon**, **Aranzazu Louro-Lopez**, **Rudiger Gerlach**, **Laurence Bennie**, **Jane Hawkins**, **Donna Fellows**, **Ian Shield**, **Chris Whitfield**, and **Daniel Rydval**. Without your helps with the lab work and the experiment work, and your helps with student affairs, I wouldn't manage to finish this thesis on time. I also want to thank my reviewers of the Annual Progress Meeting in University of Bristol: **Ian Bull**, **Penny Jones**, and **Casey Bryce** for giving me advise and comments on my research progress and making my research work better.

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Introduction

Micronutrients play an essential role in animal health and production. However, the levels of the micronutrients in forages, which may be sufficient for optimum crop yields, are not always adequate to meet the needs of livestock (Gupta et al., 2008; Lee et al., 2018). Among the micronutrients essential to ruminants, copper (Cu), selenium (Se) and iodine (I) are most likely to be inadequate for grazing livestock such as sheep (Kao et al., 2020). To prevent micronutrient deficiency, farmers in the UK typically give supplements to the animal (Fisher, 2008). Supplements are often prophylactically and routinely administered as part of standard practice rather than strategically based on the nutrient level in feeds and/or the nutritional requirements of the animal. To increase utilisation efficiency of the supplements in ruminants, the absorption of supplemental micronutrients in different chemical forms (e.g. organic or inorganic) and dose rates have been comprehensively studied. However, how different chemical forms of the supplemental micronutrients affect the fate of the micronutrients post excretion in a pasture system remains unclear. Understanding this is critical because the excreta of cattle and sheep contain large amounts of nutrients and are therefore a major source of micronutrients. Furthermore, on-farm application of animal excreta as an organic soil fertiliser has become more and more popular (Kao et al., 2020). In order to improve the recycling rate of micronutrients in a pasture system, it is therefore important to understand the potential impact of different micronutrient supplements to the flux of a micronutrient in pasture systems.

To investigate the impact of the chemical form of supplemental micronutrients, together with other potentially influential factors such as soil OM, on the flux of

micronutrients (focusing on the commonly supplemented micronutrients: zinc (Zn), Cu, manganese (Mn) and Se) in a pasture system, this research encompasses two sequential main experiments. The first is a sheep experiment studying the effects of different forms (organic or inorganic) of supplemental micronutrients, offered at a high and a low industrial concentrations, based on the regulation of (NRC, 2007), on the excretion of the micronutrients in urine and faeces. The second is a pot-lysimeter experiment studying micronutrient redistribution in soils (high OM or low OM content), uptake by perennial ryegrass (*Lolium perenne*), loss in leachate after the excreta collected from the sheep experiment was applied to the soils. It is hypothesised that the different chemical forms of supplemental micronutrients in the sheep experiment will have significant impact on the partitioning of the micronutrients in urine and faeces due to their different metabolic pathways in sheep. It is further hypothesised that the micronutrient flux in the soil-grass systems will be affected by the application of different excreta type (urine or faeces) due to their differences in nutrient composition and physical structure.

The thesis is split into five interconnected chapters. Chapter 1 reviews the literature regarding factors that influence micronutrient flux in pasture systems. Chapter 2 describes the methodologies of sample preparation and analysis used in the experiments of Chapter 3 and Chapter 4. Chapter 3 presents the set-up, results and discussion of the sheep experiment. Chapter 4 presents the set-up, results and discussion of the lysimeter pot experiment. Chapter 5 summarises the findings from the sheep and the pot experiment and discusses the implications and future research based on this study. In the chapter of the sheep experiment, I will be referring to the supplemental micronutrients provided in animal feed as 'supplemental minerals' which is the conventional terminology used in the field of animal nutrition. Other uses of the term

'mineral', e.g. in the context of the soil environment, will refer to minerals in the geological sense.

Declaration of published work

This thesis contains excerpts from work that I wrote and published during the course of this PhD. Only material that I wrote myself is included here (i.e. sections written by co-authors are not included). The publications and their inclusion in this thesis are summarised below. Further details of the publications and author contributions are given in **Appendix C**.

Kao, P.-T., T. Darch, S. P. McGrath, N. R. Kendall, H. L. Buss, H. Warren, and M. R. F. Lee. 2020. Factors influencing elemental micronutrient supply from pasture systems for grazing ruminants. Advances in Agronomy 164:161-229.

This paper constitutes my literature review for this thesis and makes up the bulk of **Chapter 1.** A small amount of content from this paper also appears in **Chapters 2-5, and Appendix B.**

Kao, P. T., Fleming, H., Buss, H. L., Warren, H., McGrath, S. P., Darch, T., & Lee, M. R. (2021). 154 Impact of supplementation dose and form on selenium partitioning and composition in urine and faeces of sheep. Animal-science proceedings, 12(1), 124.

This conference paper presented preliminary results of this thesis and makes up the bulk of **Appendix A.3 and A.4**, with a small amount of content also appearing in **Chapter 3.**

Chapter 1

Factors influencing micronutrient supply from pasture systems for grazing ruminants

1.1 The definition of micronutrients used in this thesis

A term 'micronutrient' is used in animal nutrition to include trace minerals and vitamins that are provided at the microgram or milligram per kilogram level of total body weight. In this thesis, the term 'micronutrient' is used throughout to indicate elemental micronutrients or trace minerals that are the most nutritionally relevant for ruminants, including Cu, Zn, iron (Fe), Mn, Se, cobalt (Co) and I.

1.2 Micronutrient intake and ruminant requirements

Forage grasses and legumes are the most important sources of fibrous energy for ruminants, and may be consumed directly via grazing, or eaten after conservation as hay or silage (Minson, 1990). However, the levels of micronutrient in forages, which may be sufficient for optimum crop yields, are not always adequate to meet the needs of livestock (Gupta et al., 2008; Lee et al., 2018). **Table 1.1** gives the micronutrient requirements of ruminants and typical concentrations in pastures of the UK. Concentrations of micronutrients in forage can be a useful indicator of potential micronutrient insufficiency or imbalance for grazing livestock. Based on **Table 1.1**, Cu, Se, and I are the three micronutrients with the highest likelihood of insufficient supply for grazing livestock, and there is a reasonable risk of Co insufficiency for sheep. Note that these values are approximate only, and in some cases, the balance between nutrients could be more important than the total concentrations in forage (**Chapter 1.3**). There are no currently published data on the typical Fe content of UK forages, but unpublished data from the North Wyke Farm Platform (Rothamsted Research, North Wyke, Devon, SW England) found that in 2018, the mean Fe content was 286 mg kg-DM^{-1} (**Table 1.1**). This concentration is significantly above the minimum Fe requirement of lamb and beef cattle, of between 28 to 83 mg kg-DM^{-1} and 50 mg kg-DM^{-1}, respectively (NRC, 2007, 2016). Furthermore, primary Fe deficiency has never been demonstrated

unequivocally in grazing animals, a reflection of the normally high Fe content of pastures and forages and of the opportunities for their contamination with soil and dust (Underwood & Suttle, 1999). However, it is worth noticing that a high ration of Fe can interfere with the absorption of other micronutrients, primarily Cu and Zn (NRC, 2001). Also, it should be noted that micronutrients in forage are not fully available for absorption by ruminants. The absorbability of forage-derived micronutrients varies with forage type, the nutrient composition of forage, and the chemical form of the micronutrient present in the forage. For example, the availability of Cu was 0.023 and 0.012 (mg/kg-DM) in temperate forage in summer and autumn, respectively, which was presumably due to the different ratios of Cu, Mo and sulphur (S) in the herbage (Minson, 1990). The effects of Mo and S on Cu availability to ruminants is discussed in **Chapter 1.3**.

Soil ingestion during grazing or within ensiled crops, is another source of micronutrients. Soil ingestion occurs particularly in dry weather, when the soil is dusty and herbage is short, such that animals are grazing close to the soil, or in wet weather, where soil is splashed onto the herbage being consumed. Low cut heights during ensiling or hay making on uneven fields can also increase soil contamination of conserved feed and subsequently soil intake by animals. Determined by titanium (Ti) trace analysis, it was estimated that between 0.73 and 0.99 kg-soil $day^{-1}animal^{-1}$ was ingested by grazing cattle in June and August, respectively, in the State of Idaho in US (Mayland et al., 1977). Grazing dairy cattle in New Zealand each ingested approximately 0.50 to 0.87 kg-soil day^{-1} (Healy, 1967), while another study showed 0.071-0.163 kg of soil is ingested in an arid region (Vaithiyanathan & Singh, 1994). Taking the range of soil ingestion rates estimated for sheep (0.062 to 0.163 kg-soil day^{-1}), and the typical soil concentrations of micronutrients, sheep can ingest considerable

quantities of micronutrients via this route (**Table 1.2**). However, soil ingestion is associated with poor grazing or conservation management, which is not an ideal intake strategy. Furthermore, micronutrient absorption via this route can be low – ingestion from soils labelled with stable isotopes has indicated that sheep absorbed only 34 % of ^{75}Se, 14 % of ^{65}Zn, 1 % of ^{60}Co, and 0.4 % of ^{54}Mn present (Healy et al., 1970).

1.3 Micronutrient deficiencies and supplementation of ruminants

Despite being presented a very small proportion of total body weight, micronutrients play critical roles in metabolic reactions. Due to the large number of enzymes and hormones that a micronutrient may activate, deficiency/imbalance of a given micronutrient can manifest in a range of signs (**Table 1.3)**. For example, Zn is well-known for its critical role in gene expression where the coordination of Zn to cysteine (Cys) and histidine (His) creates the DNA-binding proteins that influence transcription, and hence cell replication (Underwood & Suttle, 1999). Therefore, deficiency of Zn can lead to dysfunction of digestion, DNA synthesis and protein metabolism. In another example, the Se-dependent enzyme, glutathione peroxidase, plays a critical role in the protection of the cell membrane against peroxides (Hefnawy & Tortora-Perez, 2010). Therefore, deficiency of Se can damage cellular and mitochondrial membranes, which may result in white muscle disease, degenerative changes in skeletal muscles and in the myocardium of young animals, and ruminants are particularly susceptible to these diseases (Hefnawy & Tortora-Perez, 2010).

Besides the insufficient nutrient supply from feed, micronutrient deprivation in livestock can also occur during digestion due to nutrient antagonism, of which there are two basic types. The first type occurs when the elements are physically or chemically similar, which leads to isomorphous replacement at the absorption sites (Davies, 1979).

Table 1.1 Micronutrient densities of dietary feed required by ruminants and concentrations of micronutrients in typical UK pastures

Animal Type	Micronutrient densities of dietary feed required by typical ruminant livestock (mg kg-DM^{-1})*						
	Fe	Co	Cu	I	Se	Zn	Mn
Dairy cattle[a]	24[d]	0.1	10	0.2	0.3	30	25
Beef cattle[b]	n.d.	0.1	10	0.5	0.1	30	40
Sheep[c]	25-40[e]	0.2	11	0.8	0.2	33	40
	Micronutrient concentrations of typical UK pastures (mg kg-DM^{-1})**						
Range	n.d.	0.05-0.25	2-15	0.1-0.5	0.02-0.15	20-60	25-250
Mean	n.d.	0.1	8	0.15	0.07	50	100
RRes-NW farm platform	286[f]	N.A.	7.4[f]	N.A.	0.041[g]	30.0[f]	165[f]

N.A.: Data not available. *data referring to Lee et al. (2018) except for Fe. **Peers and Phillips (2011). [a]Dairy cattle weighing 650 kg and producing 30 L of milk per day. [b]Beef cattle weighing 300-400 kg, gaining 1kg per day. [c]Lamb of 25-40 kg live weight. [d]Dairy cow producing 25 kg of milk per day at 205 days of gestation NRC (2001). [e]Garmo et al. (1986); [f]Unpublished data; values are mean values of 63 grass samples collected in 3 periods, April-May, July-August, and October, in 2018 from 21 fields of the farm platform of Rothamsted Research North Wyke (Devon, South-West England). [g]Unpublished data. Values are the mean of 75 forage samples taken from 5 permanent pasture fields of the North Wyke farm platform.

Table 1.2 Micronutrient consumption by sheep through ingestion of soil and pasture forage

Element	Mean topsoil concentration (England and Wales) †	Micronutrient intake through soil ingestion (mg day^{-1})*	Typical concentrations in UK pastures (mg kg-DM^{-1})**	Micronutrient intake through forage by sheep (mg day^{-1})¥	Total (soil + pasture) micronutrient intake amount (mg day^{-1})	Daily requirement of Micronutrient by sheep (mg day^{-1})***
Fe	2.9% (w/w)	2059-4727	264ǂ	256ǂ	2315-4983	N.A.
Mn	0.077% (w/w)	55-126	100	97	152-223	64
Cu	24.0 mg kg^{-1}	1.7-3.9	8	8	9.5-11.7	17.6
Zn	91.0 mg kg^{-1}	6.1-14.8	50	49	54.6-63.3	53
Co	11.0 mg kg^{-1}	0.8-1.8	0.1	0.1	0.9-1.9	0.3
Se	0.71 mg kg^{-1}	0.05-0.12	0.07	0.07	0.12-0.18	0.3
I	7.5 mg kg^{-1}	0.5-1.2	0.15	0.15	0.7-1.4	1.3

N.A.: Data not available. †Rawlins et al. (2012). *Assuming each sheep ingest 0.062-0.163 kg of soil day^{-1} (Vaithiyanathan & Singh, 1994). **Peers and Phillips (2011). *** Lee et al. (2018). ǂFe concentration in forage refers to mean value of grass from Thomas et al. (1952). ¥ Based on that voluntary intake of temperate forage by sheep is 61 g kgW$^{-0.75}$ (Minson, 1990) so a 40 kg sheep intakes approximately 970 g DM of temperate forage day^{-1}

For example, Zn^{2+} and Cu^{2+} ions tend to act antagonistically due to their similarities in electronic structure, and when dietary ratio of Cu:Zn are very high (50:1), Cu can interfere with the absorption of Zn (NRC, 2001). The second type of antagonism occurs due to other reactions hampering the utilization of a micronutrient within the animal. The most well-known example is the antagonism with Cu. A dietary excess of Mo and S (as sulphate or sulphide) decreases the absorption of Cu mainly due to the formation of thiomolybdate compounds that will readily bind with Cu in the rumen (Gould & Kendall, 2011). Furthermore, in the absence of available Cu in the rumen, thiomolybdate compounds can be absorbed through the rumen wall and intestines, and then bind to Cu-containing substances, including enzymes, and cause clinical signs of Cu deficiency (Gould & Kendall, 2011). Hence, a low Cu:Mo ratio (<2) has been reported to result in Cu deficiency or Mo toxicity in cattle (Gooneratne et al., 1989). In addition to interacting with Mo and S, Cu can also interact with Fe and S in rumen and, although the mechanisms remain unclear, the interaction also acts to reduce Cu availability to animals or availability to detoxify thiomolybdates (Gould and Kendall, 2011). As ratios of micronutrients differ across forages, ruminant weight gain/growth can vary across forages due to these antagonisms (Minson, 1990).

The signs of micronutrient deprivation can be sub-clinical or clinical. Clinical signs are obvious, and easily diagnosed as being problematic (**Table 1.3**). On the other hand, while a sub-clinical deprivation can still result in a loss in productivity, the lack of obvious outward signs makes it hard to diagnose. For example, in sheep with induced Co deficiency, ewes produce fewer lambs and had more stillbirths and neonatal mortalities than the Co-sufficient ewes, without showing significant changes in live-weight, body condition score and conception rate (Fisher & MacPherson, 1991). Similarly, lambs that suffered weeks of subclinical Co-deficiency were reported to have

ultimately lower weight gain and survival rates (Vellema et al., 1997). It should be noted that excessive provision of micronutrients can cause toxicity and can even lead to similar clinical signs as those manifested by micronutrient deficiency. Cu in particularly is especially easily oversupplied to sheep because the differential between requirement and toxicity is very narrow, and if the level of Mo is extremely low (<1 mg kg^{-1}), forage with a normal Cu content of 8 to 11 mg kg^{-1} in the diet can also produce toxicity (NRC, 1985).

Table 1.3 Clinical signs of micronutrient deficiency in ruminants

Element	Clinical signs of deficiency
Cu	Anaemia[1,2,3], bone disorders[1,2,3], abnormal gait in ruminants[1], deficiency in pregnant ewe can lead to neonatal ataxia or 'swayback disease' of offspring[1,2], low fertility associated with depressed oestrus and aborted fetuses[1], cardiovascular disorders[1], poor immune system[3] and discoloration of hair[2,3]
Mn	Ataxia[1,3], skeletal disorders when deficiency occurs during embryonic development or early postnatal life[1,2] and reproductive disorders[1,2]. Requirements by ruminants are low, so deficiency is rare.
Zn	Anorexia[1], abnormalities of skin and its appendages[1], skeletal disorders[1] and impairment of immune system[1,2].
Fe	Anaemia[1,2], cognitive dysfunction[1], reduced appetite[2] and weight loss[2].
Se	Muscular degeneration[1], white muscle disease[1,2], poor immune ability[2] and infertility[2]
Co	Poor growth and weight loss[1], anaemia[1], poor disease resistance[1], reduce appetite[2,] poor quality wool[2] and white liver disease[4]
I	Enlarged thyroid gland[1,3], lethargy[2], impaired brain development[1], disorders of hair and wool[1,3], low milk yield in mammals[1] and reproductive dysfunctions[5].

[1]Underwood and Suttle (1999) [2]Lee et al. (2018) [3]NRC (2001) [4]Kennedy et al. (1994) [5]Hidiroglou (1979).

There are many ways of supplementing to optimize the intake of micronutrients by livestock, including the direct application to soil or foliar fertilizers/spray on pasture, or direct supplementation of the animal via: salt licks, boluses, feed supplements,

drenches or injections (Fisher, 2008; Underwood & Suttle, 1999). The choice of supplement depends on the production system, the nutrient(s) of interest, the season, the cause of nutrient deficiency, and the cost-benefit ratio. In a 2004 survey of 200 UK grassland farmers (mean head of livestock: dairy or beef cattle = 343, ewes = 387), on average, farmers used between two and three different methods to correct nutrient deficiencies in their livestock (Fisher, 2008). In order, from the most to the least popular, these were: licks (73 %), boluses (53 %), injections (40 %), supplementing water (37 %), supplementing feed (20 %), using (soil/foliar) fertilisers (16 %), and drenching (2 %) (Fisher, 2008). An appropriate diagnosis of micronutrient status in livestock, and an evaluation of the economic benefit of supplementing versus its cost, are recommended before supplements are used, and available methods for diagnosis of need have been summarised previously (Corah, 1996; Kincaid, 2000). However, optimal supplementation is difficult considering the variable micronutrient concentrations in forages, the variable requirements at different animal growth stages of animal and the difficulty of precise assessment of the degree of deficiency (Fisher, 2008). Therefore, micronutrient supplements to livestock are often used prophylactically and routinely as part of standard practice, rather than strategically based on the nutrient level in feeds and/or the nutrition level of animals.

1.4 Micronutrient inputs to pasture

Inputs of micronutrients to pasture systems include weathering from soil parent materials, soil amendments (fertilizer, manure, lime or biosolids) and atmospheric deposition. National data from England and Wales show that the annual inputs of Cu and Zn to agricultural soils were about 1248 and 3336 tonnes, respectively, in 2008 (Nicholson et al., 2010). For Cu and Zn respectively, livestock manures contributed 30 and 31 % of total inputs to agricultural land, whereas atmospheric deposition

contributed 27 and 31 %, and application of biosolids comprised 29 and 21 %, the rest of the Cu and Zn came from other sources such as compost, digestate, fertilisers and irrigation water (Nicholson et al., 2010). However, to date, global statistics pertaining to the major input of micronutrients to pasture are lacking. This is not only due to the difficulty of separating data of pasture systems from that of other agricultural systems, but also due to the lack of national data from all countries worldwide.

The parent rock from which a soil is formed can influence not only the quantity of micronutrients to plants, but also the availability of applied micronutrients (Reid & Horvath, 1980). For example, there is a strong correlation between the concentration of Se in geological parent materials and the soils derived from them in most circumstances. The Se concentration in most soils is low (world mean 0.4 mg kg^{-1}), but high concentrations of Se (up to 1200 mg kg^{-1}) have been reported in some seleniferous areas (Fordyce, 2007). However, in a review of the concentrations of micronutrients in soils and rocks, Reid and Horvath (1980) revealed that soil parent materials are highly variable in composition, and soil-forming processes vary substantially from one climate regime to another, such that, in some cases, the parent materials of soils can have weak relationship with the concentrations of micronutrients in the deriving soils. This may be particularly true for soils formed on sedimentary rocks, which are formed from the transported debris of other rocks of all types and which were identified as the most important and widespread parent materials of the agricultural soils of the world (Reid & Horvath, 1980). In another special case, I in soils is generally much richer than in the parent materials, since most I is derived from the oceans and transferred onto soils via the atmosphere (Fuge & Johnson, 1986).

Another key source of micronutrients to pasture is the application of livestock manure, as a common fertilizer intended to supply nitrogen (N), phosphorus (P) and potassium

(K). National data for the UK reported that in 2018, 68 % of farms used organic manures, with 51 % of them using cattle manures, on at least one field of the farm (Defra, 2019). Global statistics from the Food and Agricultural Organization of the United Nations (FAOSTAT) show an increasing trend in the quantity of N from animal manure applied to pasture (**Figure 1.1**), where manure from ruminants, i.e. cattle and sheep, are the dominant sources (**Figure 1.2**), reflecting the on-farm application of ruminant manure in pasture systems.

Although applying inorganic fertilizers to soil to supply micronutrients is rare in pasture systems, the applied manures are a major source of micronutrients. The excess micronutrients taken in by the animal, as well as endogenous waste, are mostly excreted through animal urine and faeces (Lee et al., 2002; Minson, 1990). Micronutrient contents in livestock manure differ depending on the sources and the concentrations in feeds (**Table 1.4**). In general, micronutrient concentrations in manure are two- to fourfold higher than those in feeds, with a few exceptions for beef cattle (Sheppard & Sanipelli, 2012). The associated data for sheep manure is lacking, even though sheep manure is the second largest sources of N from livestock globally, which is a knowledge gap that needs addressing.

Micronutrient excretion from livestock is affected by the nutrient composition and forms in feed and supplements, as different micronutrients may have different bio-availabilities from different feed sources. For example, 62.1 and 79.4 % of the total Zn in bermudagrass (*Cynodon dactylon*) and alfalfa (*Medicago sativa*), respectively, were released in the rumen, and different release percentages between forage types were also found for Cu (Spears, 1994). The chemical forms in the mineral supplement affect the absorption of micronutrients in animals. For example, Se supplied as selenomethionine (SeMet) and selenized yeast were shown to be more bioavailable than selenite (SeO_3^{2-})

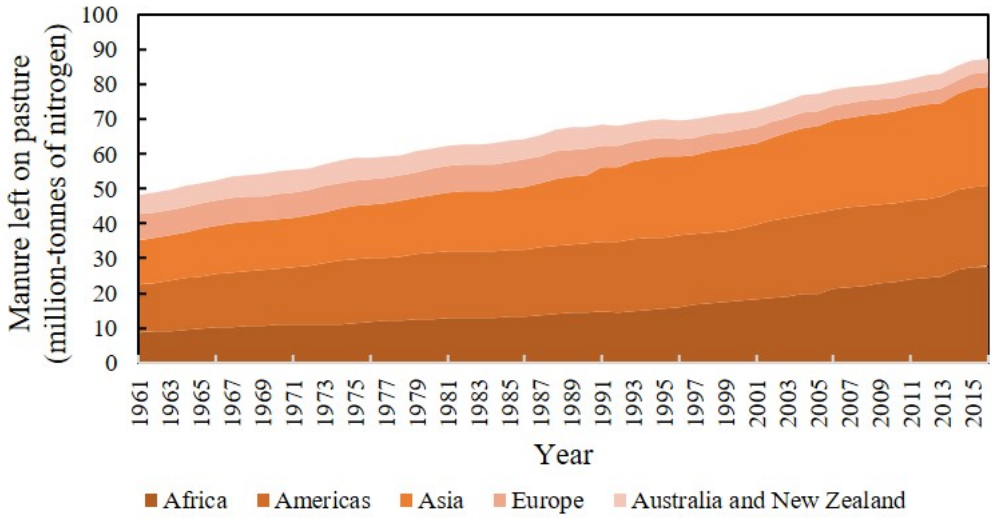


Figure 1.1 The global trend of N input from animal manures to pasture. This trend along with the data shown in Table 4 indicates animal manures as the significant source of micronutrient input in pasture systems. Data is downloaded from FAOSTAT. Data category: Manure left on pasture/Regions/All animal/Manure (N content)/Year from 1961 to 2017.

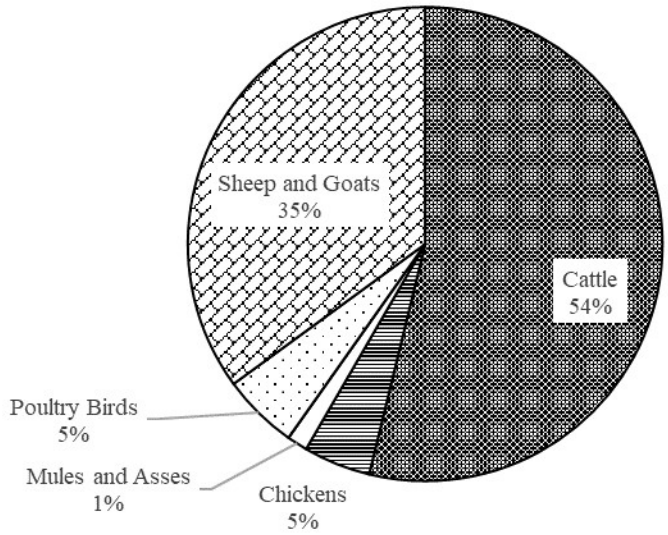


Figure 1.2 The proportion (year 2017) of animal manure sources of N input (million tonnes-N-1 yr-1) on pasture globally. Cattle and sheep are the dominant manure source of N, reflecting potential on-farm application of ruminant manure in pasture systems. Data is downloaded from FAOSTAT. Data category: Manure left on pasture/World/Cattle+ Sheep and Goats+ Chicken+ Poultry Birds+ Mules and Asses/Manure (N content)/Year 2017.

in ruminants (Spears, 2003). Also, despite previous reports that elemental Se is not available to ruminants, recent research has shown absorption in sheep when SeO_3^{2-} is converted by silage lactic acid bacteria inoculants into nano-elemental Se (M. Lee et al., 2019). Manganese sulphate ($MnSO_4$), a commonly used mineral supplement of Mn, has better availability than two other commonly used sources, manganese (II) oxide (MnO) and manganese carbonate ($MnCO_3$), that show only a 35 and 30 % relative absorption in sheep compared to $MnSO_4$ (Underwood & Suttle, 1999). Compared to zinc sulphate ($ZnSO_4$) supplementing with zinc-methionine (Zn-Met) was found to increase the daily gain of lambs and the Zn concentration in their serum (Garg et al., 2008). Compared to copper sulphate ($CuSO_4$), the most used Cu supplement, copper (II) oxide (CuO) and copper monosulphide (CuS) are poorly available (Underwood & Suttle, 1999). However, despite our assumption that greater absorption equates to reduced micronutrient loss from livestock, some absorbed micronutrients will still be excreted through endogenous excretion such as bile and sloughed epithelial cells. Therefore, studies on the effect of the composition of micronutrients supplied from feed and supplements on micronutrient excretion are required for understanding micronutrient in manure, and subsequent micronutrient availability in pasture systems.

1.5 Accumulation of micronutrients from livestock excreta in soil and uptake by forage

Despite the substantial quantity of micronutrients applied to pasture systems from livestock excreta, not all of it is readily available to plants. Sequential extraction procedures (SEP) are widely used as an indirect technique for understanding micronutrient fractionation in a solid phase (Bacon & Davidson, 2008; Gleyzes et al., 2002) such as animal manure. Micronutrients that are extracted early in a SEP are generally recognized as weakly bound to the solid phase, and hence could potentially

Table 1.4 Concentrations of macro and micronutrients in livestock manure and ratios of micronutrient concentrations in manure and in feed

Nutrient concentrations	Manure sources			
	Dairy cattle	Beef cattle	Broiler	Layer
		Macronutrients[1]		
N (%)	0.6	1.1	0.3	2.1
P_2O_5 (%)	0.3	1.0	2.6	3.3
K_2O (%)	0.7	1.5	1.8	1.7
		Micronutrients[2]		
Fe (mg kg^{-1})	897	657	618	1134
Mn (mg kg^{-1})	311	129	497	630
Cu (mg kg^{-1})	75.7	16.2	206	75
Zn (mg kg^{-1})	350	81.5	549	518
Co (mg kg^{-1})	1.61	0.65	0.78	1.52
Se (mg kg^{-1})	1.16	0.54	2.21	2.94
I (mg kg^{-1})	1.66	0.73	2.55	3.31
		Manure/feed concentration ratios[2]		
Fe (mg kg^{-1})	3.1	6.5	2.3	
Mn (mg kg^{-1})	3.2	2.6	3.4	
Cu (mg kg^{-1})	3.4	2.0	3.3	
Zn (mg kg^{-1})	2.9	2.2	3.4	
Co (mg kg^{-1})	3.3	4.5	3.3	
Se (mg kg^{-1})	2.1	1.4	2.8	
I (mg kg^{-1})	2.4	1.5	3.3	

[1]The systems from which the manure were collected were FYM for dairy cattle, feedlot for beef cattle, solid with litter for broiler and layer. The unit is weight percentage on a wet basis (Wilkinson, 1979). [2]Sheppard and Sanipelli (2012).

have greater mobility and environmental impact compared to the later fractions (Bacon & Davidson, 2008). Among the different fractions in the SEP designed by the Bureau Community of Reference (BCR) (**Table 1.5**), water-soluble and exchangeable fractions are considered to be bioavailable; oxides-, carbonate-, and OM-bound fractions may be potentially bioavailable; and the residual fraction is generally not available either to plants or microorganisms (He et al., 2005). Bolan et al. (2004) reviewed studies into

the forms of some micronutrients in livestock manures as determined by SEPs. They found that metallic micronutrients including Cu and Zn mostly exist in the OM fraction, but the SEP fractionation depends on the individual mineral and manure type. For example, a greater proportion of the total Cu (31.6 %) and Zn (16.1 %) extracted was present in the water-soluble fraction, compared to that of Mn (5.1 %), in dairy cattle manure (Bolan et al., 2004). For Zn, 16.1, 7.4 and 1.9 % was present in the water-soluble fraction of manures from dairy cattle, poultry, and pigs, respectively. The non-metallic micronutrients, Se and I, are poorly studied and require further research.

Table 1.5 Revised-BCR sequential extraction procedure*

Step	Extraction reagents	Targeted phases
1	0.11 mol L^{-1} CH_3COOH	Water soluble, exchangeable cations and carbonate bound
2	0.5 mol L^{-1} $NH_2OH.HCl$ at pH 1.5	Fe-Mn oxide bound
3	H_2O_2 (85 °C) then 1.0 mol L^{-1} $CH_3CO_2NH_4$	OM and sulphide bound
4	*Aqua regia*	Residual

*This method was originally proposed by the Community Bureau of Reference (BCR) and revised by Rauret et al. (1999).

Micronutrients in manure may accumulate in soils or be lost through leaching or surface runoff (**Table 1.6**). In past studies, Cu, Zn and Mn were the micronutrients of most interest due to their relatively high concentrations in livestock excreta and their potential toxicity to plants if accumulating in soil. However, the accumulation and movement of manure-borne Co, Se and I in soil are rarely studied, which may be due to their relatively low concentrations in manure compared to in soil. Similarly, studies on manure-borne Fe are also limited, which may be due to the high background concentration of Fe in soil.

Regarding accumulation of micronutrients in soil after applying manures, most studies report total concentrations of micronutrients at the soil surface, and a few report total concentrations at depth (**Table 1.6**). After a ten-year application of cattle or dairy manure, the concentrations of Fe, Mn and Cu in surface soil were found to be significantly elevated (Benke et al., 2008; Brock et al., 2006; Sheppard & Sanipelli, 2012). Similar observations were indicated by some studies applying pig manure (Xu et al., 2013) or poultry litter (Gupta & Charles, 1999). However, for Zn, some studies showed raised Zn concentrations (Benke et al., 2008; Brock et al., 2006) whereas others also indicated reduced Zn concentrations of surface soil after ten-year cattle manure application (Zhao et al., 2014) or no significant difference after seven-year cattle manure application (Lipoth & Schoenau, 2007) or more than fifteen-year poultry litter application (Gupta & Charles, 1999). Downward movement of micronutrients is more obvious in soil profiles after longer term manure application, and with higher application rates. For example, the downward movement of Cu was limited to <15 cm after a three-year application of broiler litter to the soil surface (Adeli et al., 2007) whereas significant movement of Cu and Mn, down to 60 cm, was observed after a >15-year application of poultry litter at 8.98 tonnes $ha^{-1}y^{-1}$ (Gupta & Charles, 1999). More significant downward movement of Cu and Zn to 90 cm and 30 cm depth, respectively, was observed at a higher rate (180 tonnes $ha^{-1}y^{-1}$) than at lower application rates of cattle manure (Benke et al., 2008). No significant soil accumulation or downward movement of Co was observed after 25-years of the application of cattle manure. There is a lack of studies investigating the vertical movement of Se and I in soil profiles.

To evaluate the potential availability of micronutrients to plants, some studies carried out diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid

(EDTA) extraction on manured soils for metallic micronutrients whereas others reported the concentrations or total uptake in plants directly (**Table 1.6**). The effects of manure application on micronutrient concentrations in plants can vary due to plant type, manure type and the duration of manure application. For example, both a pot and a field experiment found increased Mn uptake in cowpea (*Vigna unguiculate*) and in maize (*Zea mays*), respectively, after soil application of cattle manure (Abebe et al., 2005; Zhao et al., 2014). Conversely, the application of poultry litter seemed to lower Mn uptake by certain clovers (*Trifolium* spp.) (Bomke & Lowe, 1991). For Cu and Zn, most studies found increased EDTA- or DTPA-extractable concentrations after manure application (Benke et al., 2008; Cambier et al., 2014; Lipoth & Schoenau, 2007; Xu et al., 2013); only Benke et al. (2008) observed decreased concentrations of EDTA-extractable Cu in soil-surface applied cattle manure over 25 years. An interesting observation was made that despite the increased total soil Cu, the DTPA-extractable Cu in surface soil (0-15 cm) was significantly decreased (Benke et al., 2008), indicating that the application of cattle manure led to the accumulation of Cu in the less labile soil fractions and was therefore less available to plants. For studies reporting micronutrient accumulation in manured soils, the proportion of plant available micronutrients relative to the total concentrations in soil should also be reported. Only a few studies have been reported such results for Co, Se and I, including a two-year field study which showed that the application of poultry manure (40 tonnes $ha^{-1}y^{-1}$) can increase Se concentrations in clover forage.

1.6 Factors influencing soil micronutrient availability to plants

According to data from the North Wyke Farm Platform, total soil concentrations of Fe, Mn, Cu and Zn were poorly related to the total concentrations in forage (**Table 1.7**). The coefficients of determination (R^2) between soil and forage concentrations of Cu,

Zn, Fe and Mn on three different forage treatments (permanent pasture, grass-legume mixtures, and regularly reseeded grass ley) were statistically insignificant. Total Se concentrations in soil may also be a poor predictor of associated vegetation Se concentrations (Bowley et al., 2017). The key reason for these discrepancies is that the availability of a micronutrient in soil depends upon its effective concentration in soil solution (Reid & Horvath, 1980). The equilibrium concentration of micronutrients in soil solutions are regulated by several chemical and biological processes, including plant removal, sorption-desorption from sorption sites, complexation with inorganic/organic complexes, mineral precipitation, microbial activity and leaching/surface runoff (Bolan et al., 2004; Loneragan, 1975; Reid & Horvath, 1980).

Together with the effective concentration, the speciation of a micronutrient in soil solution determines its plant availability (Loneragan, 1975). For Fe, since the concentration of ionic Fe (Fe^{2+} and Fe^{3+}) in a typical aerated soil is low, Fe chelated with phytosiderophores, such as nicotianamine and mugineic acid, or with fungal siderophores, such as ferricrocin, become the predominant forms of available Fe to plants (Boiteau et al., 2018). Mn, Cu, Zn and Co are predominately taken up as the divalent cations, Mn^{2+}, Cu^{2+}, Zn^{2+} and Co^{2+}, respectively (Antoniadis et al., 2017; Palit et al., 1994). For Se, oxyanionic Se (IV), Se (VI) and Se in amino acids, e.g. SeMet and selenocysteine (SeCys) are plant-available (Kikkert & Berkelaar, 2013; Sors et al., 2005). Selenate (SeO_4^{2-}) uptake by plants is typically greater than SeO_3^{2-} uptake, since the former can be transported actively through sulphate transporters, whilst the latter can only be passively absorbed (Fernández-Martínez & Charlet, 2009; Sors et al., 2005). However, Kikkert and Berkelaar (2013) indicated higher uptake rates of SeO_3^{2-} than SeO_4^{2-} by both canola (*Brassica napus*) and wheat (*Triticum aestivum*), which may be

Table 1.6 Accumulation and movement of micronutrient in soil and availability to forages post manure application

Element	Manure type	Application method in field/pot experiment	Movement and accumulation in soil profile	Plant availability or plant uptake	Reference
Fe	Cattle manure	Annual application at 0, 20, 40 tonnes ha^{-1} on field for 10 years	Significant higher Fe soil content in 0-20 cm depth.	Significant higher contents of Fe in stems and seeds of corn were observed.	1
	Poultry manure	Manures at rate of 0, 10, 20 and 40 tonnes ha^{-1} were applied in pot soils.	N.A.	No significant effects on Fe concentrations in tomato plants	2
	Pig slurry	The slurry was applied six times a year. A total of 4571 $m^3 ha^{-1}$ of slurry was spread by broadcast on the field for 5 years.	Low concentrations (10 μg L^{-1}) of Fe was found in leachate.	N.A.	3
Mn	Cattle manure	Annual application at 0, 20, 40 tonnes ha^{-1} on field for 10 years	Significant higher Mn soil content in 0-20 cm depth was observed.	Significant higher contents of Mn in stems and seeds of corn were observed.	1
	Cattle manure or poultry litter	The manures were applied at rates of 0, 20, 40, 60, 100 tonnes ha^{-1} in pot soils.	N.A	Significant increased Mn content in cowpea at application rates (20 to 100 tonnes ha^{-1})	4
	Dairy manure	No history record available but the author indicated that the fields all had a history of heavy manure application.	Significant higher Mn accumulation in surface soil (0-15 cm) than subsoil (>30cm) was observed.	N.A.	5
	Poultry litter	8.97 tonnes/ha of self-dried manure were applied annually on field for 15-20 years (broadcast or disk to 10 cm depth).	Significant higher Mn content than un-manured lands in soils down to 60 cm depth.	N.A.	6
	Poultry manure	Annual application at 0, 1.25, 2.5, 5, 10, 20 and 40 tonnes/ha (fresh weight) on field for 2 years.	N.A.	Significant lowered uptake and concentrations in 1st-cut clover forage.	7
	Pig slurry	The slurry was applied six times a year. A total of 4571 $m^3 ha^{-1}$ of slurry was spread by broadcast on the field for 5 years.	Little influence from slurry application on Mn fractionation was observed. Significant higher concentrations in leachates over the control were observed.	N.A.	3

Cu	Cattle feedlot manure	The manure was applied annually at 0, 30, 60, 90 tonnes ha^{-1} on a rainfed field and at 0, 60, 120, 180 tonnes ha^{-1} on an irrigated field for 25 years.	Significantly higher Cu soil content was observed in the 0-15 cm depth. At high application rate (180 tonnes ha^{-1} y^{-1}), significant movement was observed down to 90 cm depth.	Decreased concentrations of EDTA-extractable Cu in 0-15 cm depth of soil.	8
	Poultry litter	8.97 tonnes ha^{-1} of self-dried manure were applied annually on field for 15-20 years (broadcast or disk to 10 cm depth).	Significant higher Cu content than un-manured lands in soils down to 60 cm depth.	N.A.	6
	Poultry manure	Annual application at 0, 1.25, 2.5, 5, 10, 20 and 40 tonnes ha^{-1} (fresh weight) on field for 2 years.	N.A.	Significant higher Cu uptake and concentrations in 1st and 2nd cuts of clover forage was observed.	7
	Broiler litter	The litter was annually applied at 0, 2.2, 4.5 and 6.7 tonnes ha^{-1} on field for 3 years.	Downward movement of Cu was limited to 0-15 cm depth.	N.A.	9
	Pig manure	Annual application at 0, 100, 250 and 500 kg total-N ha^{-1} for 7 years and at 0, 10, 25 and 50 tonnes/ha (fresh weight) for 3 years on field. The applied manure was incorporated (15 cm depth).	Significantly increasing Cu accumulation in soil (0-15 cm) with times of application was observed.	Significantly increasing DTPA-extractable soil Cu with times of application. Decreased Cu concentrations in stems and grains of soybean.	10
	Pig slurry	The slurry was applied six times a year. A total of 4571 m^3 ha^{-1} of slurry was spread by broadcast on the field for 5 years.	Cu accumulation in soil occurred in 0-20 cm, 20-40 cm and 40-60 cm depth. Major soil Cu fraction shifted from residual fraction to OM-bound fraction. Very low concentrations (3 μg L^{-1}) of Cu was found in the leachate.	N.A.	3
	Liquid pig or solid cattle manure	One field was applied liquid pig manure for 5 years (injected to 10-13 depth) and the other field was applied the cattle manure for 7 years (broadcast and incorporate into soils).	At high application rate of pig or cattle manure, total concentration of Cu in surface soil increased.	At high application rate of pig or cattle manure, DTPA-extractable Cu concentration in surface soil and Cu plant uptake increased. The proportion of DTPA-Cu of total Cu in soil decreased on the treatment of cattle.	11

	Dairy manure or solid poultry layer manure	The manures were annually applied on the fields for up to 40 years.	Cu accumulated in the plow layer only. Cu accumulation rate was 10 times greater for dairy manure fields than that for poultry manure field.	N.A.	12
Zn	Cattle feedlot manure	The manure was applied annually at 0, 30, 60, 90 tonnes ha^{-1} on a rainfed field and at 0, 60, 120, 180 tonnes ha^{-1} on an irrigated field for 25 years.	Significantly higher Zn soil content was observed in the 0-15, and15-30 depth at high application rate (120 and 180 tonnes ha^{-1} y^{-1})	Increased EDTA-extractable fractions om 0-15, 15-30 cm depth of soil.	8
	Cattle manure	Annual application at 0, 20, 40 tonnes ha^{-1} on field for 10 years	Significant lower Zn soil content in 0-20 cm depth was observed.	Significant higher contents of Zn in stems and seeds of corn were observed.	1
	Poultry litter	8.97 tonnes ha^{-1} of self-dried manure were applied annually on field for 15-20 years (broadcast or disk to 10 cm depth).	No significant higher Zn content than un-manured lands in soil profile (to 60 cm depth).	N.A.	6
	Poultry manure	Annual application at 0, 1.25, 2.5, 5, 10, 20 and 40 tonnes ha^{-1} (fresh weight) on field for 2 years.	N.A.	Significant higher Zn uptake and concentrations in 1^{st} to 4^{th} cuts of clover forage was observed.	7
	Poultry litter	Annual application on field for 25 years	Accumulate close to surface and dominantly in reducible fraction (47.3%).	N.A.	13
	Broiler litter	The litter was annually applied at 0, 2.2, 4.5 and 6.7 tonnes ha^{-1} on field for 3 years.	Downward movement of Zn was limited to 0-15 cm depth.	N.A.	9
	Liquid pig or solid cattle manure	One field was applied liquid pig manure for 5 years (injected to 10-13 depth) and the other field was applied the cattle manure for 7 years (broadcast and incorporate into soils).	At high application rate of pig manure, total concentration of Zn in surface soil increased, whereas there was no significant increased Zn on the treatment of cattle manure.	At high application rate of pig or cattle manure, DTPA-extractable Zn concentrations in surface soil, proportions of total soil Zn and plant uptake were all increased.	11
	Dairy manure or solid poultry layer manure	The manures were annually applied on the fields for up to 40 years.	Zn accumulated in the plow layer only.	N.A.	12

	Pig manure	Annual application at 0, 100, 250 and 500 kg total-N ha^{-1} for 7 years and at 0, 10, 25 and 50 tonnes/ha (fresh weight) for 3 years on field. The applied manure was incorporated (15 cm depth).	Significantly increasing Zn accumulation in soil (0-15 cm) with times of application was observed.	Significantly increasing DTPA-extractable soil Zn with times of application. Decreased Zn concentrations in stems and grains of soybean and increased Zn concentrations in maize grains were observed.	10
	Pig slurry	The slurry was applied six times a year. A total of 4571 $m^3 ha^{-1}$ of slurry was spread by broadcast on the field for 5 years.	Significant soil accumulation of Zn was observed in 0-20 cm and 20-40 depth. Significant shift of main soil Zn fraction from residual fraction to oxidizable fraction was observed in surface soil (0-20 cm). Significant higher concentrations in leachate over the control were observed.	N.A.	3
Co	Cattle feedlot manure	The manure was applied annually at 0, 30, 60, 90 tonnes ha^{-1} on a rainfed field and at 0, 60, 120, 180 tonnes ha^{-1} on an irrigated field for 25 years.	No significant increase in soil total Co or downward moving was observed.		
	Poultry manure	Annual application at 0, 1.25, 2.5, 5, 10, 20 and 40 tonnes ha^{-1} (fresh weight) on field for 2 years.	N.A.	Concentrations of forage were below detection limits.	7
	Pig slurry	The slurry was applied six times a year. A total of 4571 $m^3 ha^{-1}$ of slurry was spread by broadcast on the field for 5 years.	No significant change of soil total Co or Co fractionation was observed. Significant higher concentrations in leachate over the control were observed.	N.A.	3
Se	Dairy manure	No history record available but the author indicated that the fields all had a history of heavy manure application.	Significant higher Se accumulation in surface soil (0-15 cm) than subsoil (>30cm) was observed.	N.A.	5
	Poultry litter	The field had a history of intensive litter application for 30 years before the experiment. The litter was applied on field	Se was detectable in the leachate at day 15 after application despite it was not significantly higher than the control. No	N.A.	14

	at 6.7 tonnes ha^{-1} by broadcast, by disk tillage or by injected in 8 cm depth.	detectable Se was in leachate at day 42 after application.		
Poultry manure	Annual application at 0, 1.25, 2.5, 5, 10, 20 and 40 tonnes ha^{-1} (fresh weight) on field for 2 years.	N.A.	Manure application increased 1st-cut clover forage Se concentrations by an average of 39% over the control.	7

References: 1. Zhao et al. (2014) 2. Demir et al. (2010) 3. L'Herroux et al. (1997) 4. Abebe et al. (2005) 5. Sheppard and Sanipelli (2012) 6. Gupta and Charles (1999) 7. Bomke and Lowe (1991) 8. Benke et al. (2008) 9. Adeli et al. (2007) 10. Xu et al. (2013) 11. Lipoth and Schoenau (2007) 12. Brock et al. (2006) 13. Han et al. (2000) 14. Kibet et al. (2013).

Table 1.7 Coefficients of determination (R^2) of the correlations between total concentrations in surface soils* and the concentrations in forages* for Fe, Mn Cu and Zn under three forage management practices from Rothamsted Research North Wyke Farm Platform (Southwest, England)

	Cu	Zn	Fe	Mn
Grass-legume mixture	0.0010	0.0792	0.0320	0.0971
Permanent pasture managed	0.0000	0.0001	0.0973	0.2382
Reseeded grass monoculture swards	0.0023	0.0323	0.0159	0.0332

*The forage and soil samples were collected in three seasons (April-May, July-August and October) in 2018; in each season, seven bulked samples, composed of 20 sub-samples collected at random at each field) were collected from seven fields of the same forage treatment.

due to the presence of sulphate in the hydroponic system. Like SeO_4^{2-}, seleno-amino acids (SeMet and SeCys) can be actively taken up by plants through amino acid transporters, and were shown to be taken up by plants more efficiently than inorganic Se (Kikkert & Berkelaar, 2013). Iodine as I^-, at lower pH, or IO_3^-, at higher pH, are both plant-available, but I^- is the more prominent form at typical pasture soil pH (Fuge, 2013).

Micronutrient speciation and concentrations in soil solution are related and influenced by many of the same soil properties and processes. **Figure 1.3** illustrates the physical, chemical and biological reactions in soil that potentially influence micronutrient availability to plants. Chemical and biological reactions occur in soil solution and at the solid-water interface (Johnston & Tombacz, 2002). Soil properties or agricultural management that affect these reactions are wide ranging and the major factors will be discussed in the following sections in brief. The effects of these factors vary considerably from one element to another as well as in their relative degree of effectiveness (Fageria et al., 2002).

1.6.1 Soil acidity and soil redox status

Soil pH and soil redox potential (Eh) are the two major geochemical processes controlling the solubility and mobility of an element (Bourg & Loch, 1995). These parameters refer to the concentration of H^+ ions, and the potential for electrons to be transferred from one substance to another, respectively. In soil, where considerable numbers of redox reactions occur, Eh measurement aids in the estimation of the oxidation status of soil, with more positive values representing more oxidising conditions. The variation of soil pH and Eh, either singularly or together, can make a significant impact on the chemical behaviour of an element. As a result, chemical processes such as sorption-desorption, precipitation-dissolution, and complexation are

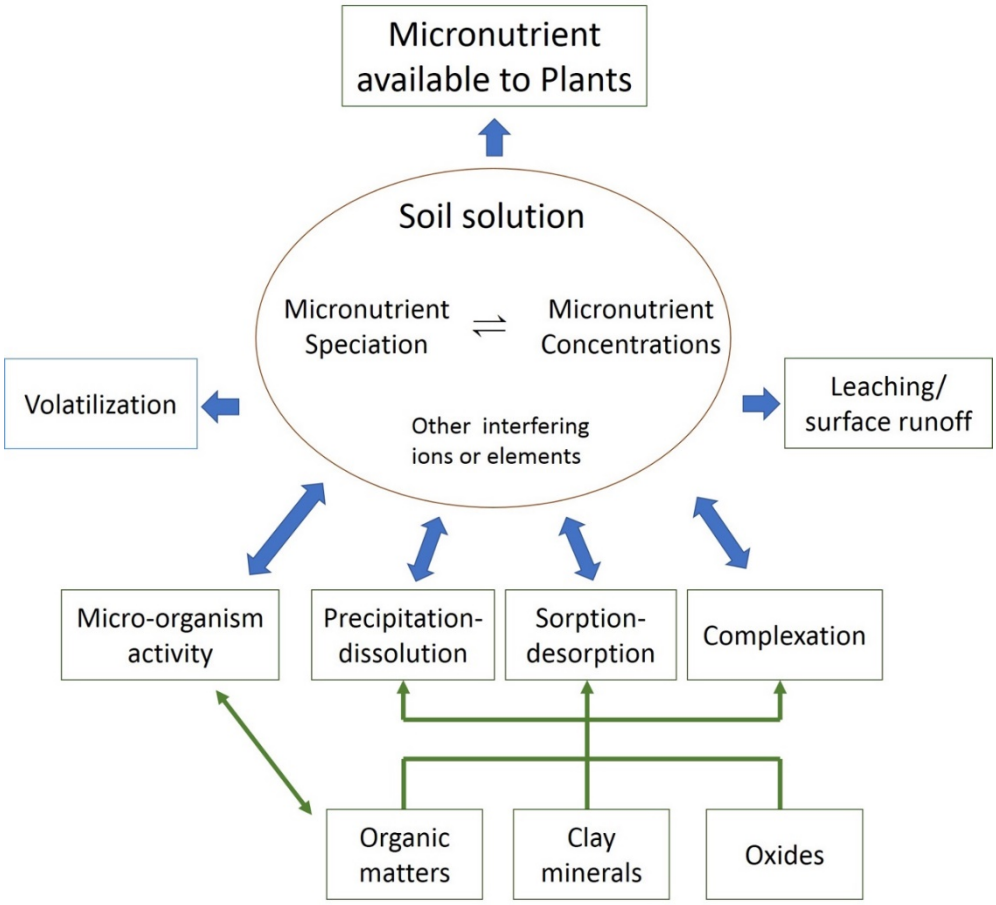


Figure 1.3 Major physical, chemical and biological reactions and factors in soil system that influence micronutrient availability to plants. The blue-wide arrows symbolize micronutrient movement or chemical and biological reactions and the green-fine arrows symbolize factors that affect the chemical or biological reactions.

all affected, and each of these chemical processes can influence the availability of a micronutrient to plants.

In solution chemistry, using an Eh-pH diagram is a simplified but useful way to predict the species change of an element under specific pH and Eh conditions. Simple Eh-pH diagrams for Cu, Zn, Fe, Cu, Co, Se and I, as summarized by Brookins (2012), are presented in **Figure 1.4**. In an Eh-pH diagram, a line represents equilibrium between the two species on each side of the line at certain pH and Eh. Soil Eh normally fluctuates between -300 and +900 mV depending on the aeration of a soil, and cultivated soils are most frequently in the range of +300 and +500 under aerobic conditions (Husson, 2013).

Most cultivated soils have a pH between 4 and 9 (Husson, 2013). According to the Eh-pH diagrams, in most cultivated soils, speciation of Cu and Zn is more likely to be affected by a change of pH than Eh. For Fe, Mn, Co and Se, the variation of pH and Eh equally influence their chemical speciation, and for I, the variation of Eh dominates the speciation.

Soil pH and Eh largely govern micronutrient speciation change in soil solution, which in turn affects the availability of a micronutrient to plants. For example, when soil pH and Eh increase, Fe tends to be present in Fe (III)-oxides/hydroxides, which are less soluble forms of Fe, similar to Mn (II) in Mn oxides (Husson, 2013). Zn and Cu are more mobile at lower soil pH in the form of Zn (II) and Cu (II), respectively(Husson, 2013). The lowered mobility of Cu and Zn at higher soil pH is mainly due to their stronger sorption to oxides and hydroxides, OM, and clay minerals (Fageria et al., 2002), whereas Mn and Fe mobility is due to both a sorption effect and an oxidation effect. The mobility of Co under various soil Eh-pH conditions has been poorly studied. However, in general, low pH and moderate to high Eh are the most favourable for the solubilisation of many heavy metals (Bourg & Loch, 1995).

In contrast to metals, Se and I exist as anions under common soil conditions (Winkel et al., 2015). In general, it is agreed that under oxic and moderately reducing conditions, Se solubility is governed by adsorption (Nakamaru & Altansuvd, 2014). In contrast, under the reducing conditions found in wetlands, the oxyanions of Se are reduced to Se (0) within weeks, and the reduced Se can complex with OM or sulphides, and are therefore less available; these forms can comprise more than 50 % of the total Se in wet soils (Nakamaru & Altansuvd, 2014). I^- is the major form of soluble I in acid and waterlogged soils, while IO_3^- is the dominant species of soluble iodine in dry, oxidizing and alkaline soils (Yuita, 1992). Similar to Se, the sorption of ionic forms of I by Fe

and Al oxides is strongly sensitive to soil pH, with the sorption greatest in acid conditions (Fuge, 2013). In most cases, I in soil is strongly sorbed, especially onto organic substances and Al and Fe oxides, and is not easily desorbed. However, under reducing conditions, such as those found in waterlogged soils (Muramatsu et al., 1996), I tends to be more solubilized than under dry and oxidizing conditions (Ashworth, 2009; Fuge, 2013; Yuita, 1992). The desorption of I under anoxic conditions may be due to the speciation shift from IO_3^- to I^-. However, this assumption contradicts a study showing that both species in soil solution were increased under anoxic conditions (Ashworth, 2009). The underlying mechanism of I sorption-desorption under various redox status remains unclear, but it is presumed that it is highly associated with microbial activity (Amachi, 2008).

The chemical changes of sulphide (S^{2-}) and OM in soil under various redox states may also affect micronutrient speciation and thus, availability. For example, bonding to S^{2-} and to OM dominates the fluctuation of the concentrations of Cu and Zn in the exchangeable phase under variable soil Eh and pH (Calmano et al., 1993). Similarly, the frequent low Zn concentration in flooded soils may be associated with the precipitation of ZnS or the formation of OM-Zn complexes under anaerobic conditions (Fageria et al., 2002). These observations revealed the ternary relation between soil pH, redox status and S^{2-}/OM. Some agricultural management practices, such as applying inorganic or organic fertilizers, liming and irrigation can also alter soil pH and Eh, which will be reviewed further in **Chapter 1.6.7**.

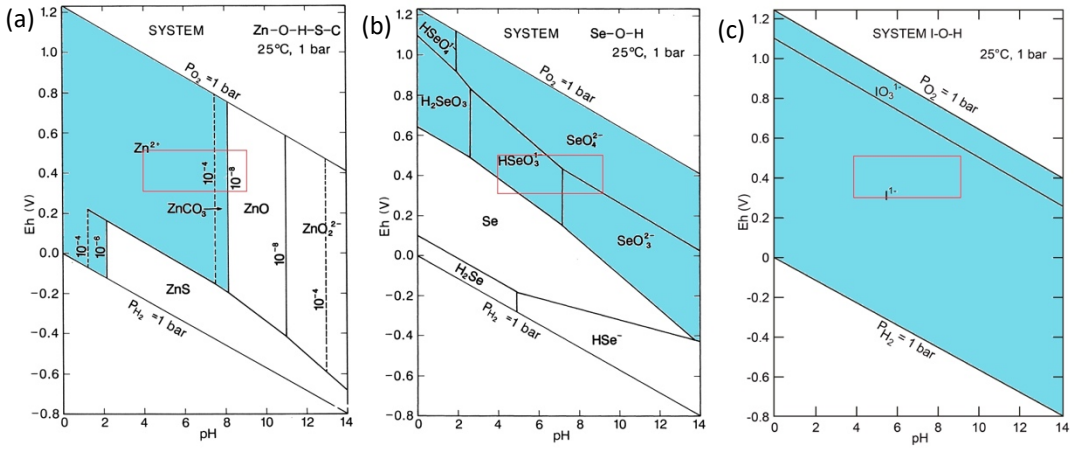


Figure 1.4 Eh-pH diagrams of micronutrients for (a) Zn, (b) Se, (c) I from Brookins (2012). The coloured areas are where plant-available chemical species of a micronutrient locate in its diagram. The red boxes with dotted-lines represent the Eh-pH range, Eh=+300~+500 mV and pH=4~9, where typical soils fluctuate under aerobic conditions (Husson, 2013).

1.6.2 Organic matter

Soil OM interacts with nearly all soil properties and functions, affecting the availability of a micronutrient to plants. Due to the multiple effects of OM on soil chemistry and physics, its role in micronutrient flux in soil and the underlying mechanisms can be extremely complex. Some major effects of OM are discussed here. The formation of OM-metal compounds generally enhances the mobility and plant availability of metallic micronutrients (Fageria et al., 2002) but the extent is element dependent. In a 21-year tillage experiment, the quantity of soil Mn, Cu and Zn extracted by DTPA was significantly (P<0.01) and positively correlated with the OM content of soil (de Santiago et al., 2008). However, Cu is known to be more tightly bound to OM compared to other metals, according to the order of the affinity for metal cations complexed by OM: Cu^{2+}>Cd^{2+}>Fe^{2+}>Pb^{2+}>Ni^{2+}>Co^{2+}>Mn^{2+}>Zn^{2+} (Adriano, 2001).

The properties of OM are also significant with regards to the availability of the complexed micronutrients. Soil OM can be classified into 'operationally-defined' groups, i.e., water-insoluble humic acids or humin, or water-soluble fulvic acids and

low-molecular weight microbial products (Fageria et al., 2002). Metal complexation with humic substances is normally strong, while ionic-bonding with low-molecular-weight organic acids (LMWOAs, e.g., acetic, citric, malic) is relatively weak (Fageria et al., 2002). The humic substances with low molecular-mass, like fulvic acid (FA), can contribute to the available pool of Fe present in soil solution due to their solubility and capability in forming dissolved Fe-OM complexes, while the humic substances with a high-molecular-mass, which have lower solubility, can remove large amounts of Fe from available pools, lowering Fe availability (Colombo et al., 2014). Similarly, Wang et al. (2010) indicated that higher complexation stability with Cu (II) was found in the OM fraction with molecular weight, determined by means of sequential-stage ultrafiltration technique (Burba et al., 1998), of more than 1 kDa, which resulted in a lower availability of Cu to the plant, while in the OM fraction of molecular weight lower than 1 kDa, the opposite effect was seen. For Co, there is little research on the effect of soil OM on its mobility and availability but its affinity to OM is assumed to be less than Cu (Lange et al., 2016).

Selenium is likely to be bound to OM and sulphides under reduced soil conditions. The soil adsorption coefficient (*K*d) of Se for OM is about twice that for soil clays, with a value Kd_{SeOM} = 1800 L kg^{-1} (Fernández-Martínez & Charlet, 2009). The exact mechanisms of OM-Se binding are still unclear. However, three possible retention mechanisms of Se by OM were summarized by Winkel et al. (2015) including: (i) direct OM complexation, (ii) indirect OM-complexation via OM-metal complexes, and (iii) microbial reduction and incorporation into amino acids, proteins, and natural OM. Plant uptake of Se can be either positively or negatively correlated with soil OM (Li et al., 2017; Winkel et al., 2015) . Qin et al. (2012) indicated that FA-Se was the predominant form of Se in all the soils studied, accounting for 62 % of OM-Se, and that of the total

FA-Se, weakly bound FA-Se was most prevalent, indicating OM-Se as a significant source of bioavailable Se. Wang et al. (1996) indicated that FA is likely to be a buffer that stimulates the bioavailability of Se in low concentrations, while mitigating the toxicity of Se in high concentrations. For I, OM is one of the most influential soil components (Fuge, 2013; Medrano-Macias et al., 2016), with most I occurring as organic-bound in soil profiles analysed by XANES and micro-XRF (Shimamoto et al., 2011). The degree of humification does not significantly affect I sorption in soil (Duborska et al., 2019).

1.6.3 Inorganic constituents of soil

In addition to soil OM, the inorganic constituents, such as clay minerals, oxides, carbonates and hydrous oxides of Fe, Al and Mn, that usually comprise more than 90 % of the mass of soils (Alloway, 2013), encompass the chemically reactive surface sites for ions and molecules in soil (Alloway, 2013; Johnston & Tombacz, 2002). The reactive surface sites of a constituent can be divided into polar and nonpolar sites, of which their relative distribution on each inorganic constituent of soil was summarized by Johnston and Tombacz (2002). Of these, the polar sites are where reactions relevant to the mobility and plant availability of micronutrients normally occur.

The cumulative negative charges of a clay mineral are comprised of permanent charges created by isomorphous substitution in phyllosilicate, and temporary, pH-dependent charges developed on the edges and surfaces of the layer sheets of phyllosilicates (Brady & Ray, 2000). pH-dependent charges in a clay mineral vary with soil pH, and ultimately affect the ability of a clay mineral to provide sorption sites. As soil pH increases, these pH-dependent negative charges on the mineral clays increase, resulting in higher sorption of metal cations, like Cu^{2+} and Zn^{2+}. Selenium, commonly present as

an anion, is less retained by clay minerals than by OM (Fernández-Martínez & Charlet, 2009). Sorption of Se by anionic clays and layered double hydroxides through electrostatic interactions is highly pH dependent (Winkel et al., 2015). As pH increases, Se adsorption decreases due to more negatively charged clay surfaces and sesquioxide edges, which results in weaker electrostatic interactions (Winkel et al., 2015). Selenium solubility in the presence of kaolinite and montmorillonite is governed by adsorption. The adsorption decreases with a pH increase between pH 4 and 8 and becomes negligible above pH 8 (Bar-Yosef & Meek, 1987). Therefore, the mobility and bioavailability of inorganic Se in the environment increases with increasing pH, and with decreasing clay content in the soil (Winkel et al., 2015).

Oxides and hydroxides in soil can scavenge heavy metals, either as strong adsorbents or as coprecipitating matrices (Bourg & Loch, 1995). The presence of Fe oxides/hydroxides increase Cu and Zn sorption, lowering the availability of Cu and Zn to plants (Al-Sewailem et al., 1999; Suhr et al., 2018). At low soil pH, more of the bound metals can be solubilized from Fe and Al oxides, while at higher soil pH, sorption of metals on those oxides increases (Al-Sewailem et al., 1999). In soil, the sorption of Se and I (both commonly present as anions) onto Fe or Al oxides/hydroxides, which have net positive surface charges at typical pH ranges, is more dominant than adsorption onto clays (Fuge, 2013; Nakamaru & Altansuvd, 2014). The sorption of Se has been reported for many kinds of Al and Fe hydroxides, such as gibbsite and goethite (Nakamaru & Altansuvd, 2014). Using ^{75}Se as radioactive tracer, Nakamaru et al. (2005) found that 80-100 % of an added SeO_3^{2-} solution was sorbed by Al or Fe oxyhydroxides. The sorptive properties of oxides and carbonates for Se are pH-dependent. At low soil pH, higher retentions of Se can be found as these surfaces develop more positively charged sites than negatively charged sites. The mobility and bioavailability of the

sorbed Se is associated with the sorption mechanism of Se onto (hydro)oxides, is strongly affected by the ionic strength of soil solution as well as soil pH (Fernández-Martínez & Charlet, 2009). Lower ionic strength is predicted to favour outer-sphere (electrostatic) sorption, and higher ionic strength to favour inner-sphere sorption by (hydro)oxides to form complexes (Fernández-Martínez & Charlet, 2009). There are few studies investigating the effects of oxides and carbonates on I. However, it is generally agreed that Al and Fe oxides play a role in I retention, and that IO_3^- is more strongly and rapidly retained by Al and Fe (hydro)oxides than I^- (Whitehead, 1974). Couture and Seitz (1983) found that at soil pH up to 9, IO_3^- was strongly absorbed by hematite (Fe_2O_3), which presumably occurred by replacement of OH^- ions on the surface. The sorption of IO_3^- on hematite was found to be rapid and reversible. I^- was found to be less strongly sorbed than IO_3^- by hematite at high pH, and by kaolinite at low pH. The presence of carbonates, e.g., calcite ($CaCO_3$) and dolomite ($CaMg(CO_3)_2$), affects soil pH, and ultimately influences the mobilities of micronutrients (Alloway, 2013). Studies of liming on micronutrient availability in soil will be further reviewed in **Chapter 1.6.7**.

1.6.4 Soil moisture and soil temperature

The availability of most micronutrients in soil tends to decrease at low soil temperature and low moisture content, due to reduced root activity and low rates of dissolution and diffusion of nutrients (Fageria et al., 2002). On the other hand, soil moisture directly influences soil oxygen content, which, as discussed in **Chapter 1.6.1**, can impact soil redox status and ultimately the bioavailability of micronutrients. For example, high soil moisture status reduces insoluble manganese dioxide (MnO_2) and releases freely available Mn^{2+}, and co-precipitated Co (Brookins, 2012). Because of this, plant concentrations of these two elements can increase markedly during wet periods and

vice versa. In flooded soils, Zn, Co and Cu may form precipitates with sulphides in soil (Calmano et al., 1993), which lowers their availability to plants.

Dominant climatic factors that affect soil Se concentrations, such as aridity, precipitation and evapotranspiration, are essentially related to soil moisture and these factors are predicted to increase in importance due to climate change (Jones et al., 2017). The aridity index and precipitation, although inversely related, can both have negative effects on topsoil Se concentration (Jones et al., 2017). Precipitation can enhance the diffusion and transport of Se in soil, while soil tends to be more oxic under arid conditions, which results in more mobile Se species. For I, soil moisture affects soil redox status and hence I availability as discussed in **Chapter 1.6.1**. On the other hand, rainfall can deposit a marked amount of I volatilized from the ocean and carried by winds (Reid & Horvath, 1980). Soil moisture which is too high can also lead to soil saturation and, therefore, potential nutrient loss through surface runoff and leaching (Haygarth et al., 1999).

1.6.5 Soil microbial activities

Studies on the effect of microbial activity on the geochemical behaviour of micronutrients in the agricultural context are generally lacking. However, numerous studies have been carried out to facilitate the microbial remediation of heavy metals in contaminated soils (Gadd, 2004; Khan, 2005; Sessitsch et al., 2013). Gadd (2004) reviewed the biogeochemical processes of trace elements that were associated with microbial activities including: acidification, oxidation, reduction, chelation and biomethylation. Some of these processes mobilize metals in soil. For example, oxidation by *Thiobacillus* spp., a S-oxidizing bacterium, can solubilize metals by acidification resulting from the production of sulphuric acid (Gadd, 2004). Similarly, coordination of metals by bacterial chelates, such as carboxylic acids and siderophores,

can increase the mobility of metals in the soil. For instance, several carboxylic acids, (e.g., citric, oxalic and acetic) have been shown to mobilize metals from insoluble metal-containing minerals (Sessitsch et al., 2013). Siderophores, generally known as high-affinity Fe-chelating compounds, are released by organisms to complex Fe (III) under Fe-limiting conditions. Divalent cations, such as Fe^{2+}, Zn^{2+} and Cu^{2+}, were also found to be complexed by siderophores; however, these complexed cations formed extracellularly are less likely to be transported across the cell membrane than their ionic forms and are less likely to be recognized by siderophore receptors due to conformational mismatching (Sessitsch et al., 2013). In grasslands with high soil pH, where metals tended to have low mobilities, grasses produce phytosiderophores for metal acquisition. Similar strategies have evolved in many soil microbes, such as ferricrocin, a fungal siderophore that is known as an important source of bioavailable Fe in soil (Boiteau et al., 2018). Higher plants can take up Fe (II) from ferricrocin via the reduction of the Fe (III)-siderophore complex, which links the fungal activity in soil to Fe availability for plants (Boiteau et al., 2018). However, due to the diverse species of microorganisms and the multiple metal chelating compounds they produce, to date it remains difficult to identify the predominant microorganisms and the associated chelation mechanisms in grassland. On the other hand, some microorganisms may immobilize metals. For example, sulphate-reducing bacteria can reduce sulphate to sulphide and, as this reaction consumes H^+ and increases soil pH, the result is that metals may be precipitated as hydroxides (Gadd, 2004).

For Se and I, biomethylation, the dominant volatilization mechanism, has been well studied. Organic-forms of Se, like Se-Cys and Se-Met, or inorganic forms of Se can be converted into volatile methylated Se, e.g., dimethyl selenide (DMSe), dimethyl diselenide (DMDSe) and selenenyl sulphide (DMSeS), in microorganisms and plants

(Chasteen & Bentley, 2003; Winkel et al., 2015). Once volatile methylated species of Se are formed, they may diffuse out of the cell and be released into the atmosphere (Winkel et al., 2015), lowering Se concentrations in soil or in plants. Mechanisms of Se methylation involve several reductive steps and the addition of methyl groups that result in methylated Se compounds (Chasteen & Bentley, 2003). Se methylation in soil is generally agreed to be a biological process driven by bacteria, e.g., *Enterobacter* sp. (Dungan & Frankenberger, 2000) and *Rhodobacter* sp. (VanFleetStalder et al., 1997). Due to the high solubility of DMSe, the major form of volatilized Se (Sors et al., 2005), the volatilization rate of Se decreases with increasing soil moisture (Fernández-Martínez & Charlet, 2009). Other soil factors that affect microbial activities, such as pH, Eh and OM content, can also influence the volatilization rate of Se. Vriens et al. (2014) quantified Se volatilization from a peat soil, by gas-trapping in the field, as 190-210 ng Se m^{-2} d^{-1}, and about 70 % of the volatilized Se was converted by methylation. This number may be equivalent to 0.77 kg-Se ha^{-1} y^{-1}, if we assume that the depth of soil involved in Se volatilization is 0.2 m and the bulk density of soil is 1600 kg m^{-3}. However, the mean Se concentration of 0.71 mg kg^{-1} in topsoils across England and Wales (Rawlins et al. 2012) would mean that < 0.04% of the total surface Se is volatilized annually, a nearly negligible proportion. Conversely, Zhang and Frankenberger (2000) reported that 65 % of Se was volatilized through methylation in 15 d from soil spiked with high concentrations of SeMet (16-80 μg Se g-$soil^{-1}$), which is a precursor of DMSe. These seemingly contradictory results show that experimental conditions and the forms of Se in the soil substantially affect the outcome. Furthermore, Winkel et al. (2015) pointed out that there is a high spatial and temporal variability in Se volatilization rates. Therefore, the volatilization rate of Se in terms of various soil conditions still needs further research and evaluation.

Methylation of I in the soil is mainly driven by microbial activity, producing compounds, such as methyl iodide (CH_3I) (Amachi, 2008). Aerobic bacteria that can convert I^- to CH_3I include *Alteromonus macleodii*, *Vibrio splendidus*, *Rhizobium* sp. and *Methylosinus trichosporium* (Amachi, 2008). Fungi, including several strains of basidiomycetes, strains of imperfect fungi (e.g., *Penicillium* spp. and *Aspergillus* spp.) and a strain of ascomycete (*Hormoconis resinae*), are also capable of volatilizing I as CH_3I (Ban-Nai et al., 2006). Using sodium iodide as the source of I, the cultivated basidiomycete (*Lentinula edodes*) in culture medium had higher rates of I volatilization (3.4 %) than the other fungal strains tested (Ban-Nai et al., 2006). Biomethylation of I occurs more readily under anaerobic conditions, such as in flooded soils (Muramatsu & Yoshida, 1995). However, there is a lack of research evaluating the volatilization rate of I in terrestrial systems.

Apart from biomethylation, microorganisms also influence the mobility of Se and I through several biochemical processes, such as the microbial reduction of Se (Fernández-Martínez & Charlet, 2009) and the microbial immobilization of I (Amachi, 2008). The reduction of Se that transforms the Se oxyanions, SeO_3^{2-} and SeO_4^{2-}, into elemental Se (0) or reduced forms of Se, can lower the mobility and bioavailability of Se over a wide range of geochemical conditions. There are two types of Se reduction, dissimilatory reduction and assimilatory reduction, and both are driven by microorganisms. In dissimilatory reduction, microorganisms use the oxidized Se (VI) and Se (IV) as the terminal electron acceptors during respiration of organic carbon, or during the oxidation of S produced by sulphate-reducing bacteria (Fernández-Martínez & Charlet, 2009). In assimilatory reduction, the microorganisms incorporate inorganic Se into organic compounds, such as SeMet and SeCys, and is generally assumed to be similar to the pathways of S incorporation in microorganisms due to the chemical

similarities between S and Se (Fernández-Martínez & Charlet, 2009). A similar conversion of Se into organic forms and full reduction to elemental Se has also been reported in other biological media, e.g. in silage as driven by *Lactobacillus* bacteria (M. R. F. Lee et al., 2019). In the microbial immobilization of I, although the mechanism remains unclear, it is generally known that bacterial activity can immobilize I through two possible pathways: oxidation of I^- into I_2, which increases the incorporation of I with organic compounds, and microbial assimilation (Amachi, 2008).

1.6.6 Antagonisms between ions for plant absorption

As stated initially in **Chapter 1.6**, the correlation between the total concentrations of micronutrients in soil and that in forage was found to be insignificant, which may due to the chemical and biological processes affecting the availability of micronutrients to forages. Antagonisms between ions can be one of these processes. For example, high provision of Cu and Zn decreased Fe accumulation in the leaves of sea purslane (*Halimone portulacoides*) but increased Fe accumulation in the roots (Siedlecka, 1995). Some of the Fe-Zn antagonism is explained by the similar ionic radii of hydrated cations and cell regulatory mechanisms between Fe and Zn (Siedlecka, 1995). Cu and Zn were also found to be antagonistic in their accumulation in roots and leaves of the same plant (Siedlecka, 1995). Loneragan (1975) reported Mn^{2+} absorption by legumes was inhibited by the addition of Ca^{2+} into the nutrient solution in a hydroponic study. Absorption of Se by perennial ryegrass (*Lolium perenne L.*) and of strawberry clover (*Trifolium fragiferrum L.*) is also hindered by antagonisms between SeO_3^{2-} and phosphate (PO_4^{3-}) and between SeO_4^{2-} and sulphate (SO_4^{2-}) (Hopper & Parker, 1999). More field and pot studies with Se-S antagonism are reviewed in **Chapter 1.6.7.1**. There is limited research on the antagonisms between I and other ions.

1.6.7 Agricultural management practices

1.6.7.1 Fertilizer application

The influence of fertilizer application on soil micronutrient availability is affected by soil properties like pH and OM content. Inorganic fertilizers provide nutrients from inorganic mineral components with N, P and K fertilizers by far the most common. Organic fertilizers derive nutrients from organic materials, such as animal manure, sewage sludge or agricultural by-products, which vary in their nutrient composition.

Long-term application of inorganic fertilizers can raise the concentrations of some micronutrients in soil. For example, Kuppusamy et al. (2017) reported that a 45-year application of inorganic fertilizers raised the concentrations of Co, Cu, Fe, Mn, Se and Zn in soil of a paddy rice (*Oryza sativa*) field, which presumably was due to amounts of these micronutrients in the fertilizers. Similarly, a long-term experiment initiated in 1856, the Park Grass Experiment at Rothamsted Research in the UK, showed that the yearly input of Se and I was greater than their yearly offtake which was attributed to the application of chalk and fertilizer that contained trace amounts of Se and I (Bowley et al., 2017). As for the effect on micronutrient availability, an 18-year field experiment by Wei et al. (2006) revealed that long-term application of N in the form of urea or animal manure can decrease soil pH, affecting availabilities of micronutrients as discussed in **Chapter 1.6.1**. On the other hand, the application of N and P together may cause a 'dilution effect', resulting in reduced micronutrient concentrations in plants due to the increased crop growth in response to fertilizer application. For example, a long-term (30 years) experiment found that the application of N (urea) and P fertilizers increased DTPA-extractable Fe, Mn, and Cu in the soil (Fan et al., 2012). However, despite the increased total uptake of Fe, Mn and Cu, the concentrations of Cu in maize grains were lowered, and only Mn was found in increased concentrations (Fan et al.,

2012), reflecting the dilution effect from inorganic fertilizer on Fe and Cu. The increased DTPA-extractable Fe, Mn and Cu was likely due to a decrease in pH caused by the application of the fertilizers (Fan et al., 2012). Similar results were reported in a nine-year field experiment by Wang et al. (2017), in which the application of N fertilizer increased the DTPA-extractable Fe, Mn and Cu in a grassland soil by up to 262, 150 and 55 %, respectively, which were negatively correlated with soil pH.

Laser (2007) indicated that the effect of N fertilizer on Se accumulation in plants was insignificant due to the low background soil Se concentrations in the studied grasslands, which ranged between 0.31-0.45 mg kg^{-1}. Such Se levels are in the typical range for soils, implying that in normal grassland, N fertilizer application might have little effect on Se taken up by plants. Also, liming had no significant effect on Se uptake from soils without additional Se fertilizer (Laser, 2007). Soil application of S fertilizer has been shown to decrease Se uptake by ryegrass, alfalfa (*Medicago sativa*) and wheat (Cartes et al., 2006; Liu et al., 2015; Mackowiak & Amacher, 2008). Fan et al. (2008) showed that the temporal fluctuation of Se concentrations in wheat grain was opposite to atmospheric S concentrations, and that the Se concentrations were always low when sulphate fertiliser or farmyard manure (FYM), which mineralises producing sulphate, was applied. Comparative research attributed these observations to the elemental antagonism between Se and S (Cartes et al., 2006; Mackowiak & Amacher, 2008), since SO_4^{2-} and SeO_4^{2-} are presumed to share the same transporter in plants (Sors et al., 2005). Another study attributed the antagonism to acidification provided by the applied elemental S, according to the observed increase in oxide-bound Se and decrease in soluble Se in soil after S application (Liu et al., 2015). The impact of inorganic fertilizer on the mobility or bioavailability of I is poorly studied.

The effect of the organic fertilizers on soil OM content and soil pH varies with the type of organic fertilizer (do Carmo et al., 2016), diversely influencing micronutrient availability to plants. Organic farms (n=11) in northern Switzerland that used compost had significantly higher soil organic carbon (SOC) than conventional farms (n=7) that did not (Schweizer et al., 2018). An 18-year application of P, N and manure together significantly increased the available Zn, Mn and Fe in the soil, which can be attributed to the high OM brought by the manure. However, no significant change in available Cu was observed (Wei et al., 2006), which may be attributed to the high chelation of Cu by OM as discussed in **Chapter 1.6.2**. In a six-year experiment, the application of compost led to increased concentrations of EDTA- or DTPA- extractable Cu, Zn, Fe and Mn, but an insignificant effect on the total concentrations of these micronutrients in soil, demonstrating that the increased micronutrient extractability was not due to higher total input (Herencia et al., 2008). In general, the application of an organic fertilizer can increase the concentrations of extractable Zn, Mn and Fe in soil (Baldantoni et al., 2016; Maqueda et al., 2015; Shahid et al., 2016), while its effect on Cu extractability varies. The application of organic fertilizers can also change soil redox status, leading to species conversion. For example, the application of manure was observed to increase the concentration of Fe (II) and Mn (II) ions, which are most mobile in reduced conditions, such as drainage effluent (Bolan et al., 2004). Furthermore, applying organic fertilizers can facilitate the mobility of micronutrients by increasing dissolved organic carbon (DOC), which tends to complex with certain micronutrients in the soil solution (Bolan et al., 2004). However, DOC-complexed metals are not always readily available to plants and can be more mobile and susceptible to leaching (Bolan et al., 2004).

Application of organic materials to soil facilitates Se volatilization (Li et al., 2017). Volatilization has been shown to decrease adsorption of Se by soil, which was attributed to the increase in low-molecular-weight organic acids from organic materials, which could lead to higher mobility of Se in soil (Øgaard et al., 2006). Soil application of fertilizers or FYM was found to decrease Se concentrations in wheat grains, which was attributed to the sulphate from the applications (Fan et al., 2008). The effect cannot be attributed to a dilution effect on Se concentrations in wheat grain from applying fertilizers or FYM, as there was no significant correlation (R^2= 0.0009) between the grain yield and the grain Se concentration (Fan et al., 2008). Qingyun et al. (2016) also showed that 20-year application of organic compost to soil led to lower Se accumulation in wheat and maize compared to all other applications including control, inorganic N, P and K plus organic compost, and inorganic N, P and K application. Despite having the highest soil Se concentration, the application of organic compost did not bring about correspondingly higher Se in the exchangeable fraction. Instead, higher oxidizable Se was reported compared to the other treatments (Qingyun et al., 2016), resulting in lower Se availability from the soil. Another field study carried out on a seleniferous soil in India showed that Se accumulation by wheat and by oilseed rape (*Brassica napus*) were reduced significantly by the application of poultry litter, sugar cane (*Saccharum spp*.) press mud and FYM (Sharma et al., 2011). Few studies have assessed the impact of organic fertilizer on the mobility or bioavailability of I.

1.6.7.2 Liming

Liming is carried out on land where low soil pH may impede the growth of crops, including grass, due to the reduced mobility of most metals. However, Heal (2001) found that more Mn was leached from limed soils than un-limed soils but the reason

for this remains unclear. Apart from changing soil pH, liming can also reduce Cu availability in soil by enabling precipitation of Cu-hydroxides and/or Cu-carbonates (Brunetto et al., 2016). For non-metallic elements, liming may reduce available I due to the increased pH (Lidiard, 1995). A long-term grassland experiment showed that the limed soil had less TMAH (tetramethylammoniumhydroxide) extracted I and Se than the un-limed soil (Bowley et al., 2017). The TMAH extractable fraction should include humus-bound Se and reactive inorganic Se associated with Fe, Al and Mn oxides/hydroxides in soil, resulting in the assumption that the accumulation of TMAH-extractable I and Se in the un-limed soil was due to stronger inorganic adsorption at low pH (Bowley et al., 2017). The data showed that there was a significant correlation between TMAH-extractable I from soil and that from the plant ($R = 0.464$). By contrast, a negative correlation between TMAH-extractable Se from soil and that from the plant ($R = -0.457$) was found, implying that liming can decrease the availability of I but can increase that of Se to plants (Bowley et al., 2017).

1.7 Highlights and knowledge gaps

Livestock manure is a major source of micronutrients in UK pastures, and the excretion of micronutrients from animals is associated with the absorption of the given feed and supplements. After the manure is applied to soil, the accumulation of micronutrients in the soil and plant uptake of micronutrients from the soil varied with manure type, and application rate and duration. Current acknowledged factors that have potential impacts on the availability of soil micronutrients to plants include soil pH and Eh, soil OM and oxides, soil microbial activities, element antagonisms and soil fertiliser application.

Although there are numerous studies investigating the micronutrient absorption in animals given different feed and supplements, high micronutrient absorption does not

necessarily equate to low micronutrient excretion because some absorbed micronutrients will still be excreted through endogenous excretion. Therefore, a 'excretion-focus' investigation of factors affecting the excretion of micronutrients in manures, such as feed composition and the forms of supplemental micronutrients is needed. Whilst there are substantial studies on the effects of soil properties on the availability of micronutrients to plants, research associated with the effects on the availability of micronutrients derived from manure applications to pasture forage is lacking. It is also clear that our knowledge of the micronutrient concentrations in pasture forage and factors that affect these concentrations, such as climatic, soil properties and farm management practices, is in its infancy.

1.7 Study objective and hypotheses

The understanding of micronutrient flux in a ruminant-soil-grass system is still in its infancy. The objective of the current study was to investigate factors in pasture systems that might have significant impact on the return of micronutrients (Zu, Cu, Mn and Se) to pasture forages from the animal feed. The micronutrients given to ruminants go through their digestion systems and some are excreted to urine or faeces. Once the animal excreta are applied to soils, the micronutrients can go through different biogeochemical processes, such as microbial decomposition, leaching, and soil fixation. It was hypothesized that supplemental minerals to ruminants composed of different chemical forms (organic or inorganic) of micronutrients (Zn, Cu, Mn and Se) could significantly influence their flux in the system through altering: (1) micronutrient partitioning between urine and faeces; (2) the concentrations or chemical form of micronutrients in urine and faeces; (3) the nutrient balance in urine and faeces, due to the different metabolic pathways that the supplemental minerals of different forms of

micronutrients might go through in ruminants. These hypotheses were tested by a sheep experiment in **Chapter 3**.

Once the excreta (urine and/or faeces) was applied to soils, the flux of micronutrients was hypothesized to be influenced by: (1) different excreta collected from the animals of different treatments due to their differences in micronutrient species and nutrient composition; (2) different types of excreta (urine and/or faeces) that might have different decomposition rates and redistribution in soils due to their different physiochemical properties; (3) soils of different OM contents that might interacts differently with the nutrients in the different types of excreta collected from animals given different the supplemental minerals. These hypotheses were tested in a consecutive pot experiment after the sheep experiment, and were discussed in **Chapter 4**.

Chapter 2

Sample preparation and analytical methods

2.1 Total element analysis by ICP-MS or ICP-OES

2.1.1 Sample preparation of sheep faeces for total element analysis

The samples of sheep faeces were dried at 80°C in an oven for 3 d until the weight of samples remained constant. This method was based on a preliminary test carried out for the selection of a drying method (**Appendix A.2**). The dried samples were finely ground using a coffee grinder (BR-CG3-UK, Brewberry®) with stainless steel grinder cup and blade, and digested using microwave digestion (MARS, CEM Corporation, 3100 Smith Farm Road, Matthews, NC, USA). A sample of 0.25 g was loaded into a Teflon vessel and 3 mL concentrated nitric acid (HNO_3) was added and left for 60 min to pre-digest and prevent caking. Following the pre-digestion, an aliquot of 3 mL ultra-pure water (18 MΩ) and 2 mL hydrogen peroxide (H_2O_2) were added into the tube and shaken gently. Afterward, Teflon lids were put on the tubes, which were put into insulation sleeves and loaded onto the microwave system. The details of the digestion program are shown in **Table 2.1**. After the program finished, each of the digested samples was washed into a 50 mL Greiner tube and made up to 50 mL using the 18 MΩ water. The samples were then ready for total analysis by ICP-MS or ICP-OES. Certified standard samples (**Appendix Table B.1**), provided by Wageningen Evaluating Programs for Analytical Laboratories (WEPAL), were digested and analysed using the same method for quality control (QC) of the analysis. The recovery rate of acid digestion of an element was calculated according to **Equation 2.1**. The recoveries were acceptable, being within 100% ± 10% of the certified sample values (**Table A.5**). The final result of element concentration in faecal samples were calculated according to **Equation 2.2.**

$$R\ \% = \frac{C_a \times V_a}{C_{STD} \times W_{STD}} \times 100\% \qquad \textbf{(2.1)}$$

$$C\ = \frac{(C_a - C_{BK}) \times D}{R\ \%} \times \frac{V_a}{W_s} \qquad \textbf{(2.2)}$$

In **Equation 2.1**, R=recovery rate of the acid digestion, C_a = the concentration of the element in the final liquid analyte (mg L^{-1}), V_a = the total volume made to of the analyte (L), C_{STD} = the concentration of the element in the original solid standard sample (mg kg^{-1}), W_{STD}= the weight of the solid standard sample used for the analysis (kg).

In **Equation 2.2**, C= the concentration of the element in the original faecal sample (mg kg^{-1}), C_{BK}= the concentration of the element in the blank sample (sample following the same sample preparation protocol of the faecal sample with no addition of faecal sample into the Teflon vessel) (mg L^{-1}), D=dilution times of the final analyte. W_s = the amount of faecal sample in weight of dry matter that was used for analysis (kg).

Table 2.1 Method program of the microwave system used for digesting faecal samples

Power		Ramp	Temperature	Hold
Max (w)	%	(minutes)	(°C)	(minutes)
1600	100	12	115	1
1600	100	8	175	10

2.1.2 Sample preparation of silage, grass and feed pellets for total element analysis

The samples of silage and grass were freeze-dried and finely ground with a CT293-Cyclotec grinder. The feed pellets were dried in an oven at 80°C for 3 d and finely ground using a coffee grinder (BR-CG3-UK, Brewberry®) with stainless steel grinder cup and blade, and digested using the method of **Chapter 2.1.1** before the analysis by ICP-MS or ICP-OES.

2.1.3 Sample preparation of soil for total element analysis

An aliquot of 0.25 g of each air dried and finely ground soil sample was weighed into a Pyrex® test tube. A volume of 5 mL aqua regia (4 mL HCl and 1 mL HNO_3) was then added into each tube. The tubes were placed in a Carbolite® heating block to digest the soil samples. The digestion program is described in **Table 2.2**. After the digestion was

finished and the heating block was cooled, a volume of 5 mL 25% HNO_3 was added to each tube, and the heating block was reheated to 80°C and the temperature maintained for one hour. Afterwards, the total volume was made up to 25 mL with ultra-pure water (18 MΩ). Each sample was then filtered through a Whatman No. 40 filter paper and sent to ACU for analysis.

Table 2.2 Method program of Aqua regia digestion of soil in Carbolite heating block

Ramp (minutes)	Temperature (°C)	Hold (hours)
1	35	3
1	60	3
2	105	1
2	125	2
-	50	5

2.1.4 Sample preparation of liquid samples for total element analysis

Samples of urine were filtered through 0.45 µm syringe filter. An aliquot of 1 mL urine sample was diluted 20 times in 0.5% HNO_3 and 1% methanol and analysed by ICP-OES for P, S and Zn and by ICP-MS (Mn, Fe, Cu, Se, Mo, cadmium (Cd)). Samples on the ICP-MS were analysed twice and the results were averaged to deal with the instrument instability due to matrix effects. Samples of drinking water were taken were filtered through Whatman No. 42 filter paper and acidified in 5% (v/v) HNO_3 before ICP-MS analysis. Samples of leachate were defrosted and filtered using 0.45 µm syringe filter and acidified in 5% (v/v) HNO_3 before ICP-MS analysis.

2.1.5 Sample analysis using ICP-MS or ICP-OES

All the prepared analytes for total element analysis were sent to Analytical Chemistry Unit (ACU) of Rothamsted Research, and analysed using ICP-MS (Perkin Elmer® NexION 300X) or ICP-OES (Perkin Elmer® Optima 7300DV and Agilent® 5900 SVDV). The ICP-MS was equipped with an introduction system (a CETAC ASX-520

auto-sampler, an ESI SC-FAST valve, a Meinhardt concentric glass nebulizer (type C0.5) and a glass cyclonic spray chamber), a torch (for plasma formation), lens and a quadropole mass analyser. The ICP-MS settings were: sample loop size = 1 ml; nebulizer gas flow = 0.91 L min^{-1}; auxiliary gas flow = 1.2 L min^{-1}; plasma flow = 18 L min^{-1}; radio frequency (RF) power = 1600 Watts, kinetic energy discrimination (KED) mode at 3 mL min^{-1} He. The ICP-OES consisted of a sample introduction system (CETAC ASX-520 autosampler, peristaltic pump, Meinhardt concentric glass nebulizer (Type C1) and cyclonic spray chamber), a torch for plasma formation, transfer optics and an echelle polychromator with a solid state segmented-array charged-coupled detector (SCD). The ICP-OES settings were: sample uptake = 1 mL min^{-1}; nebulizer gas flow = 0.7 L min^{-1}; auxiliary gas flow = 0.3 L min^{-1}; plasma flow = 17 L min^{-1}; RF power = 1400 Watts.

The ICP-OES was used for concentrations above ca. 50 µg L^{-1} in solution, and the ICP-MS below ca. 50 µg L^{-1}. Cd, Mo and Se were always analysed by the ICP-MS. The isotope mass and wavelength used and the estimated detection limit of each element in the ICP-MS and ICP-OES are shown in **Table 2.3**. If an extraction or a digestion was performed before the analysis, blanks and (in-house) standards together with the samples were prepared following the same protocol to sample preparation to check whether results were within an acceptable range (± 2 standard deviations). In-house standards have been verified using certified reference materials. During the analysis using ICP-MS or ICP-OES, every 10th sample was repeated to check the repeatability of the extraction/digestion (acceptable range is 5-10% depending on the element). Instrument calibration was verified for each element using various certified reference solutions, and instrument stability was determined by re-analysing each 10th sample at the end of a run.

Table 2.3 The isotope mass and wavelength used and detection limit for each element in the ICP-MS and ICP-OES

Element	ICP-MS		ICP-OES		
	Isotope mass (amu)	Detection limit (μg L^{-1})	Wavelength (nm)	Detection limit plant digests (mg L^{-1})	Detection limit soil digests (mg L^{-1})
Cd	111	0.01	-		
Co	59	0.05	228.616	0.002	0.002
Cu	63	0.03	327.393	0.005	0.006
Fe	57	0.10	238.204	0.031	0.695
Mn	55	0.02	257.610	0.001	0.020
Mo	95	0.02	-		
P	-		213.617	0.256	0.132
S	-		181.975	0.098	0.670
Se	78	0.04	-		
Zn	-		206.200	0.007	0.029
Na	-		589.592	0.170	0.149
Ca	-		315.887	0.027	0.283

2.2 Sequential extraction of sheep faeces

Sequential extraction procedures (SEP), using different chemical solutions sequentially to extract elements from soil samples, are commonly used in soil science to study the chemical fractionation of an element in the soil. According to the purpose of a SEP, extraction steps using different chemical solutions are adopted. A modified three-step sequential extraction procedure proposed by the Bureau Community of Reference (BCR) (Rauret et al., 1999) was used to extract heavy metals, e.g., Cu and Zn from sediments and soils. Since there was no established SEP for extracting faeces, the revised-BCR SEP was adopted in this research for extracting metallic elements (**Table 2.4**). Note that the final step for extracting the residual elements is the same as the digestion for total element analysis of faeces (**Chapter 2.1.1**). For Se, a non-metallic element, a SEP proposed by Shetaya et al. (2012) for extracting iodine (I) from soil was revised and adopted here (**Table 2.5**). The concentration of KH_2PO_4 used in step 2 was changed from 0.15 M to 0.016 M in order to align with the extraction of 'exchangeable Se' according to (Stroud et al., 2012). And instead of using ~4.5 g of moist sample, 1

g of dried faeces, approximately equal to 4.0~4.5 g moist faeces, was used for extraction. The sample preparation is described in **Chapter 2.1.1**. The recovery rates of the adopted extractions of faeces can be found in **Appendix A.3**.

Table 2.4 The extraction reagents in the revised BCR SEP

Steps	Reagents	Nominal target phase(s)
1	0.11 M CH_3COOH	Exchangeable, water- and acid-soluble
2	0.5 M NH_2OH-HCl at pH=1.5	Reducible
3	H_2O_2 (85°C) then 1 M CH_3COONH_4	Oxidizable
4	(revised) HNO_3/H_2O_2 acid digestion	Residual

Table 2.5 The extraction reagents for Se SEP

Steps	Reagents	Nominal target phase(s)
1	0.01 M KNO_3	Water- and acid- soluble
2	(revised) 0.016 M KH_2PO_4	Exchangeable
3	10% TMAH	OM-bound a/o specific sorption on Fe/Al hydroxides
4	HNO_3/H_2O_2 acid digestion	Residual

2.2.1 Protocol for the sequential extraction of metallic elements

Step1: An aliquot of 20 mL of 0.11 M CH_3COOH solution was added to 0.5 g of dried faeces in a 50 mL centrifuge tube. The mixture was shaken for 16 h at 20 ± 0.5°C, then centrifuged at 3000 *g* at 20°C for 20 min. The supernatant was decanted and passed through a Whatman No. 42 filter paper (pore size: 2.5 μm) in a polyethylene funnel into a 30 mL polyethylene container for storage. To the residue 10 mL of ultra-pure water (18 MΩ) was added and shaken for 15 min to wash the carry-over away. The sample was then centrifuged again at 3000 *g* at 20°C for 20 min. The supernatant was carefully decanted and discarded.

Step 2: An aliquot of 20 mL of 0.5 M NH_2OH-HCl was added to the residue from step 1. The residue in the tube was resuspended by manually shaking and extracted by

mechanical shaking for 16 h at 20 ± 0.5°C. The subsequent centrifugation and decantation, supernatant storage and residual washing procedures were as in step 1.

Step 3: An aliquot of 5 mL of H_2O_2 was added to the residue. The tube was covered loosely and sat for 1 h at room temperature with occasional manual shaking. The volume was then reduced to less than 3 mL by heating the uncovered tube at 85 ± 2°C in a heating block for 1 hour. A further aliquot of 5 mL of H_2O_2 was then added and the vessel was covered and heated at 85 ± 2°C for 1 h. The volume was then reduced again to about 1 mL by placing the uncovered tube in the heating block at 85 ±2°C for another 1 hour. Next, an aliquot of 25 mL of 1M CH_3COONH_4 was added and the tube shaken for 16 hours at 20 ± 0.5°C. Afterwards, the centrifugation, decantation, extract storage and residual washing procedures were as the same as in step 1.

Step 4: The residue from step 3 was washed into a porcelain evaporating dish, and oven-dried at 80°C overnight. The following day, the dried residual was weighed and transferred into a microwave vessel and digested using the same method adopted in **Chapter 2.1.1.** All the stored extracts from step 1, step 2 and step 3 were acidified by adding an aliquot of 0.5 mL concentrated HNO_3 into a 9.5 mL extract for sample preservation before ICP-OES or ICP-MS analysis.

2.2.2 Protocol of the sequential extraction of Se

A 1 g of faeces sample was shaken with 20 mL of 0.01M KNO_3, followed by extraction with 20 mL of 0.016 M KH_2PO_4 and then 20 mL of 10% TMAH in a 40 mL centrifuge tube. At each stage of extraction, the samples were shaken for 16 h on a mechanical shaker, followed by centrifugation at 3000 *g* at 20°C for 20 min. Supernatant decantation and filtration and the residual washing procedure are the same as that used in the revised-BCR extraction described previously except that the residual was washed

in a volume of 20 mL, instead of 10 mL, of ultra-pure water due to the starting weight of the faecal samples. The final residual went through the same procedure as the final step of the revised-BCR extraction for total analysis. Samples from step 1, step 2 and step 3 were stored at -20°C freezer before analysis using HPLC-HG-AFS (**Chapter 2.3**).

2.3 Total Se and Se speciation analysis by HPLC-HG-AFS

2.3.1 HPLC-HG-AFS

Total Se concentration and Se speciation was analysed using an HPLC-HG-AFS system, which involves high-performance liquid chromatography (HPLC) for Se species separation, and a hydride generator (HG) coupled with atomic fluorescence spectrometry (AFS) for Se detection (**Figure 2.1**). The HPLC includes a Thermo Scientific Dionex 3000 series® pump (pump rate = 1mL min^{-1}), a 6-port injector (PSA®), a heater block (PSA® Model S570U100), a C18 column (Gemini®, 250 x 4.60 mm, particle size = 5 μm). The detection system (Millennium Excalibur-PS Analytical®, UK) includes cassettes that control the flow rates of reductant (4.5-5.5 mL min^{-1}), pre-reductant (at 30% the flow rate of the reductant), blank (9 ± 1 mL min^{-1}) and sample (9 ± 1 mL min^{-1}) and includes a Se vapour generator and an AFS detector with a Se hollow cathode lamp (Photron®, PTY Ltd.). The argon gas flows at rate = 250 ± 5 mL min^{-1}. The limit of detection (LOD) of this instrument was 2.0 μg L^{-1}, pre-determined according to the method described in **Appendix A.1**.

2.3.2 Sample preparation of sheep faeces for Se speciation analysis

Since only liquid samples are analysable in HPLC-HG-AFS, a P solution was used to extract Se from the sheep faeces. The influence of the pH and concentration of the P solutions, and the effect of the sample drying method were tested in advance (**Chapter**

A.5). For each sample, an aliquot of 5 g of 80°C oven dried faeces was weighed into a 50 mL polypropylene tube, and then 30 mL of PB was added to the tube to make a slurry with a ratio of faeces to extractant of 1:6 (w/v). The slurry was placed on a mechanical shaker and shaken at room temperature for 1 h. After shaking, the slurry was centrifuged at 2500 rpm for 5 min. The supernatant was filtered through a 0.45 μm Nylon syringe filter and analysed immediately. It should be noted that the P extractant used could only extract part of the Se in the sheep faeces, which was about the same quantity as the first two steps of the Se SEP (**Table 2.4**). To analyse the Se speciation change after breaking down the proteins in the extracted sample, 100 mg Pronase® Protease, from *Streptomyces griseus* was added to the mixture of faeces sample and the P extractant before the extraction.

2.3.3 HPLC-HG-AFS analysis protocol and quality control

Before the analysis, the analyte was filtered through 0.45 μm syringe filter, and diluted, if necessary, with phosphate buffer (PB, NaH_2PO_4/Na_2HPO_4, pH= 7.4) or the same background solution of the sample, if no matrix effect of the background solution was

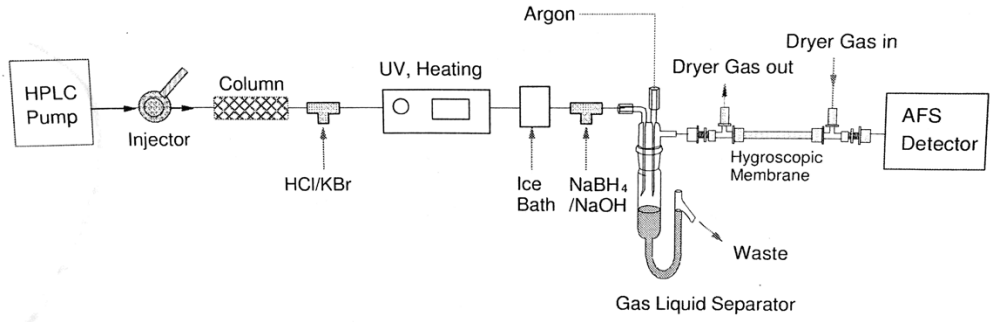


Figure 2.1 Schematic diagram* of the HPLC-HG-AFS system for the analysis of Se speciation. *This diagram was copied from the manual of the instrument.

observed in advance (**Appendix A.1**). The final analyte was transferred into a 15 mL polypropene tube and placed in the autosampler of the instrument. For the analysis of total Se concentration, 2 reagent blanks were run first followed by 4 external standards

of different Se concentrations formulated by sodium selenite (Na_2SeO_3) (≧ 98%, Sigma®) and ultra-pure water (18 MΩ) or the background solution of samples to produce the calibration curves (R^2 ≧ 0.995). The slope of the calibration curve was recorded before analysing samples. New standard solutions were made once the variation of slopes of different batches of analysis was over one order of magnitude. Samples were run following the calibration. A standard was run every 10 samples for QC of analysis, and the variation of each analysis of the same standard sample was ensured to be controlled within 10 % of the previous detected concentration. The matrix effect of different background solutions on the Se detection and the QA were examined before the analysis (**Appendix A.1)**. For the analysis of Se speciation, 2 reagent blanks were run first followed by 2 external standards of different Se concentrations formulated by Na_2SeO_3 ((≧ 98 %, Sigma®), sodium selenate (≧ 98 %, Sigma®), seleno-L-methionine (≧ 98 %, Sigma®), and se-(methyl)selenocysteine hydrochloride (≧95 %, Sigma®) and the background solutions to produce the calibration curve of 0, 10, 20 μg L^{-1} (R^2 ≧ 0.995). The QA and QC methods are the same as total Se analysis described above.

2.4 Silage quality analysis

2.4.1 Sample preparation and analysis of fibre component

The fibre component analysis included the quantification of modified acid detergent fibre (mADF), acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL). Samples for the analysis of fibre composition were freeze-dried and coarsely ground with a grinder (Retech-SM-300). To prepare the samples for the analysis, two different subsamples of 0.45-0.50 g were weighed into two different filter bags. For neutral detergent fibre (NDF) analysis, the filter bag ANKOM® F58 was used, while the bag ANKOM® F57 was used for the sequential analysis of modified

acid detergent fibre (mADF), acid detergent fibre (ADF) and acid detergent lignin (ADL). The bags with the samples were sealed using a heat sealer within 4 mm of its open end. After this, they were placed in a beaker and soaked with enough acetone to cover the bags for 10 min. Afterwards, the acetone was poured out and the bags were placed on a wire rack to air-dry. The bags were then properly placed on bag suspender trays with up to three bags per tray.

To start the analysis of NDF, mADF, ADF and ADL, the bag suspender trays stacked and placed in an ANKOM^{2000}® Fibre Analyser. The analysis of the NDF fraction was conducted using a heat-stable bacterial alpha-amylase and sodium sulphate (Na_2SO_4), as specified in the methodology (ANKOM method 1). For the determination of mADF and ADF fractions, an acid detergent solution (ADS) was used for the extraction of the fractions. The solution was made following the methodology for ADF analysis (ANKOM method 2). For the mADF analysis (first step in the sequence), the ADS was diluted with 1 N sulphuric acid (H_2SO_4) (1:1 v/v ratio). After the ADF analysis, bags were submerged in 72% H_2SO_4 for 3 h, as described in the method of the beakers recommended by ANKOM (ANKOM method 3).

After each procedure described above, the samples were submerged again in acetone for 3-5 min and placed on a wire screen to air-dry before being oven-dried at 102 ± 2°C for 2-4 h. After this time, samples were placed into a desiccant pouch, prior weighing, to cool down and to remain protected from moisture gain. The determination of each fraction was conducted by gravimetric analysis (weight before and after extraction), as described in the different methodologies for the different fibre fractions, and the values were expressed as % dry OM.

2.4.2 Sample preparation and analysis of silage pH, VFAs and ammoniacal nitrogen

For the analysis of silage pH, VFAs and ammoniacal nitrogen, samples were kept at -20°C before analysis. For the analysis of silage pH, an aliquot of 10 g of the frozen silage and 90 mL of ultra-pure water (18 MΩ) were mixed in stomacher bag and processed through an ultra- stomacher (400 Circulator-Seward®) at 230 rpm for 2 min (according to the instrument setting). Afterwards, the pH value was measured from the supernatant of the stomached sample using a pH/ORP meter (Seven2Go, Mettler Toledo®) coupled with a pH microelectrode (InLad Micro, Mettler Toledo®).

For the analysis of VFAs and ammoniacal nitrogen, an aliquot of 20 g of the frozen silage and 100 mL of ultra-pure water (18 MΩ) were mixed in a stomacher bag. After gently agitating the bag to ensure the water mixed well with the silage, the bag was then sealed and stored at 4°C overnight. On the next day, the extract was filtered through Whatman No.1 filter paper in a cold room (4°C). The filtered extract was stored at -20°C before the analysis. Analysis of VFAs was carried out by HPLC (1260 infinity-Agilent®) with an Agilent Hi-PLEX H^+ column at 40°C and the eluent was 0.005 M H_2SO_4 at a flow rate = 0.6 mL min^{-1}. The VFAs were detected using a diode array detector (DAD) at wavelength = 210 nm. The peaks were identified by retention times and quantified against a range of the individual standards. The ammoniacal nitrogen was detected using method described in **Chapter 2.5**.

2.5 Total nitrogen and carbon analysis

2.5.1 Sample preparation of urine for liquid TN analysis

Samples were taken from a -20°C freezer and defrosted in a 4°C fridge the day before the analysis. On the day of analysis, the defrosted urine samples were filtered through

a 0.45 μm Nylon syringe filter and diluted 50 times with distilled water to become the final analytes.

2.5.2 Sample preparation of solid samples for TN analysis

The drying and grinding method of faeces samples for TN analysis were as the method described in **Chapter 2.1.1**. Samples were stored at a -20°C freezer before analysis. Samples of grass and silage were freeze-dried and finely ground with a CT293-Cyclotec® grinder and stored at a -20°C freezer before analysis. Samples of soil were air dried and finely ground using a grinder (RM200-Retsch®) and stored in a 4°C fridge before analysis.

2.5.3 Instruments used for the liquid and the solid TN analysis

The concentrations of total nitrogen (TN) in liquid samples and solid samples were determined using a photometric analyzer (Aquakem 250, Thermo Scientific®) and an elemental analyser (NA-1500, Carlo-Erba®), respectively, at the analytical unit of North Wyke, Rothamsted Research.

2.6 Soil extractable nitrogen analysis

An aliquot of 40 g of air dried and finely ground soil was extracted with 2 M potassium chloride (KCl) solution on a shaker for one hour. The final analyte was the filtrate of the extract passing through a Whatman no.1 filter paper. The analyte was frozen immediately after extraction and defrosted prior to analysis. Samples were sent to the North Wyke analytical unit and analysed using a photometric analyzer (Aquakem 250, Thermo Scientific®). An in-house quality control of analysis was applied and all of the values were within the control limits. The results were calculated according to **Equation 2.3**.

$$C = \frac{(C_{ex} - C_{KCl}) \times V_{ex}}{\frac{W_{soil}}{M\%}} \quad \textbf{(2.3)}$$

In **Equation 2.3**, C=the concentration of N in the soil sample (mg-N kg-DM^{-1} soil), C_{ex}=the concentration of N in the liquid sample of extract (mg-N L^{-1}), C_{KCl}=the concentration of N in the blank sample (background KCl solution used for the extraction) (mg-N L^{-1}), V_{ex}= the volume of the KCl solution used to extract the soil sample (L), W_{soil}= the weight of the soil sample used in the extraction (kg), M%=soil moisture content.

2.7 Soil pH

An aliquot of 25 mL ultra-pure water (18 MΩ) was added to 10 g of each air-dried soil in a plastic vial. The vial was shaken for 10 s and left to stand with the lid on for 30 min. The vial was shaken again and left to stand for another 30 min. The vial was shaken again, and the pH measurement was taken immediately after shaking. The pH was measured using a pH/ORP meter (Seven2Go, Mettler Toledo®) coupled with a pH microelectrode (InLad Micro, Mettler Toledo®). During the measurement, the electrode was inserted to the same depth, ca. 1 cm from the surface, in each sample.

2.8 Soil solution pH analysis

The sample collection method is described in Chapter **4.2.4.2**. To measure the pH of the soil solution collected during the pot experiment, a pH electrode (InLad Micro, Mettler Toledo®) connected to the pH/ORP meter (Seven2Go, Mettler Toledo®) was calibrated using standard solutions of pH 4, pH 7, and pH 11, and a redox buffer (220 mV). After the calibration, the electrodes were inserted directly into the vacutainer tube to take the measurement. The final pH value is the value given by the reader.

2.9 Soil extractable Se and S

The soil extractable Se and S were determined using two different P solutions (0.016 M KH_2PO_4 and 0.016 MPB (NaH_2PO4/Na_2HPO_4)). KH_2PO_4 is known as a good reagent for extracting plant-available S (Zhao & McGrath, 1994). Due to the chemical similarity between SO_4^{2-} and SeO_4^{2-}, the reagent has also been used to extract Se from soil previously (Stroud et al., 2010). The use of PB is a new attempt in this study to investigate the effect of a different pH environment on Se sorption in the soil.

A 5 g soil sample (2 mm; using a stainless-steel sieve) was weighed into a 50 mL sample tube followed by an addition of 25 mL extractants (0.016 M KH_2PO_4 (pH=4.8) or PB (pH=7.5), and extracted for 1 h at 25°C. After extraction, samples were filtered through Whatman No.42 filter papers. The supernatants were acidified in 5% HNO_3 (v/v) and sent to ACU for total (organic + inorganic) Se and S analysis by ICP-OES or ICP-MS (**Chapter 2.1**).

2.10 Soil particle size distribution

The particle size distribution of the two soils (Great Harpenden and Weighbridge Piece) used in **Chapter 4** were determined by NRM® analytical laboratories (United Kingdom) using their standard method of pipette sampling (NRM Advice sheet 30). The sand was removed by sieving and then the resulting sample was mixed to form a suspension with water. Afterwards, the fractions of clay and silt were determined by sampling from the suspension using a pipette at different sedimentation times according to Stokes Law. The soil texture is defined by the proportion of sand, silt and clay sized mineral particles, and utilising the UK/ADAS classification system where sand particles are those between 2.00-0.063 mm, silt particles are those between 0.063-0.002 mm and those particles which are less than 0.002 mm are the clay particles.

2.11 Soil amorphous and organically bound aluminium and iron

Amorphous and organically bound Al and Fe in finely ground soil was sent to and determined by ACU. An aliquot of 0.500 g air dried (<0.5mm fine ground) soil was extracted with 50 mL extractant reagent (0.114 M ammonium oxalate and 0.086 M oxalic acid) in a 100 mL brown/foil covered HDPE bottle. Afterwards, the bottle was the placed upright in a reciprocating shaker and shake for 4 h at 25°C at 120 rpm. In a dark room, using red light to see by, the extracts were filtered through Whatman No. 42 filter papers to produce a solution which was clear when viewed through reflected light. The filtrates were diluted 10 times in HNO_3 (1 mL sample to 9 mL 5% HNO_3). The analytes were submitted for ICP-OES analysis. Prior to instrumental analysis, samples were kept cool and stored in the dark (cover with foil), to prevent photo-induced decomposition of oxalate, as this may result in loss of Fe by precipitation. This method was referring to and modified from (Rayment & Lyons, 2011; Schwertmann, 1964; Sparks et al., 2020).

2.12 Soil available P (Olsen)

Soil (sieved to 2 mm) available P was determined by ACU following the standard analytical protocol of Olsen-P proposed by (Olsen et al., 1954).

2.13 Soil exchangeable cations

Soil (sieved to 2 mm) exchangeable cations was determined by ACU following the standard analytical protocol of extraction using 1M NH_4NO_3 as the extracting reagent (Metson, 1957).

2.14 Statistical analysis

Different ANOVA models were performed to analyse the significance of factors to the interested response variables. QQ-plots were performed, and outliers were removed to

ensure that the residuals from the ANOVA models followed a normal distribution. In **Chapter 3**, the impact of the forma and dose level of the supplemental minerals on micronutrient excretion and partitioning in urine and faeces was tested using an ANOVA model: (y~ Block + Mineral form (F) + Supplementary dose (D) + Interaction (F*D)). To compare the difference of mineral intake and excretion across different sampling days, an ANOVA model: (y~ Block + Day) was applied, based on the assumption that the effect of time was independent from the effect of treatment. In **Chapter 4**, an ANOVA model: (y~ Block + Excreta type (ET) + Form of supplemental mineral (Form) + Soil + Interactions (ET x Form + ET x Soil + Form x Soil + ET x Form x Soil) was performed to test the significance of excreta type, supplemental mineral form, soil type and their interactions on grass DM, nutrient uptake by forage, nutrient leaching, and pH of soil solution at different sampling time. A principal component analysis (PCA) was performed to analyse the difference of grass harvested at different cutting cycles based on the nutrient components of the grass. Under the circumstances that different cutting cycles can be a significant factor to the nutrient uptake by grass, a modified ANOVA model: (y~ Time + ET + Form + Soil + ET x Form + ET x Soil + Form x Soil + ET x Form x Soil) was performed to include the evaluation of temporal effect. This modified statistical model was used based on the assumption that temporal effect has no significant interaction with the treatment factors. Post-hoc tests were performed by means of Fisher's least significant difference (LSD) at the significant level of $P<0.05$. All the statistical analyses were conducted in R (R Core Team, 2018).

Chapter 3

The effects of chemical form and dose of supplemental trace minerals on the partitioning of micronutrient excretion to urine and faeces in sheep

3.1 Introduction

The dose and form of a supplemental mineral affect its absorption at a micronutrient in livestock, which might in turn influence the excretion of micronutrients and their recycling in the environment. For example, some organic forms of Se (selenized yeast or SeMet) lead to higher Se absorption in ruminants than inorganic forms, e.g. SeO_3^{2-} (Pechova et al., 2012; Spears, 2003). Similarly, compared to $ZnSO_4$, Zn-Met was found to increase Zn concentrations in the serum of lambs (Garg et al., 2008). Despite some studies reporting the retention and excretion of minerals under different supplementation strategies (Ehlig et al., 1967; Lopez et al., 1969), to what extent the nutrient composition and chemical form of micronutrients in the excreta are affected by different forms of mineral supplements are not clear and need to be studied in detail. The possible pathways through which the mineral supplement form can make impact on the ultimate cycling of micronutrients via animal excreta include (1) altering micronutrient partitioning between urine and faeces (2) altering the concentrations or chemical form of micronutrients in urine and faeces (3) altering the nutrient balance in urine and faeces, due to the potentially different metabolic pathways of the mineral supplements of different forms.

To investigate these three potential impact pathways, 24 sheep were given organic or inorganic forms of supplemental minerals (Se, Zn, Cu, Mn) for two weeks following a 2-week acclimatization period. The range of supplementary doses adopted in previous studies of mineral absorption in animal is often wider, mostly with a higher dose, than the range a farmer would adopt in practice. Therefore, two supplementary doses typically used by European industry based on the recommendation of (NRC, 2007) were adopted in this study. The total amount and the concentrations of nutrients

excreted in urine and faeces were measured and analysed. A further analysis of chemical forms of Se, Zn, Cu and Mn in faeces and Se species in faeces were approached via sequential extractions of faeces and via P extractions coupled with instrumental analysis of HPLC-HG-AFS, respectively.

3.2 Experiment setup

3.2.1 Experimental design

For two weeks, concentrates containing the inorganic minerals: $NaSeO_3$, ZnO, $CuSO_4 \cdot 5H_2O$ and MnO or concentrates containing the organic minerals: selenised yeast (Selplex®, Alltech Inc., USA) and Cu, Zn, Mn chelates of protein hydrolysate (Bioplex®, Alltech Inc., USA) were given to male Charolais x Suffolk-Mule sheep offered a ration of grass silage and concentrate on a 60:40 DM basis using a pre-determined *ad libitum* silage DM intake. The grass silage was big bale silage sourced from the North Wyke farm cut from permanent pasture which was predominatly perennial ryegrass (*Lolium perenne*). The concentrate contained: barley (*Hordeum vulgare*), wheatfeed, soya (bean; *Glycine max*) meal, wheat (*Triticum aestivum*), maize (*Zea mays*) distillers dark grains, dried beet pulp; molasses, soya hulls, linseed (*Linum usitatissimum*) expeller, calcium carbonate, and sodium chloride. Premixed minerals were added to the concentrate prior to pelleting, as either organic or inorganic forms at either a typical European industrial dose of inclusion (IND) or a dose that was slightly lower than IND (80% IND for Cu, Zn and Mn; 0.2 mg kg-DM^{-1} for Se). The dose of 80% IND was based the recommendation of (NRC, 2007). In practical administration of supplementing the minerals in industry, the given dose is typically higher than the dose of regulation, hence the dose of 100% IND used in the current study. The experiment design resulted in four treatments: organic minerals at a higher (OH) or lower (OL) level and inorganic minerals at a higher (IH) or lower (IL) level. The

chemical forms and the concentrations of the added minerals in the concentrates are shown in **Table 3.1**. The nutrient and energy contents of the provided concentrates are shown in **Table 3.2**.

Table 3.1 Concentrations of the added supplemental minerals in the concentrates for the different treatments

Organic minerals			Inorganic minerals		
Mineral forms	Supplementary doses (mg-element kg^{-1})		Mineral forms	Supplementary doses (mg-element kg^{-1})	
	High (OH)	Low (OL)*		High (IH)	Low (IL)*
Selenised yeast (Selplex®)	0.6	0.2	Sodium selenite	0.6	0.2
Copper (II) chelate of protein hydrolysate (Bioplex®)	17	13	Copper (II) sulphate pentahydrate	17	13
Zinc chelate of protein hydrolysate (Bioplex®)	104	84	Zinc oxide	104	84
Manganese chelate of protein hydrolysate (Bioplex®)	60	48	Manganese (II) oxide	60	48

*The doses of the OL and IL treatments were based on the regulation of (NRC, 2007). For Cu, Zn and Mn, the lower doses were 80% of the high doses. The dose for Se was not 0.8IND treatments because the maximum permitted allowance of the inclusion of organic Se was 0.2 mg-Se kg-DM^{-1} diet at 12% moisture in 'Commission Implementing Regulation (EU) 2019/804'.

Sheep (n=24) were pre-weighed (mean weight = 57 ± 2.9 kg; Body Condition Score (BCS) = 3.3 ± 0.20) and allocated into six blocks according to body weight. Each of the four sheep in each block were individually penned and offered silage with one of the four different treatments of concentrate in individual feeding buckets, resulting in one sheep per block for each of the four concentrate feeds. The ranges of the sheep liveweights across the allocation blocks and the treatment groups are shown in **Tables 3.3** and **3.4**, respectively. The sheep experiment was carried out at the Robert Orr Small Ruminant Facility at North Wyke, Rothamsted Research, using a Biocontrol System (**Figure 3.1**) for automatic feeding and data recording. Complete urine and faecal

collection were performed separately and individually before the morning feed. The collected urine and faeces were quantified and stored for further chemical analysis.

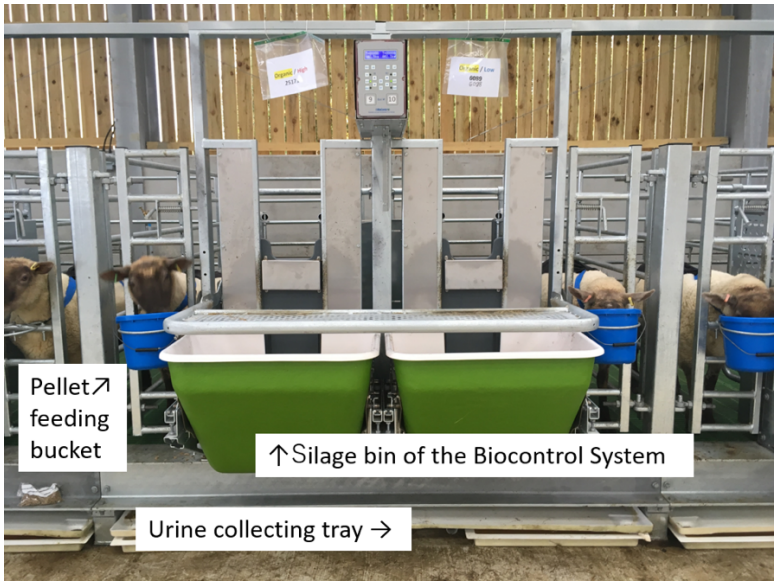


Figure 3.1 The Biocontrol System used for automatic feeding and data recording. Each sheep had an individual room and feeding bins. The green bin carried the feed silage and recorded the consumption. The blue bin carried the pre-weighed concentrate of treatment. The tray set underneath the slat was used to separate and collect the urine.

3.2.2 Sheep acclimatization, feeding and monitoring of animal growth

The sheep were acclimatized in the facility for two weeks before the start of supplementation (day 0). A control concentrate (**Table 3.2**; no additional premixed minerals) was offered as a wash-out and control basal period to all sheep for one week before the start of the supplementation period (day 0). During this pre-experiment period the quantity of control concentrate was slowly increased, with an increment of ca. 100 g d^{-1}, to the required level of inclusion (60:40 silage: concentrate (DM basis)) with the day before the first sampling (day -1) used to present the baseline level of excretion from the basal diet. The supplementary feeding started from day 0 when the control concentrate was replaced with the concentrate containing the targeted supplemented minerals (**Table 3.2**). The concentrate was offered in the morning in a separate feeding bucket from the silage bin, which was on a weigh-cell (**Figure 3.1**).

The silage was fed twice a day at 09:00 and 16:00. Water access was *ad libitum* via an automatic drinker with no assessment of intake performed. All animals were weighed weekly prior to morning feeding and BCS assessed at the same time.

3.2.3 Sample collection and storage

An aliquot of 1-2 mL of drinking water was taken from the individual water troughs and bulked as one sample and three samples in total were taken of each at each sampling time. The sample was carried out on a weekly basis. A sample of 'fresh' water from the tap was also taken and treated the same as the samples taken from the trough. Approximately 100 g fresh weight of the silage was sampled from each feed bin before the daily morning feed and bulked as one sample on a daily basis. Urine and faecal samples were collected separately on an individual animal basis before the morning feed, using a bespoke faecal collection diaper and a urine collection tray set underneath the pen (**Figure 3.2**).

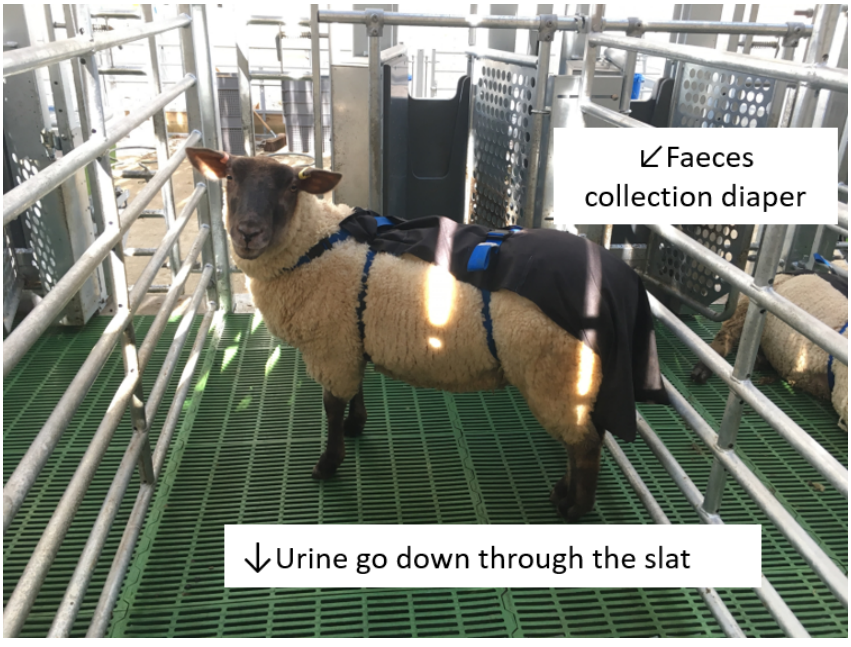


Figure 3.2 Facilities used for total collection of faecal and urinary samples. The faeces sample was collected in the collection diaper and the urine sample went down through the slat and was collected in the collection tray (Figure 3.1).

The total volume of the urine was measured and poured into a 4 L plastic storage bucket (bulked urine sample per animal). The faeces collected was put into a plastic bag and weighed (bulked faecal sample per animal). A 50 mL sample of urine and ca. 200 g of faeces was taken from the bulked urine and faecal samples, respectively, for further chemical analysis. The rest of the bulked samples were stored at -18°C for future analyses and studies (**Chapter 4**). The methods of sample preparation for analysis are presented in **Chapter 2**.

3.2.4 Ethics approval

Animals were assessed daily for health and well-being, as determined by alertness, feed and water intake. All animal procedures and the care for the animals were carried out under strict regulations described in the Animals (Scientific Procedures) Act 1986 issued by the Home Office of Her Majesty's Britannic Government under the Project License number P592D2677.

3.2.5 Calculations

The total outputs of the targeted elements were calculated by multiplying the concentration of an element by the total excretion amount of urine or faeces. Since the total intake amount varied by individual animal, although the total intake of silage and concentrate was not significantly different across treatments and experimental time (**Tables B.4 and B.5**), the total output of an element was corrected by total element intake from silage and concentrate, according to **Equations 3.1, 3.2 and 3.3**.

$$Urine\ \%\ in\ total\ element\ intake = \frac{Ex_U}{In_S+In_C+In_M} \times 100\% \quad \textbf{(3.1)}$$

$$Faeces\ \%\ in\ total\ element\ intake = \frac{Ex_F}{In_S+In_C+In_M} \times 100\% \quad \textbf{(3.2)}$$

$$Retention\ \%\ in\ total\ element\ intake = \frac{(Ex_U+Ex_F)-(In_S+In_C+In_S)}{In_S+In_C+In_M} \times 100\% \quad \textbf{(3.3)}$$

Where in the **Equations 3.1, 3.2 and 3.3**, Ex = excretion on day (mg) 14, In = intake on day 13 (mg), U = urine, F = faeces, S = silage, C = background concentrate, M = supplemented mineral.

The ratios of Se/P and Se/S in urine and faeces were calculated following the **Equations 3.4 to 3.9**:

$$R_U(Se{:}S) = \frac{U_{Se}}{U_S \times 1000} \quad \textbf{(3.4)}$$

$$R_F\ (Se{:}S) = \frac{F_{Se}}{F_S \times 1000} \quad \textbf{(3.5)}$$

$$R_{F:U}\ (Se{:}S) = \frac{R_F(Se{:}S)}{R_U(Se{:}S)} \quad \textbf{(3.6)}$$

$$R_U(Se{:}P) = \frac{U_{Se}}{U_P \times 1000} \quad \textbf{(3.7)}$$

$$R_F\ (Se{:}P) = \frac{F_{Se}}{F_P \times 1000} \quad \textbf{(3.8)}$$

$$R_{F:U}\ (Se{:}P) = \frac{R_F(Se{:}P)}{R_U(Se{:}P)} \quad \textbf{(3.9)}$$

In **Equations 3.4 to 3.9**, R_U= ratio of different elements in the urine sample, R_F= ratio of different elements in the faeces sample, U_{Se}=Se concentration in the urine sample (μg L^{-1}), U_S=S concentration in the urine sample (mg L^{-1}), U_P=P concentration in the urine sample (mg L^{-1}), F_{Se}= Se concentration in the faecal sample (μg kg^{-1}), F_S= S concentration in the faecal sample (mg kg^{-1}), F_P= P concentration in the faecal sample (mg kg^{-1}).

3.2.6 Statistical analysis

An ANOVA model (y~ block + mineral form (F_{form}) + supplementary dose (F_{dose}) + F_{form}*F_{dose}) was performed to test the influence of the two main factors (F_{form} and F_{dose}) and their interaction on the response variables, including total urine and faeces, micronutrient concentrations in urine and faeces and micronutrient partitioning in

Table 3.2 Nutrient and energy contents in the concentrates.

Component*	Control	IL	IH	OL	OH
ME (MJ/kg)	10.9	10.9	10.9	10.9	10.9
Crude ash (%)	7.59	7.58	7.58	7.63	7.60
Crude fibre (%)	7.77	7.76	7.76	7.76	7.76
Crude protein (%)	17.0	17.0	17.0	17.0	17.0
Dry matter (%)	88.0	88.0	88.0	88.0	88.0
Crude oils and fats (%)	3.65	3.65	3.65	3.65	3.65
Starch and sugar (%)	30.3	30.3	30.3	30.2	30.2
Starch (%)	22.9	22.9	22.9	22.9	22.9
Sugar (%)	7.35	7.35	7.35	7.35	7.35
NDF (%)	20.8	20.7	20.7	20.7	20.7
Sodium (%)	0.45	0.47	0.47	0.47	0.45
Calcium (%)	1.11	1.12	1.12	1.11	1.12
Magnesium (%)	0.23	0.23	0.23	0.23	0.23
Salt (%)	1.30	1.35	1.35	1.35	1.30
DUP (g kg^{-1})	42.4	42.6	42.4	42.4	42.4
ERDP (g kg^{-1})	107	107	107	107	107
Vitamin A (IU kg^{-1})	-	8000	8000	8000	8000
Vitamin B12 (mcg kg^{-1})	-	60.0	60.0	60.0	60.0
Vitamin D3 (IU kg^{-1})	-	2000	2000	2000	2000
Vitamin E (IU kg^{-1})	-	10.0	10.0	10.0	10.0

*The data were provided by the feed company HJ Lea Oakes proving the service of making the concentrate feed. N.A.: No data are available due to no leftover concentrate sample of OL group.

Table 3.3 Summary of sheep liveweights by allocation block

Block	Minimum weight (kg)	Maximum weight (kg)
1	50.0	52.0
2	52.5	54.0
3	55.5	56.5
4	57.0	58.0
5	58.5	60.0
6	60.5	61.5

Table 3.4 Summary of sheep liveweights by treatment

Treatment	Minimum weight (kg)	Maximum weight (kg)
OH	50.5	61.0
OL	50.0	61.5
IH	51.5	60.5
IL	52.0	61.5

excreta. This factorial ANOVA was only applied to compare data collected on the same day. To compare the difference of mineral intake and excretion across different sampling days, an ANOVA model (y~ block + day) was applied, based on the assumption that the effect of time was independent from the effect of treatment. The post-hoc test was performed by means of Fisher's least significant difference (LSD) at the significant level of $P<0.05$. All the statistical analyses were conducted in R (R Core Team, 2018).

3.3 Results

3.3.1 Changes in sheep body weight and body condition score

Body weight and body condition score were measured on a daily basis throughout the experimental period. There was no significant difference from treatments in the sheep body weight and the body condition score at the different times of measurement throughout the experiment (**Table 3.5 and 3.6**). According to the manual of 'Managing

ewes for Better Return' by (AHDB, 2019), the scoring scale is from 1 to 5, with 1 being thin and 5 being very fat, and 2.25 or 3.5 being the half or the quarter scores. The recorded average body condition score in the current study was between 3.2 to 3.4 (**Table 3.6**), which is deemed in in a good health condition for the sheep.

Table 3.5 Sheep body weights during the penned period

Treatment	Allocation	6 days before day 0	Day 1	Day 8	Day 14
		kg ± SE			
IL	56.5 ± 1.40	57.0 ± 1.30	57.1 ± 1.39	58.7 ± 1.46	57.0 ± 1.73
IH	56.4 ± 1.37	57.9 ± 1.39	58.7 ± 1.60	59.1 ± 1.47	57.1 ± 1.60
OL	56.1 ± 1.72	58.1 ± 2.13	56.3 ± 2.08	56.8 ± 2.19	53.8 ± 1.69
OH	56.5 ± 1.61	59.2 ± 1.86	59.3 ± 1.75	59.2 ± 1.79	58.3 ± 1.71
		P level			
F_{form}	0.5567	0.2517	0.9431	0.3234	0.2453
F_{dose}	0.5567	0.3230	0.0600	0.1354	0.0178
F_{form} x F_{dose}	0.3814	0.9332	0.5231	0.2829	0.0216

'*' indicatea statistical significances at p-value< 0.05.

Table 3.6 Sheep body condition scores during the penned period

Treatment	Allocation	6 days before Day 0	Day 1	Day 8	Day 14
		mean score ± SE			
IL	3.3 ± 0.09	3.3 ± 0.08	3.3 ± 0.08	3.3 ± 0.05	3.3 ± 0.05
IH	3.4 ± 0.06	3.4 ± 0.06	3.4 ± 0.06	3.4 ± 0.06	3.4 ± 0.06
OL	3.3 ± 0.09	3.3 ± 0.08	3.3 ± 0.06	3.2 ± 0.10	3.2 ± 0.08
OH	3.3 ± 0.13	3.3 ± 0.11	3.3 ± 0.11	3.4 ± 0.09	3.3 ± 0.11
		P level			
F_{form}	0.4446	0.7658	0.5495	0.2790	0.0911
F_{dose}	0.4446	0.3772	0.2396	0.0808	0.0911
F_{form} x F_{dose}	0.4446	0.7658	1.0000	0.2790	0.2957

3.3.2 Intake sources of Se, Cu, Zn and Mn

The intake sources of nutrients in this experiment include silage, the background and supplementary minerals in the concentrate and drinking water. The total intake of the silage and concentrate are shown in **Tables B.4** and **B.5**, and no statistical difference was found across treatments and time throughout the supplementation period. The total intake of micronutrients from drinking water is not available because the amount of water intake was not quantified. The concentrations of trace elements in drinking water are shown in **Table 3.7.** The concentration of Se in the drinking water was no different

from the tap water and was close to the LOD value, meaning the provision of Se from the drinking water was negligible. The contents of Mn, Cu in the drinking water, although significantly higher than the LOD values, were all in the level of μg L^{-1}, which, compared to their provision from feed and supplements in mg L^{-1}, was negligible. The content of Zn in the drinking water was significantly higher than the tap water and was provided in the mg L^{-1} range. The high input of Zn from the drinking water can be attributed to the galvanised troughs used in the experiment. Further discussion is in **Appendix A.3**.

The basal diet (silage + concentrate-without mineral premix) is a major micronutrient source in the IH and OH diets (**Table 3.8**), especially for Mn (over 70% from the basal diet), Cu and Zn (ca. 50% from the basal diet). For Se, the basal diet at the higher-dose treatments (IH and OH) accounted for less than 30% of total intake, whereas the basal diet accounted for ca. 50% of Se intake in the diet of the lower- dose treatments (IL and OL). This result is aligned with previous findings (**Table 1.1**), indicating that the grazing animal typically in the UK are able to get their required Mn intake from forage alone, whereas the Cu and Zn concentrations in the UK pasture can sometimes be insufficient, and less than 50% of the required Se for sheep is typically available from UK pasture intake. This not only shows that the basal diet is an important source of the cation micronutrient but also suggests that the micronutrient intake from sources other than the supplemental minerals might mask the effects of mineral supplements *per se* in a supplementation trial to a lesser or greater degree, depending on the mineral.

3.3.3 Silage quality

To ensure that the silage quality remained consistent throughout the experimental period, the five silage bales used in the experiment were sampled and sent for analysis. The results show that only the pH, the mADF and ADF of the silage were significantly

Table 3.7 Mineral contents in the drinking water for sheep collected on different experimental days and mineral contents of the tap water from location of the sheep facility

Mean ± SE (n=3)	Day 0	Day 7	Day 14	Tap water (background)	p-values	LOD#
Se (μg L^{-1})	0.06 ± 0.016	0.04 ± 0.009	0.02 ± 0.006	0.02 ± 0.010	0.074	0.020
Mn (μg L^{-1})	2.37 ± 0.216^{a}	1.89 ± 0.034^{b}	2.54 ± 0.010^{a}	0.23 ± 0.015^{c}	<0.001***	0.050
Cu (μg L^{-1})	3.42 ± 0.187^{b}	4.29 ± 0.565^{b}	3.78 ± 0.388^{b}	6.08 ± 0.255^{a}	0.005**	0.050
Zn (mg L^{-1})	1.07 ± 0.047^{c}	1.18 ± 0.036^{b}	1.98 ± 0.025^{a}	0.02 ± 0.002^{d}	<0.001***	0.010
Ca (mg L^{-1})	7.27 ± 0.892^{b}	8.56 ± 0.066^{ab}	8.25 ± 0.034^{b}	9.85 ± 0.104^{a}	0.023*	0.020
Cd (μg L^{-1})	0.12 ± 0.044^{a}	0.03 ± 0.010^{b}	0.02 ± 0.005^{b}	< LOD#	0.018*	0.010
Fe (mg L^{-1})	0.01 ± 0.006^{a}	0.01 ± 0.003^{a}	0.01 ± 0.002^{a}	< LOD#	<0.001***	0.020
Mo (μg L^{-1})	0.04 ± 0.012	0.03 ± 0.004	0.05 ± 0.009	0.05 ± 0.003	0.548	0.010
Na (mg L^{-1})	7.85 ± 0.975	8.69 ± 0.053	8.90 ± 0.030	8.05 ± 0.015	0.417	0.020
P (mg L^{-1})	1.09 ± 0.024^{a}	1.11 ± 0.007^{a}	1.01 ± 0.012^{b}	0.83 ± 0.039^{c}	<0.001***	0.020
S (mg L^{-1})	6.11 ± 0.646	7.07 ± 0.035	7.05 ± 0.026	6.67 ± 0.050	0.207	0.020

The lowercase letters behind numbers represent the results of LSD test across a row post a significant result in general ANOVA test (the symbols: *, **, *** indicate statistical significances at p-value< 0.05, 0.01, 0.001, respectively). #LOD: the limit of detection of the analysis instrument.

Table 3.8 Element contents on day 13 from the silage, background concentrate and supplemental minerals in the concentrate with percentage of total intake in parentheses (mineral intakes from drinking water not included here)

Element	Treatment	Silage	Concentrate		Total intake	Mineral requirement range of growing lambs of different body weight#
			Background	Premix*		
Se (mg animal^{-1} day^{-1})	IL	0.02 ± 0.001 (9%)	0.09 ± 0.005 (38%)	0.13 ± 0.007 (53%)	0.249	0.18-1.05
	IH	0.02 ± 0.001 (4%)	0.010 ± 0.008 (19%)	0.37 ± 0.029 (77%)	0.506	
	OL	0.02 ± 0.002 (10%)	0.08 ± 0.007 (37%)	0.11 ± 0.010 (53%)	0.209	
	OH	0.02 ± 0.001 (5%)	0.10 ± 0.005 (20%)	0.37 ± 0.020 (76%)	0.486	
Cu (mg animal^{-1} day^{-1})	IL	5.10 ± 0.349 (28%)	5.05 ± 0.268 (28%)	8.06 ± 0.427 (44%)	18.21	3-15
	IH	4.72 ± 0.388 (24%)	5.09 ± 0.403 (26%)	10.1 ± 0.80 (51%)	19.95	
	OL	4.97 ± 0.436 (31%)	4.19 ± 0.381 (26%)	6.67 ± 0.607 (42%)	15.82	
	OH	5.36 ± 0.276 (26%)	5.12 ± 0.283 (25%)	10.2 ± 0.57 (49%)	20.69	
Zn (mg animal^{-1} day^{-1})	IL	20.6 ± 1.45 (20%)	32.2 ± 1.70 (31%)	50.5 ± 2.68 (49%)	103.3	13-95
	IH	19.1 ± 1.57 (17%)	32.4 ± 2.57 (28%)	63.5 ± 5.04 (55%)	115.0	
	OL	20.1 ± 1.76 (23%)	26.7 ± 2.43 (30%)	41.8 ± 3.81 (47%)	88.56	
	OH	21.6 ± 1.11 (18%)	32.6 ± 1.80 (28%)	64.0 ± 3.53 (54%)	118.2	
Mn (mg animal^{-1} day^{-1})	IL	100 ± 7.1 (63%)	30.8 ± 1.63 (19%)	29.0 ± 1.54 (18%)	159.9	12-54
	IH	92.6 ± 7.61 (58%)	31.0 ± 2.46 (19%)	36.5 ± 2.90 (23%)	160.1	
	OL	97.5 ± 8.57 (66%)	25.5 ± 2.32 (17%)	24.0 ± 2.19 (16%)	147.0	
	OH	105 ± 5.4 (61%)	30.7 ± 1.73 (18%)	36.8 ± 2.03 (21%)	173.1	

*The premix contained all the supplemental minerals and vitamins to make the treatment concentrates. #Full data is in Appendix B Table B.17; the data refers to (NRC, 2007).

different across bales of silage (**Table 3.9**). The values of the pH, mADF, ADF across different bales are: 4.50 ± 0.20, 30.0 ± 0.20%, 28.0 ± 0.60%, which are within a reasonable range. As the sheep on the different treatment groups were offered the same silage bale at a time, these differences are not expected to influence the effect of treatments.

3.3.4 Concentrations of elements in the excreted faeces and urine

The concentrations of Mn in faeces plateaued after day 7 (**Figure 3.3a**); Se and Zn in faeces reached a plateau after day 3 (**Figures 3.3e and 3.3g**). Although the concentration of Cu in faeces was variable and no statistical plateau was observed, a trend of becoming stable after day 3 was observed (**Figure 3.3c**). The concentrations in urine were more variable and lower than in faeces, and no significant temporal trend was observed (**Figures 3.3b, 3.3d, 3.3f and 3.3h**). The dose of supplementation showed a significant effect on Se concentrations in faeces after day 1 (**Figure 3.3g**). However, there was no effect of either form or dose of the supplemental minerals for other added minerals (Zn, Cu, Mn) on their concentrations in faeces after day 3, when the concentrates had plateaued (**Figures 3.3a, 3.3c and 3.3e**).

No clear temporal trend in the concentrations of un-supplemented elements was observed in faeces except for Cd (**Figure 3.4**). The supplementary dose of minerals significantly affected the concentration of Cd in faeces, with lower Cd excretion in the groups of higher dose and higher Cd excretion in the groups of lower dose (**Figure 3.4i**). In urine, the concentration of S reached a statistical plateau after day 7 (**Figure 3.4b**); P showed a steadily decreasing trend and reached a plateau after day 7 (**Figure 3.4d**); Mo and Cd had similar trends: the concentration significantly increased during the first three days and became steady after day 7 (**Figures 3.4f and 3.4j**); Fe increased

Table 3.9 Analyses of silage bales used during the experiment

Item		Different bales of silage for different feeding dates (mean ± SE; n=3)					p-value
		12 to 11 days before day 0	10 to 6 days before day 0	5 days before day 0	Day 1 to day 7	Day 8 to day 14	
pH		4.63 ± 0.006^{b}	4.40 ± 0.009^{d}	4.56 ± 0.009^{c}	4.69 ± 0.018^{a}	4.54 ± 0.006^{c}	**<0.001*** **
Volatile fatty acids (g kg-DM^{-1})	Lactic acid	48.5 ± 3.16	69.0 ± 0.75	41.8 ± 9.90	47.0 ± 3.78	64.7 ± 1.20	0.227
	Acetic acid	13.2 ± 0.75	19.0 ± 0.55	13.9 ± 2.85	12.3 ± 1.27	19.4 ± 0.71	0.248
	Propionic Acid	N.D.	N.D.	0.23 ± 0.229	0.11 ± 0.079	N.D.	0.568
	Isobutyric acid	N.D.	N.D.	N.D.	N.D.	N.D.	-
	Butyric acid	5.71 ± 0.170	0.49 ± 0.027	0.05 ± 0.038	10.3 ± 0.93	0.57 ± 0.061	0.575
	Valeric acid	N.D.	N.D.	N.D.	N.D.	N.D.	-
Ammonium (mg/L)		214 ± 2.4	211 ± 1.4	208 ± 13.8	184 ± 11.8	196 ± 2.4	0.122
Fibre compositions (% of dry OM)	Neutral detergent fibre	51.5 ± 0.33	51.9 ± 0.36	51.0 ± 0.32	52.8 ± 0.53	51.3 ± 0.62	0.109
	Modified acid detergent fibre (mADF)	29.4 ± 0.18^{c}	30.1 ± 0.24^{a}	29.4 ± 0.08^{bc}	30.0 ± 0.14^{ab}	29.1 ± 0.23^{c}	**0.016***
	Acid detergent fibre (ADF)	27.9 ± 0.03^{ab}	28.3 ± 0.14^{a}	27.4 ± 0.01^{b}	28.3 ± 0.16^{a}	27.5 ± 0.30^{b}	**0.007****
	Acid detergent lignin	3.33 ± 0.443	2.97 ± 0.135	3.03 ± 0.293	3.84 ± 0.514	3.23 ± 0.405	0.544
Cadmium (µg kg-DM^{-1})		3.99 ± 0.033^{bc}	4.84 ± 0.33^{a}	4.45 ± 0.040^{ab}	3.61 ± 0.027^{c}	4.60 ± 0.08^{a}	**0.002****
Copper (mg kg-DM^{-1})		6.37 ± 0.068^{bc}	6.82 ± 0.080^{ab}	6.22 ± 0.365^{c}	7.29 ± 0.051^{a}	6.71 ± 0.043^{bc}	**0.010***
Iron (mg kg-DM^{-1})		425 ± 8.0^{b}	498 ± 2.8^{a}	260 ± 0.4^{e}	318 ± 0.7^{d}	355 ± 3.7^{c}	**<0.001*** **
Manganese (mg kg-DM^{-1})		133 ± 2.2^{a}	131 ± 0.8^{a}	110 ± 0.5^{c}	125 ± 1.1^{b}	121 ± 0.6^{b}	**<0.001*** **
Molybdenum (mg kg-DM^{-1})		0.84 ± 0.003^{c}	0.61 ± 0.003^{d}	0.93 ± 0.006^{a}	0.83 ± 0.001^{c}	0.88 ± 0.005^{b}	**<0.001*** **
Phosphorous (g kg-DM^{-1})		3.01 ± 0.027^{a}	2.79 ± 0.018^{c}	2.94 ± 0.016^{b}	3.02 ± 0.017^{a}	3.04 ± 0.027^{a}	**<0.001*** **
Sulphur (g kg-DM^{-1})		2.37 ± 0.007^{b}	2.52 ± 0.028^{a}	2.37 ± 0.012^{b}	2.41 ± 0.016^{b}	2.45 ± 0.047^{ab}	**0.013***
Selenium (µg kg-DM^{-1})		10.2 ± 0.45^{a}	8.43 ± 1.111^{a}	8.42 ± 0.632^{a}	5.13 ± 0.738^{b}	9.69 ± 0.249^{a}	**0.008****
Zinc (mg kg-DM^{-1})		24.0 ± 0.45^{a}	23.7 ± 0.46^{b}	20.7 ± 0.36^{a}	23.3 ± 0.28^{a}	24.3 ± 0.44^{a}	**<0.001*** **
Nitrogen (%DM)		1.94 ± 0.004^{c}	2.06 ± 0.025^{a}	2.05 ± 0.008^{a}	1.99 ± 0.016^{b}	2.07 ± 0.011^{a}	**<0.001*** **

N.D. : not detectable in the analyte; the lowercase letters behind numbers represent the results of LSD test post a significant result in general ANOVA test; the symbols: *, **, *** indicate statistical significances at p-value< 0.05, 0.01, 0.001, respectively.

drastically in the first three days then dropped after day 7 (**Figure 3.4h**). For some un-supplemented elements: Cd, Mo, P, S, the supplementary dose and the form appeared to mutually influence (P<0.05) urinary excretion on some experimental days (**Figures 3.4b, 3.4d, 3.4f, 3.4h and 3.4j**).

3.3.5 The output and partitioning of Cu, Zn, Mn and Se in urine and faeces

The chemical form of the supplemental minerals had no significant impact on their excretion in urine or faeces (**Table 3.10**) nor on their retention as a percentage of total elemental intake (**Table 3.11**). The supplementary dose significantly influenced the excretion of Se in urine and faeces as a percentage of Se intake and the retention of Se as a percentage of intake. The higher dose led to higher retention percentage. A similar effect was found in faecal excretion and retention of Cu when presented as a percentage of total Cu intake. In contrast, the supplementary dose did not significantly impact on Zn or Mn excretion or retention as a percentage of total Zn and Mn intake. However, the negative retention of Zn is an artefact of the calculation of total Zn intake, which did not include the significant Zn intake from drinking water (**Appendix A.3**).

According to **Table 3.12**, the partitioning of micronutrients in different excreta is element-dependent. A high proportion of the Se (about 80% of the total excreta amount) was excreted through faeces. For Zn, Cu and Mn, faecal excretion was the dominant excretion route. These results are consistent across different treatments, meaning that there was no significant impact of the supplementary dose or form on micronutrient partitioning. Generally, there was no observable difference in the partitioning of the elements between urine and faeces across different days (**Figure 3.5**). However, the proportion of Se excreted through faeces and the proportion of S excreted through urine were both greater on day 14 than on day 1.

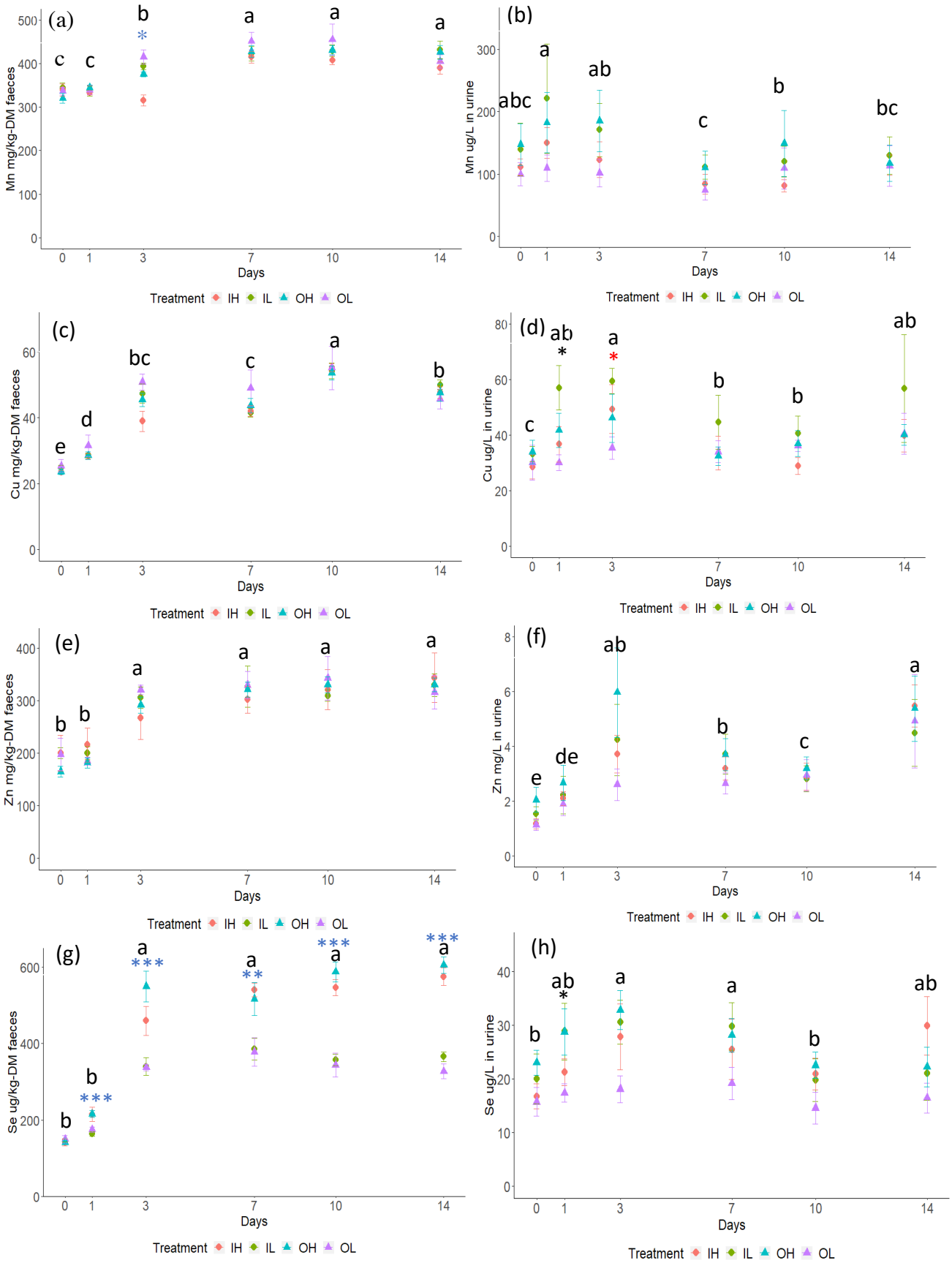
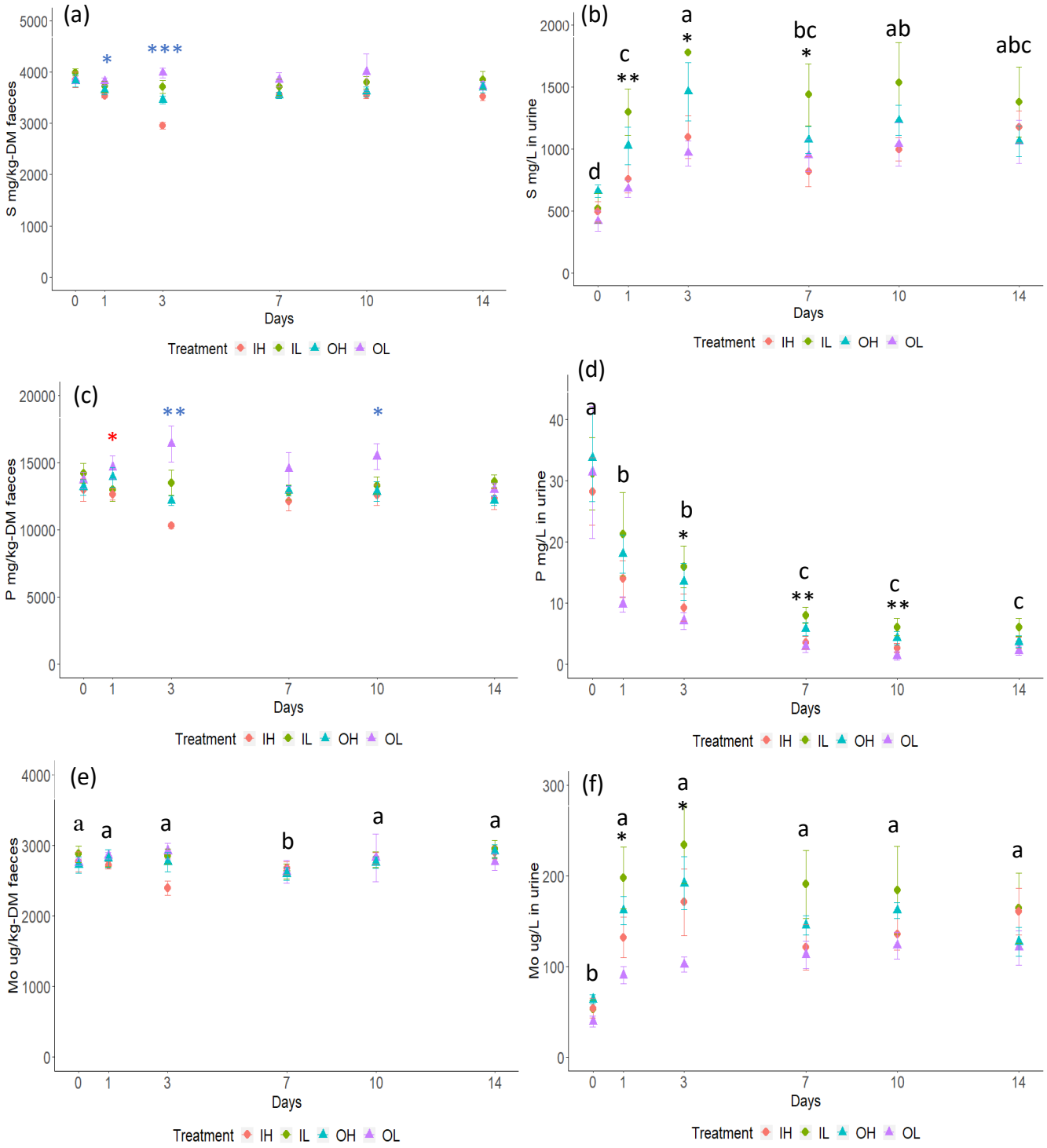


Figure 3.3 The concentrations of supplemented elements in the urine and faeces during the supplementation period. (a) Mn in faeces (b) Mn in urine (c) Cu in faeces (d) Cu in urine (e) Zn in faeces (f) Zn in urine (g) Se in faeces (h) Se in urine. The error bars are the standard errors (n=6) of the samples. The colour of the symbol: '*', '*', '*' represents the significant effect from supplemental mineral form, supplementary dose, the interaction of form and dose, respectively, within each day. The lowercase English letters represent the statistical results of post-hoc LSD test of temporal effect after the three-way factorial ANOVA test.


(a)
S mg/kg-DM faeces
Days
Treatment IH IL OH OL
(b)
S mg/L in urine
Days
Treatment IH IL OH OL
(c)
P mg/kg-DM faeces
Days
Treatment IH IL OH OL
(d)
P mg/L in urine
Days
Treatment IH IL OH OL
(e)
Mo ug/kg-DM faeces
Days
Treatment IH IL OH OL
(f)
Mo ug/L in urine
Days
Treatment IH IL OH OL

(Continued)

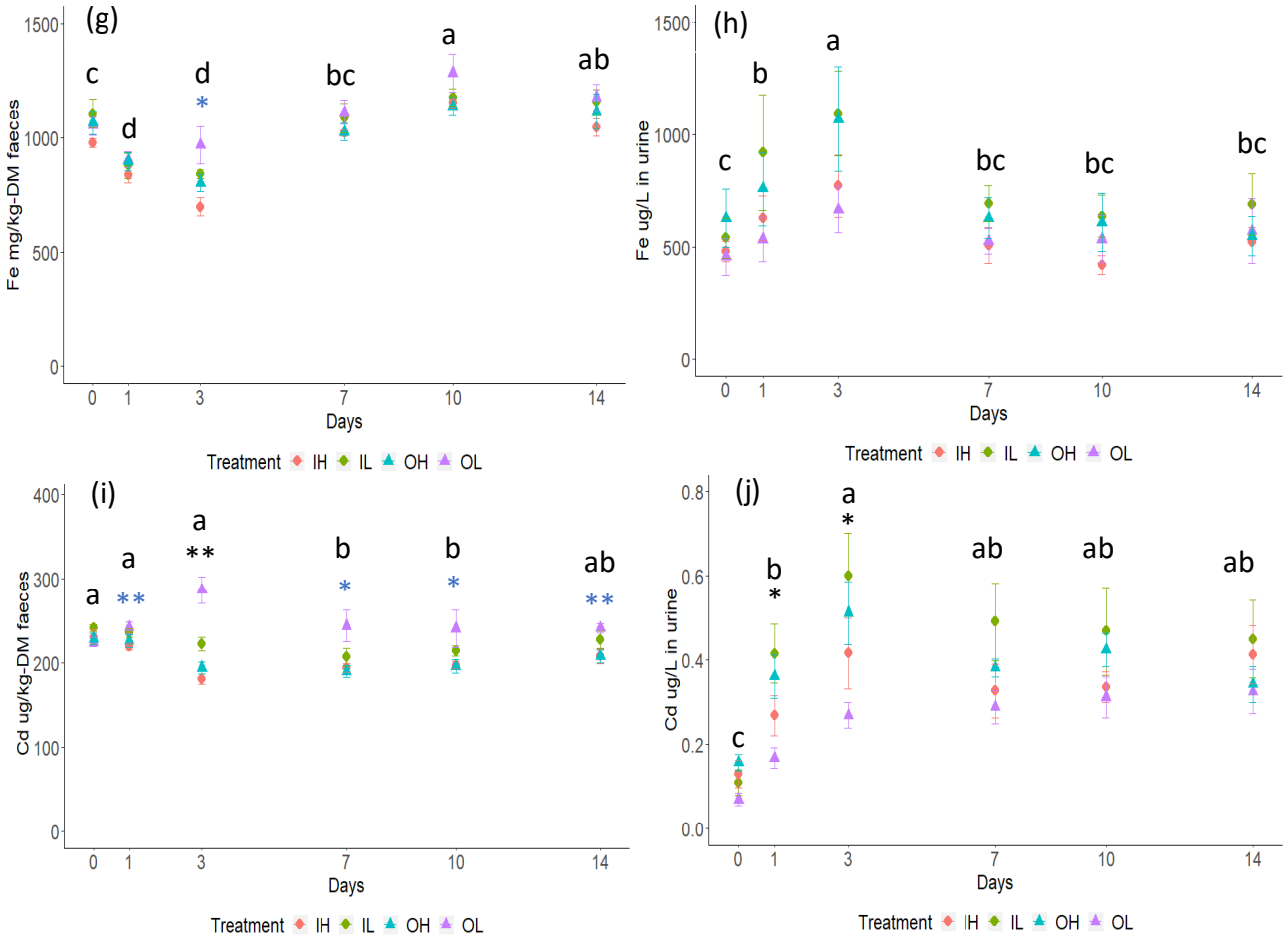


Figure 3.4 The concentrations of un-supplemented elements in the urine and faeces during the supplementation period. (a) S in faeces (b) S in urine (c) P in faeces (d) P in urine (e) Mo in faeces (f) Mo in urine (g) Fe in faeces (h) Fe in urine (i) Cd in faeces (j) Cd in urine. The error bars are the standard errors (n=6) of the samples. The colour of the symbol: '*', '*', '*' represents the significant effect from supplemental mineral form, supplementary dose, the interaction of form and dose, respectively, within each day. The lowercase English letters represent the statistical results of post-hoc LSD test of temporal effect after the three-way factorial ANOVA test

Table 3.10 Total micronutrient outputs in urine and faeces on day 14

Treatment	Se (μg day^{-1} animal^{-1} ± SE)		Zn (mg day^{-1} animal^{-1} ± SE)		Cu (mg day^{-1} animal-^{1} ± SE)		Mn (mg day^{-1} animal^{-1} ± SE)	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
IL	27.4 ± 3.22	130 ± 9.2	6.94 ± 2.238	120 ± 17.3	0.07 ± 0.014	17.8 ± 1.235	0.18 ± 0.053	152 ± 7.6
IH	44.8 ± 3.91	218 ± 22.6	9.26 ± 1.971	125 ± 13.1	0.06 ± 0.007	17.8 ± 1.078	0.18 ± 0.023	146 ± 10.7
OL	26.9 ± 1.74	105 ± 7.3	7.57 ± 1.438	102 ± 10.6	0.07 ± 0.005	14.6 ± 0.880	0.17 ± 0.027	131 ± 9.5
OH	37.5 ± 4.02	213 ± 21.4	8.97 ± 1.210	116 ± 12.5	0.07 ± 0.010	16.6 ± 1.584	0.20 ± 0.033	149 ± 14.2
				P level				
F_{form}	0.2685	0.3744	0.9235	0.3397	0.8135	0.9674	0.9585	0.4209
F_{dose}	**<0.001*** **	**<0.001*** **	0.2925	0.4890	0.8153	0.4442	0.7637	0.5930
F_{form} x F_{dose}	0.3335	0.5682	0.7911	0.7464	0.4206	0.4320	0.7415	0.2913

'***' indicates statistical significances of ANOVA test at p-value<0.001.

Table 3.11 Micronutrient excretion in urine and faecal samples on day 14 as a percentage of the total element intake on day 13

Treatment	Se			Zn			Cu			Mn		
	Urine	Faeces	Retained	Urine	Faeces	Retained	Urine	Faeces	Retained	Urine	Faeces	Retained
						% (w/w) ± SE						
IL	11.2 ± 1.39	52.2 ± 2.27	36.6 ± 1.85	6.62 ± 1.901	114 ± 10.9	-20.7 ± 12.55	0.40 ± 0.078	97.6 ± 4.33	1.99 ± 4.374	0.11 ± 0.026	95.3 ± 2.55	4.57 ± 2.562
IH	9.42 ± 0.670	45.5 ± 3.14	45.1 ± 3.11	8.20 ± 1.819	111 ± 13.8	-19.1 ± 15.15	0.32 ± 0.038	89.0 ± 2.04	10.6 ± 2.02	0.12 ± 0.017	91.1 ± 5.31	8.76 ± 5.317
OL	13.0 ± 0.33	50.9 ± 2.34	36.1 ± 2.33	8.89 ± 1.849	115 ± 9.4	-23.8 ± 9.54	0.43 ± 0.040	92.8 ± 2.06	6.74 ± 2.062	0.12 ± 0.018	90.2 ± 5.74	9.70 ± 5.744
OH	7.80 ± 0.806	43.3 ± 2.51	48.9 ± 2.14	7.63 ± 1.102	96.9 ± 6.74	-4.54 ± 6.571	0.34 ± 0.037	79.4 ± 4.82	20.1 ± 4.84	0.11 ± 0.019	85.9 ± 7.34	14.0 ± 7.34
						P level						
F_{form}	0.9361	0.5106	0.4777	0.5253	0.5218	0.5926	0.4927	0.0808	0.0829	0.8905	0.3260	0.3267
F_{dose}	**0.0010*** **	**0.0156***	**0.0004*** **	0.9051	0.3086	0.3357	0.0550	**0.0121***	**0.0118***	0.9809	0.4193	0.4197
F_{form} x F_{dose}	0.0638	0.8717	0.3765	0.2976	0.4741	0.4147	0.9059	0.5377	0.5384	0.7617	0.9945	0.9937

'*', '***' indicate statistical significances of ANOVA test at p-value<0.05 and <0.001, respectively.

Table 3.12 Total micronutrient partitioning between urine and faecal excretion as a percentage on day 14

Treatment	Se		Zn		Cu		Mn	
	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
	% (w/w)							
IL	17.7	82.3	5.1	94.9	0.4	99.6	0.1	99.9
IH	17.1	82.9	7.0	93.0	0.4	99.6	0.1	99.9
OL	15.7	84.3	6.6	93.4	0.4	99.6	0.1	99.9
OH	21.1	78.3	4.7	95.3	0.4	99.6	0.1	99.9
	P level							
F_{form}	0.8279		0.2279		0.0906		0.4383	
F_{dose}	0.1674		0.4468		0.3579		0.7323	
F_{form} x F_{dose}	0.2205		0.5464		0.9161		0.8558	

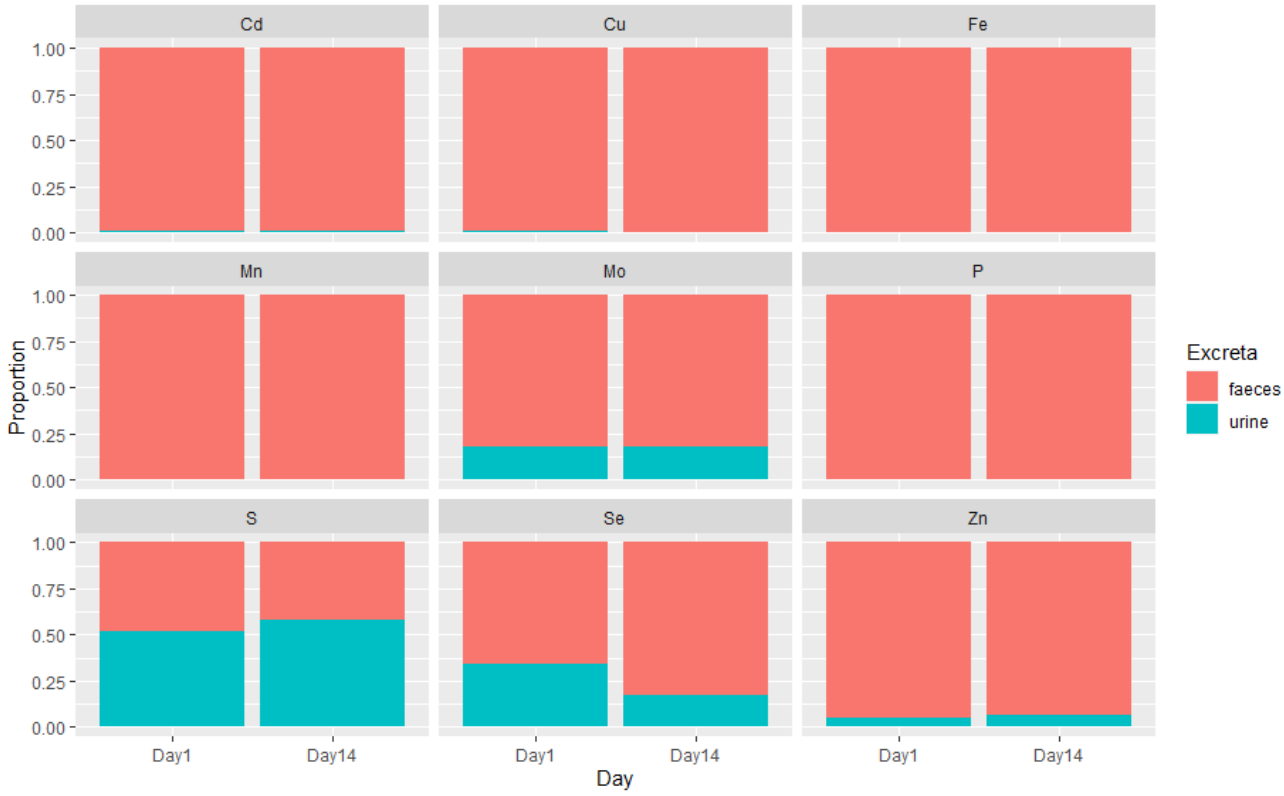


Figure 3.5 The change of micronutrient partitioning (proportion of the total excretion amount) through the supplementary period. Data are the average of the four treatments (as treatment was not significant).

3.3.7 Changes of the ratio of Se/P and Se/S in urine and faeces

The Se uptake by grass can be hindered by the antagonism between SeO_3^{2-} and PO_4^{3-} or between SeO_4^{2-} and SO_4^{2-} (Kao *et al*., 2020), therefore the ratio of Se:P and Se:S in the excreta might be an important factor for Se, P and S antagonism against Se uptake by grass.

At the beginning of supplementation period (day 0), there was no significant effect of treatment on the Se:S or Se:P ratios. After the supplementation period, the supplementary dose had a significant impact on the ratio of Se:S in both urine and faeces sampled on day 14 and had a significant impact on the ratio of Se:P in faeces sampled on day 14. The higher dose led to the higher ratios of Se:S and Se:P in the excreta (**Table 3.13**).

Table 3.13 The concentration ratios of Se:P and Se:S in the urine and faeces on day 0 and day 14

Ratio	Day	Excreta	Treatment				P level		
			IL	IH	OL	OH	Form	Dose	Form x Dose
Se:S	0	Faeces	3.68×10^{-5}	3.67×10^{-5}	3.89×10^{-5}	3.71×10^{-5}	0.4727	0.5324	0.6127
		Urine	3.83×10^{-5}	3.58×10^{-5}	4.05×10^{-5}	3.55×10^{-5}	0.4604	0.8107	0.3575
	14	Faeces	9.55×10^{-5}	1.63×10^{-4}	8.74×10^{-5}	1.64×10^{-4}	0.2815	**<0.001*** **	0.2413
		Urine	1.47×10^{-5}	2.48×10^{-5}	1.55×10^{-5}	2.05×10^{-5}	0.3353	**0.0007*** **	0.1663
Se:P	0	Faeces	1.05×10^{-5}	1.09×10^{-5}	1.09×10^{-5}	1.08×10^{-5}	0.9090	0.7695	0.7223
		Urine	1.21×10^{-3}	6.51×10^{-4}	8.30×10^{-4}	8.27×10^{-4}	0.1721	0.7691	0.4225
	14	Faeces	2.69×10^{-5}	4.73×10^{-5}	2.52×10^{-5}	4.98×10^{-5}	0.7013	**<0.001*** **	0.0598
		Urine	6.17×10^{-3}	1.08×10^{-2}	9.40×10^{-3}	7.76×10^{-3}	0.9641	0.5326	0.1989

'***' indicates the statistical significance of ANOVA test at p-value<0.001.

3.3.8 Sequential extraction of faecal samples

Because most of the Se, Cu, Zn and Mn were excreted through faeces (**Figure 3.5**), studying the chemical forms of these elements in faeces were investigated to better understand the availability of the elements to plants following manure application in the field. A chemical sequential extraction was performed to understand the fractionation of the elements and the associated chemical properties of Se, Cu, Zn, Mn in faeces (**Tables 3.14, 3.15, 3.16 and 3.17**, respectively). The supplementary dose

significantly affected the sequential extraction results of Se; the treatments of the higher dose generally had higher concentrations of Se in all the four extracted fractions (**Tables 2.3 and 2.4**). In terms of the % Se in different fraction, only the third fraction (bound to OM or sorbed to (Fe, Al)-hydroxides) showed a significant difference: the treatment of higher mineral supplement dose resulted in higher % of Se partitioning in the third fraction. For Cu, no significant difference in fractionation across different treatments groups was observed. For Zn, inorganic mineral form resulted in significantly higher Zn partitioning into the third fraction (Oxidizable). However, the Zn in the third fraction only accounted for less than 10% of the total Zn in the faecal sample. The supplementary dose and form had a significant impact on the fractionation of Mn into the fourth fraction (residual). However, the Mn in the fourth fraction accounted for less than 1% of the total Mn.

3.3.9 Se speciation analysis of faecal extracts

The chemical species of an element directly determines its' availability to plants. Although, the results of the sequential extraction of Se in faeces (**Table 3.14**) showed that the chemical form of the supplemental minerals had no effect on the chemical form of Se in the first and the second fractions of the Se SEP, which are deemed to be the most 'plant available' fractions, it remained unknown whether the chemical form of the supplemental minerals could affect the Se species in faeces and could, in turn, affect Se availability to plants. To further investigate the Se species in the plant-available fractions, faecal samples were extracted with phosphate buffer with or without the addition of a protease, and analysed Se species in the faecal extracts using HPLC-HG-AFS (**Chapter 2.2.3**). The addition of protease was used to digest and transform any Se in proteinaceous form into a detectable form by the instrument.

Table 3.14 Sequential extractions of Se in faecal samples from day 14

SEP steps		IL	IH	OL	OH	P level		
						Form	Dose	Form x Dose
*Step 1: Water- and acid- soluble	Mean ± SE (μg kg^{-1})	54.7 ± 3.89	76.3 ± 5.65	51.2 ± 4.38	71.0 ± 5.00	0.4698	**<0.001 *** **	0.9592
	% of total	33.8%	33.0%	33.5%	32.6%	0.8684	0.7882	0.8455
Step 2: Exchangeable	Mean ± SE (μg kg^{-1})	6.06 ± 0.730	11.2 ± 0.85	6.63 ± 0.431	10.8 ± 0.41	0.7103	**<0.001 *** **	0.3174
	% of total	3.7%	4.7%	4.3%	4.8%	0.2029	0.0585.	0.3630
*Step 3: OM-bound a/o specific sorption on Fe/Al hydroxides	Mean ± SE (μg kg^{-1})	45.0 ± 2.57	74.4 ± 1.94	36.6 ± 2.50	67.3 ± 2.91	**<0.001 *** **	**<0.001*** **	0.5558
	% of total	27.9%	32.3%	24.0%	29.1%	0.0670	**0.0139***	0.5964
Step 4: Residual	Mean ± SE (μg kg^{-1})	56.2 ± 6.31	76.8 ± 8.76	61.3 ± 11.42	83.1 ± 8.56	0.5885	**0.0379***	0.9192
	% of total	34.5%	30.0%	38.2%	33.5%	0.4560	0.1696	0.7773
Averaged extraction recovery % = sum of step (1, 2, 3, 4) / total analysis by ICP		44.4%	42.2%	46.1%	45.4%	-	-	-

In step 1, two outliers were removed and in step 3, one outlier was removed from the original dataset before data calculation and factorial ANOVA test. For data that have been removed, are not included in the percentage calculation. '' and '***' indicate statistical significances of ANOVA test at p-value<0.05 and <0.001, respectively.

Table 3.15 Sequential extractions of Cu in faecal samples from day 14

SEP steps		IL	IH	OL	OH	P level		
						Form	Dose	Form x Dose
Step 1: Exchangeable, water- and acid-soluble	Mean ± SE (mg kg^{-1})	2.44 ± 0.093	2.67 ± 1.112	2.32 ± 0.172	2.43 ± 0.143	0.1630	0.1854	0.6722
	% of total	6.2%	6.7%	6.2%	6.2%	0.3788	0.3153	0.4030
Step 2: Reducible	Mean ± SE (mg kg^{-1})	8.36 ± 0.500	7.91 ± 0.391	7.90 ± 0.456	7.76 ± 0.285	0.3780	0.3955	0.6500
	% of total	21.2%	19.9%	21.1%	19.9%	0.9568	0.0677	0.9579
Step 3: Oxidizable	Mean ± SE (mg kg^{-1})	22.1 ± 0.74	22.3 ± 0.85	21.1 ± 0.94	22.1 ± 0.89	0.5648	0.5512	0.7014
	% of total	55.9%	56.0%	56.6%	56.6%	0.5501	0.9298	0.9587
Step 4: Residual	Mean ± SE (mg kg^{-1})	6.61 ± 0.143	6.90 ± 0.285	6.01 ± 0.893	6.70 ± 0.362	0.3912	0.2927	0.6602
	% of total	16.7%	17.4%	16.1%	17.2%	0.6748	0.4622	0.8044
Averaged extraction recovery % = sum of step (1, 2, 3, 4) / total analysis by ICP		78.9%	83.3%	81.8%	81.7%	-	-	-

Table 3.16 Sequential extractions of Zn in faecal samples from day 14

SEP steps		IL	IH	OL	OH	P level		
						Form	Dose	Form x Dose
Step 1: Exchangeable, water- and acid-soluble	Mean ± SE (mg kg^{-1})	115 ± 12.4	119 ± 20.0	113 ± 12.8	114 ± 7.9	0.7668	0.8516	0.9000
	% of total	40.5%	39.8%	39.7%	40.5%	0.9701	0.8756	0.6285
Step 2: Reducible	Mean ± SE (mg kg^{-1})	138 ± 7.0	149 ± 23.2	147 ± 12.6	143 ± 4.0	0.9024	0.7448	0.5394
	% of total	48.4%	50.0%	51.7%	51.1%	0.0776	0.8989	0.5772
Step 3: Oxidizable	Mean ± SE (mg kg^{-1})	15.8 ± 3.29	25.4 ± 2.32	20.0 ± 1.42	19.0 ± 1.01	**0.0119***	0.7358	0.8981
	% of total	9.1%	8.5%	7.0%	6.8%	**0.0077****	0.7490	0.8619
Step 4: Residual	Mean ± SE (mg kg^{-1})	5.81 ± 0.716	5.13 ± 0.374	4.57 ± 0.47	4.54 ± 0.261	0.0540	0.4283	0.4629
	% of total	2.0%	1.7%	1.6%	1.6%	0.0984	0.3824	0.4809
Averaged extraction recovery % = sum of step (1, 2, 3, 4) / total analysis by ICP		86.4%	87.1%	90.1%	85.0%	-	-	-

'*' and '**' indicate statistical significances of ANOVA test at p-value<0.05 and <0.01, respectively.

Table 3.17 Sequential extractions of Mn in faecal samples from day 14

SEP steps		IL	IH	OL	OH	P level		
						Form	Dose	Form x Dose
Step 1: Exchangeable, water- and acid-soluble	Mean ± SE (mg kg^{-1})	307 ± 5.2	295 ± 8.0	310 ± 12.1	330 ± 16.0	0.1018	0.7321	0.1650
	% of total	79.5%	80.3%	80.2%	81.5%	0.2425	0.1346	0.8012
Step 2: Reducible	Mean ± SE (mg kg^{-1})	64.8 ± 3.77	60.1 ± 2.96	62.1 ± 2.83	63.0 ± 2.27	0.9878	0.5288	0.3550
	% of total	16.8%	16.4%	16.1%	15.6%	0.1876	0.4604	0.9668
Step 3: Oxidizable	Mean ± SE (mg kg^{-1})	12.1 ± 2.16	9.99 ± 1.425	12.6 ± 0.88	10.1 ± 0.82	0.8311	0.0956	0.8652
	% of total	3.1%	2.7%	3.3%	2.5%	0.9935	0.0875	0.5964
Step 4: Residual	Mean ± SE (mg kg^{-1})	2.46 ± 0.160	1.95 ± 0.149	1.98 ± 0.185	1.80 ± 0.130	**0.0415***	**0.0274***	0.2626
	% of total	0.6%	0.5%	0.5%	0.4%	**0.0097****	**0.0251***	0.5440
Averaged extraction recovery % = sum of step (1, 2, 3, 4) / total analysis by ICP		89.6%	94.0%	95.5%	95.0%	-	-	-

'*' and '**' indicate statistical significances of ANOVA test at p-value<0.05 and <0.01, respectively.

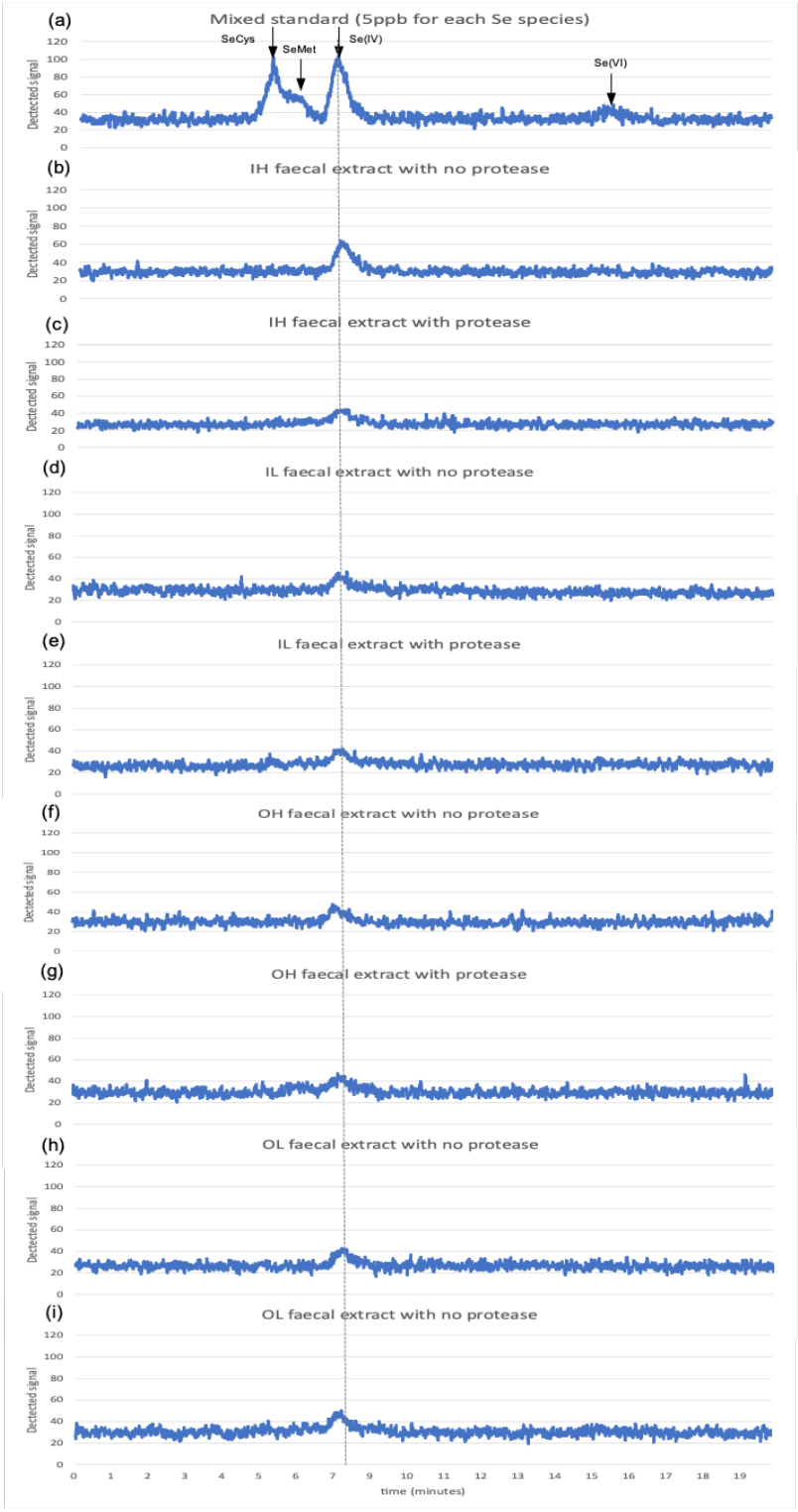


Figure 3.6 HPLC chromatographic fingerprint of the Se speciation analysis of faecal extracts. The results presented are chromatographic fingerprints of (a) mixed standard solution of selenium, and extracts of faeces of (b) IH treatment(-)protease (c) IH treatment(+)protease (d) IL treatment(-)protease (e) IL treatment(+)protease (f) OH treatment(-)protease (g) OH treatment(+)protease (h) OL treatment(-)protease (i) OL treatment(+)protease

The extracts from the faeces of IH, IL, OH and OL groups all generated a peak of SeO_3^{2-} at 7-9 minutes on the HPLC chromatographic fingerprint (**Figure 3.6**). No other significant peaks were observed. For the extracts from the faeces with an addition of protease, no new peaks other than Se (IV) were observed. The result indicates that Se (IV) was the dominant Se species in the faecal extract with the use the P solution, which is commonly used for extracting 'plant-available' Se from solid samples. The chromatographic fingerprints of the extracts with an addition of protease indicated that no proteinaceous Se was detectable in the P extracts.

3.4 Discussion

3.4.1 Micronutrient excretory partitioning between urine and faeces

The partitioning ratio of micronutrients between urine and faeces may indirectly affect micronutrient availability to forages. Urine and faeces may undergo different decomposition routes and different spatial and temporal distribution in pasture systems, which, in turn, affect micronutrients' availability.

3.4.1.1 Metallic micronutrients: Zn, Cu and Mn

In sheep, Zn, Cu and Mn in sheep are mostly excreted via faeces, which includes undigested minerals, as well as endogenous excretions from metabolised minerals, such as pancreatic secretion of Zn and bile excretion of Cu and Mn, whereas only small amounts are excreted via urine (Gooneratne et al., 1989; Grace & Gooden, 1980; Mills & Williams, 1971; Minson, 1990; Underwood & Suttle, 1999). Grace and Gooden (1980) reported that the quantities of Zn, Cu and Mn excreted through urinary losses were much lower than the losses from the digestive secretions (bile and pancreatic juice) across different Zn, Cu and Mn intake levels. The results in this study were aligned with previous studies with partitioning of Zn, Cu and Mn in faeces on day 14 at >93%, >99% and >99% (**Table 3.12**), respectively, and were not significantly affected by time

(**Figure 3.5**) nor by supplementary forms at the current supplementary doses (**Table 3.12**).

3.4.1.2 Non-metallic micronutrient: Se

Approximately 15-20% of the Se was excreted into urine across the treatments (**Table 3.12**), which aligns with previous reports of less than 50% of the Se excreted into urine by Paiva et al. (2019) and less than 30% by Koenig et al. (1997). Similar to the results reported by Koenig et al. (1997) and Paiva et al. (2019), different Se chemical forms (organic versus inorganic) did not significantly affect the partitioning of Se between urine and faeces (**Table 3.12**). However, the different Se supplementary doses adopted in the current study did not show a significant impact on the partitioning, which contradicts the results of Paiva et al. (2019) and M. Lee et al. (2019), which showed a positive correlation between Se supplementary dose and Se partitioning in urine. That is, the higher the Se supplementary dose the more Se was excreted into urine. The Se doses given in the current study are roughly between 0.1 (low) and 0.4 (high) mg d^{-1} (**Table 3.8**), which were lower than the doses supplemented in both Pavia et al. (2019) and M. Lee et al. (2019). This suggests that the partitioning of Se between urine and faeces can be different when a higher dose is supplemented (>0.4 mg d^{-1}). Both Pavia et al. (2019) and M. Lee et al. (2019) supplemented Se (0.23-1.98 mg d^{-1} and 0.17-2.03 mg kg-DM^{-1}, respectively) above the upper limit of supplementary dose (<0.23 mg d^{-1} or <0.1 mg kg-DM^{-1} in a complete feed) recommended (NRC, 2007). However, as shown in the current study, there is little difference in Se partitioning between urine and faeces when Se supplementary levels are administered below 0.4 mg d^{-1}.

In addition to the dose effect, basal diet and form of feeding may also influence the partitioning of Se between urine and faeces. Koenig et al. (1997) showed that there was a significant interaction effect between the effect of diet (forage-based versus

concentrate-based) and the effect of Se chemical form ([^{77}Se]yeast versus [^{82}Se]selenite) on Se urine partitioning. This means that both the basal diet and the chemical form can impact the Se partitioning portion in urine. However, in the present study and that of Paiva et al. (2019), Se chemical form did not show a significant effect on Se urine partitioning. Therefore, the differences in Se partitioning across different studies may be related to the variation of basal diet and dose interaction. Other factors may also have an influence, for example, different routes of administration (oral versus intravenous) of supplemental Se have also shown differences in Se partitioning into urine, faeces and exhalate (Lopez et al., 1969). As indicated in Mayland (1995), in contrast to the ingested Se, that injected either intravenously or subcutaneously into ruminants is excreted mostly in urine. Additionally, animal age (Mayland, 1995), sex, breed and Se status could also be possible additional factors that require further investigation.

Unlike Cu, Zn and Mn, Se has a third major excretion pathway as it can also be lost via exhalation (Underwood & Suttle, 1999). However, based on a preliminary test (**Appendix A.4)** undertaken before the main experiment, the Se concentration in exhalate of sheep supplemented with Se according to the IND level, using a published bag technique (Tiwary et al., 2005), was close to the detection limit of ICP-MS (0.05-0.10 μg L^{-1}). Therefore, in the main experiment, sheep exhalate samples were not taken, and the Se lost via exhalation was not considered in this study, which will result in a slight overestimation of the Se retention reported. However, Se excretion via breathing is suggested to happen only in the case of excessive dose (Pyrzyńska, 2002), which was may have not been relevant to the current study.

3.4.2 Micronutrient excretion concentration and total output in urine and faeces

The results of total output of micronutrients enable the understanding of micronutrient flux from feed to excreta. However, when studying micronutrient biogeochemical pathways after the application of manure in pasture systems, the concentration of an element in the animal excreta is also critical to look into. This is because the concentration of a micronutrient in the excreta is a determining factor in the amount of a micronutrient that a soil receives per unit area (**Equation 3.10**), assuming that urine and faecal excretion amount per unit area of soil is independent from different mineral supplement and feeding strategies.

$$Micronutrient\ recieved\ per\ unit\ soil\ \left(\frac{weight}{area}\right) = concentration\ of\ a\ micronutrient\ in\ animal\ excreta\ \left(\frac{weight}{volume\ or\ weight}\right) \times excreta\ amount\ from\ ruminant\ at\ grazing\ per\ unit\ area\ of\ soil\ \left(\frac{volume\ or\ weight}{area}\right) \quad \textbf{(3.10)}$$

3.4.2.1 Metallic micronutrients: Zn, Cu and Mn

For the metallic supplemental elements, Zn, Cu and Mn, the different form the mineral supplements given at the current doses had no significant impact on their concentrations and total output and in urine and faeces (**Figure 3.3** and **Tables 3.10**). In the current study, sheep were supplemented with 88.56-118.2 mg-Zn d^{-1} in the form of $ZnSO_4$ or Zn chelate of protein hydrolysate with no significant effect of chemical form, dose or interaction on Zn excretion in urine or faeces. This result is in agreement with the past studies: no significant difference in urinary and faecal excretion was found between $ZnSO_4$ and Zn-methionine (at ca. 35 mg-Zn d^{-1}) (Garg et al., 2008); no significant difference in faecal excretion was found between ZnO, Zn-glycine, Zn-lysine, and Zn-methionine (at ca. 80 mg-Zn d^{-1}), yet higher urinary excretions were found with ZnO

and Zn-glycine over Zn-lysine and Zn-Met (Kinal & Slupczynska, 2011); and no significant differences in urinary and faecal excretion was found between ZnO, $ZnSO_4$, Zn-Met, Zn-hydroxychloride (at ca. 65 mg d^{-1}) (VanValin et al., 2018). There are limited studies investigating the effect of different chemical form of supplemental Cu and Mn on their excretions in urine and faeces, which is expected because background levels in forage and/or concentrate feed are usually the dominant sources of Cu and Mn (**Table 3.8**). A study investigating different forms of Mn and the excretion and absorption of Mn in lambs showed that there was no significant difference in the faecal excretion of Mn between the treatment of $MnSO_4$ and the treatment of Mn chelate of glycine hydrate (Gresakova et al., 2018). The current study also found no significant effect of supplementary form on Mn excretion concentration (**Figure 3.3**), total output (**Table 3.10**) or partitioning between urine and faeces (**Table 3.12**).

3.4.2.2 Non-metallic micronutrient: Se

The different supplementary doses adopted in the current study had significant impact on both the concentrations and excretory output of Se in both urine and faeces. A higher dose led to more excreted Se. However, the chemical form of Se supplement had no significant impact. There were no interactions between mineral supplementary dose and chemical form towards micronutrient excretion in urine or in faeces in the current study.

Similarly, Paiva et al. (2019) reported that the supplementary dose of Se significantly influenced the excretion of Se in both urine and faeces, with the response consistent across different Se supplementary forms ($NaSeO_3$, Se-yeast and SeMet). This confirmed that a higher supplementary dose of Se results in an increased excretion of Se in both urine and faeces, which is independent from the chemical form of Se when offered within typical industrial levels.

Furthermore, Paiva et al. (2019) reported that at low Se supplementary doses (0.20-0.80 mg-Se kg-DM^{-1} or 0.23-1.04 mg-Se d^{-1}) no significant effect of the chemical form of Se supplements on the excretion of Se in faeces or urine; whereas at higher Se supplementary doses (1.4 mg-Se kg-DM^{-1} or 1.68-1.98 mg-Se d^{-1}), there was a significant difference between the treatment of organic Se and inorganic Se in the excretion of Se in faeces (inorganic Se > organic Se) but this was not mirrored in urine. The Se supplementary doses adopted in the current study are below 0.4 mg-Se d^{-1} (**Table 3.8**), which is in the range of the 'low Se supplementary doses' in Paiva et al. (2019). Aligned with the results of Paiva et al. (2019), there was no significant impact of chemical form of Se supplement on the excretion of Se in urine/faeces observed here. In another study (Ehlig et al., 1967), sheep supplemented with SeO_3^{2-} or SeMet at 0.4 mg-Se d^{-1} also showed no effect of Se intake form on Se excretion in faeces. This suggests that the supplementary dose of Se might be a critical factor determining whether the chemical form of the Se supplement has a significant impact on Se excretion in urine or faeces.

In the study of Koenig et al. (1997), although the doses of supplemental Se were low (0.109 and 0.114 mg-Se d^{-1} for the studied group of [^{77}Se]-yeast and [^{82}Se]-selenite, respectively), both were lower than the recommended dose of 0.23 mg-Se d^{-1} (NRC, 2007), and the effect of the chemical form of the Se was still significant. This may be attributed to the use of stable isotope tracers enabling the investigation of the effect on Se excretion caused by the difference in Se in the supplemented chemical form as opposed to Se from other sources, such as the background diet. Se sources other than supplemented Se will provide a significant contribution to the Se intake and, therefore, the route of excretion. Hence, supplementing Se at high levels (>1.68 mg-Se d^{-1}) or using isotope techniques enable the effect of different supplemental mineral forms to

show over and above the basal Se intake. However, in the 'European Commission Implementing Regulation (EU) 2019/804', the maximum provision level of supplementary organic Se is limited to 0.2 mg kg-DM^{-1} of complete intake at 12% moisture. In this study, which aimed to replicate typical farming scenarios, this equated to ca. 0.25 mg-Se d^{-1} depending on intake, at which the effect of different chemical form of supplemental Se is hardly observed without the use of an isotope technique.

3.4.3 Differences in concentrations of P and S in the excreta after mineral supplementation

Most excretion patterns of un-supplemented elements were not significantly influenced by the supplementation of Zn, Se, Cu or Mn minerals. However, P and S excretion were influenced indirectly by mineral supplementation (**Figure 3.4**). Since it is known that PO_4^{3-} and SO_4^{2-} can have antagonistic effects on SeO_3^{2-} and SeO_4^{2-}, respectively, for their availability to plants, the excretion of P and S in urine and faeces can therefore be influential to the plant availability of Se in the excreta.

3.4.3.1 Decreasing urine P after mineral supplementation

P had steadily decreasing concentrations in urine across treatments (**Figure 3.4d**). Faecal excretion is the major route of P excretion in sheep (Kebreab et al., 2008), and P intake is highly related to P excretion in faeces (Vitti et al., 2005). In the current study, faecal P did not appear to decrease as steadily as urine P. In addition, the concentration of P in silage provided across the supplementation period (day 1 to day 14) were not significantly different (**Table 3.9**). Therefore, the decreasing urine P during the experiment does not appear to be caused by variable P intake from silage.

The P concentration in urine is related to the concentration of saliva P, and P partitioning between saliva and urine is influenced by the type of diet offered

(Underwood & Suttle, 1999). The partition of the initially absorbed P in saliva increases as the roughage content of the diet increases, and therefore the partition of the absorbed P through urinary excretion decreases (Scott & Buchan, 1985; Underwood & Suttle, 1999). Salivation rate has been reported as the major factor in urinary P excretion because decreasing salivation rate increases P concentration in plasma and therefore, more P will be excreted via urine (Kebreab et al., 2008). However, in the current study the mass ratio of silage: concentrate did not decrease with experimental time, except for a slight change in the OL group, there was also no significant difference in the silage fibre concentration or form temporally, so overall there was no fibre content change leading to cause a change in salivation flow rate. Change in physical characteristics of the diet, therefore, does not appear to explain the decreasing P in urine.

Alternatively, improved metabolism in sheep by the supplemental minerals alters mineral status. Therefore, metabolic activity might be the cause of the decreasing P concentration in urine observed. Despite being a very small proportion of total body weight, micronutrients play critical roles in metabolic reactions (Kao et al., 2020). For example, Zn plays a critical role in forming DNA-binding proteins that influence transcription and, hence, cell replication (Underwood & Suttle, 1999). The increased Zn intake can increase energy metabolism, which involves the use of P as part of adenosine triphosphate (ATP) and might, in turn, increase the use of P in sheep and lead to the decreasing urine P concentration. Moreover, P is found to be directly involved in the formation *in vivo* of selenophosphate ($SePO_3^{3-}$), an intermediate product in the synthesis of SeCys, (Cupp-Sutton & Ashby, 2016; Schmidt & Simonović, 2012), which is also an ATP-requiring metabolic activity in animals. Therefore, the decreasing levels of P found in the urine may be related indirectly to the supplementary mineral intake, through improved mineral status of the animal, resulting in higher P retention

and reduced loss in urine. To ensure that the effect the supplementary mineral intake to the constant decreased P in urine was true, it would be better that there were data of P concentrations of urine collected days before day 0. However, I didn't collect the urine and faeces samples before day 0. For the future study, to investigate the effect of mineral supplementation on the metabolism of the nutrients in animal through time, samples before the administration of mineral supplements should be collected.

3.4.3.2 Increasing urine S after mineral supplementation

Increasing concentrations of urinary S were observed during the initial phase of the experimental period and they reached a plateau after day 3 (**Figure 3.4b**). The concentrations of faecal S dropped in the first three days, except for the OL group, and reached a new plateau afterward (**Figure 3.4a**). White (1980) indicated that when the intake of S increased from 0.5 g kg^{-1} to 2.0 g kg^{-1}, S tended to be excreted mainly via urine. In the current study, the S concentrations in the concentrate-feed were all over 2.0 g kg^{-1} (**Table 3.4**). **Figure 3.3** shows that urinary excretion was the major excretion route for S. However, since the concentrations of S in the basal feed were not significantly different across the supplementary period **(Tables 3.4 and 3.9)**, the increased S in urine was not attributed to changes in S content of the feed and is therefore an indirect response to the administration of the mineral pre-mix.

Selenium and S are known to be chemically and physically similar in biochemistry due to their similar configurations of electrons in the outermost valence shells and hence position within the periodic table (Shamberger, 1983). Therefore, the competitiveness between Se and S *in vivo* has been extensively studied. However, Shamberger (1983) states that Se and S cannot always substitute for one another *in vivo*, since in mammals Se compounds typically contain reduced Se, whereas S compounds typically contain oxidized S. On the other hand, White (1980) showed that the incorporation of Se in

sheep was influenced by S intake levels: a reduction in S intake resulted in an overall increase in Se retention. Although, it has been shown that S intake level can compete with Se incorporation (White, 1980), there is no study, to the author's knowledge, showing that Se intake level can influence S incorporation and hence excretion. Indeed, Se compounds tend to be more chemically reactive than S compounds (Cupp-Sutton and Ashby, 2016) and therefore, it is possible that Se supplementation can affect S metabolism and hence, excretion in animals. However, the increased S in urine during the first three days of supplementation (579 mg-S d^{-1} from day1 to day 7) was ca. 2000-5000 times higher than the dose of supplemented Se (0.2-0.5 mg-Se d^{-1}), which suggests that the increased urinary excretion of S was not solely attributed to Se-S substitution and therefore was more likely related to a wider change in metabolism.

Again, to ensure that the effect the supplementary mineral intake to metabolism of S in sheep was true, it would be better that there were data of S concentrations of urine collected days before day 0. However, I didn't collect the urine and faeces samples before day 0. For the future study, to investigate the effect of mineral supplementation on the metabolism of the nutrients in animal through time, samples before the administration of mineral supplements should be collected.

3.4.3.3 The potential impact of the change of Se:P and Se:S in urine and faeces on Se flux in the system

The ratios of Se:P and Se:S can be an indicator of the potential effect of antagonism between SeO_3^{2-} and PO_4^{3-} and between SeO_4^{2-} and SO_4^{2-}. The results showed that the form of the supplemental minerals had no significant impact on the total concentration of P and S in urine and faeces after the excretion had plateaued (**Figure 3.4**). However, since the Se excretion was significantly influenced by the supplementary doses, the ratios of Se:P and Se:S in the excreta was affected accordingly (**Table 3.13**), which

might in turn alter the availability of Se to plants. On the other hand, the different forms of the supplemental minerals had no significant impact on altering the ratios of Se:P and Se:S in urine nor in faeces (**Table 3.13**). It is therefore concluded that supplemented at the typical industrial doses, the different forms had no influence on changing the plant-availability via altering the ratios of Se:P or Se:S in the excreta.

3.4.4 The potential impact of supplemental mineral form on plant-availability of the micronutrients in sheep excreta

3.4.4.1 Chemical fractionation of Zn, Cu, Mn and Se in faeces under different treatments

It has been shown that supplementation had no influence on the partitioning of Se, Zn, Cu and Mn in between urine and faeces and over 90% of the Zn, Cu and Mn and over 80% of the Se were excreted to faeces (**Table 3.12**). Faeces is therefore an important micronutrient pool. However, not all the micronutrients in faeces was readily available to plants, as demonstrated by the SEP results (**Tables 3.14** to **3.17**). In a SEP, elements that are extracted early are generally recognized as weakly bound to the solid phase, and hence could potentially have greater mobility and environmental impact compared to the later fractions (Bacon & Davidson, 2008). Although the results of a SEP cannot be directly related to the availability to plants, it is logical that the extracts from the early steps are more available to plants than the later ones and are commonly used to estimate elemental bioavailability in soils and sediments (He et al., 2005).

The metallic elements extracted in the first SEP step were considered water-soluble and exchangeable and were therefore potentially bioavailable. Based on this assumption, the Mn (ca. 80% was in the first fraction, **Table 3.17**) and Zn (ca. 40% was in the first fraction, **Table 3.16**) in faeces was mostly available to plants. In contrast, less than 10% of Cu was in the first fraction, and over 70% of the Cu was in the last two fractions of

the BCR SEP extraction (**Table 3.15**). The different chemical form had no significant influence on the fractionation % of Zn, Cu and Mn in the first two steps of extraction. Therefore the supplemental mineral form were unlikely to have a significant impact on the plant-availability of Zn, Cu and Mn in faeces.

The fractionation of Se in different steps was relatively evenly distributed. The combination of the step 1 and step 2 of the SEP of Se can be considered comparable to the first fraction in the BCR extraction for the metallic elements (**Tables 2.3 and 2.4**), together comprising the potentially plant-available fraction of Se. Therefore, ca. 30-40% of the faecal Se was potentially available to plants (**Table 3.14**). Again, the supplemental mineral form showed no significant impact on the fractionation percentage in the first two steps. However, it should be noted that the extraction recovery rates of Se was only ca. 45%, suggesting that the results of Se may not be representative. The loss can be attributed to the fact that at every filtering process in a SEP some solids remained on the filter paper, and for those elements that are easily held onto light particles that are floating and not in the pellet after centrifugation, such as some OM, may have significant loss during this procedure.

Although no significant influence of treatment was found on the fractionation of Zn, Cu, Mn and Se in the first two steps, the supplemental mineral form showed significant influence on Se and Zn concentration in the third step (**Table 3.14, 3.16, respectively**), and of Mn in the last step (**Table 3.17**). Whether or not this difference would significantly influence the uptake of Se, Zn and Mn was investigated in the later pot experiment (**Chapter 4**).

3.4.4.2 Selenium speciation in the 'plant-available' fraction in the faeces

The results showed that there was no Se species difference across the different treatments, and only SeO_3^{2-} was detectable in the extracts (**Figure 3.6**). In case there was proteinaceous Se that was not detectable, a protease was added to the extraction. The results remained similar with and without the addition of protease, and only SeO_3^{2-} was detectable. This implies that the supplemental mineral form had no influence on Se species in the plant-available fraction. This presumption was double checked in the later pot experiment (**Chapter 4**).

3.5 Conclusions

The results of this sheep experiment did not support the hypothesis: different forms of supplemental minerals, offered at typical industrial doses, have significant impact on micronutrient partitioning and excretion in urine and faeces. The results showed that supplemented at typical industrial doses, the chemical form of the mineral supplement (organic versus inorganic) had no significant impact on partitioning of Se, Zn, Cu and Mn between urine and faeces, nor on the total output or concentration of Se, Zn, Cu and Mn in urine or faeces. Over 90% of the Zn, Cu and Mn and over 80% of the Se was excreted via faeces. The supplemental mineral form had no impact on the fractionation percentage of Zn, Cu, Mn and Se in the 'plant-available' fraction in faeces. The dominant chemical species of Se in the 'plant-available' fraction was SeO_3^{2-} and was not influenced by the different supplementary mineral treatments.

Chapter 4

Factors influencing grass uptake of micronutrients from soils applied with excreta of sheep given different forms of supplemental minerals

4.1 Introduction

In grazing pasture systems, the excreta from a grazing animal is an important source of nutrients and can play a critical role in micronutrient cycling in the environment (Kao et al., 2020). Whilst there are studies that have investigated the soil accumulation and the extractability of micronutrients in soil after the application of animal excreta (Abebe et al., 2005; Adeli et al., 2007; Benke et al., 2008; Bomke & Lowe, 1991; Brock et al., 2006; Demir et al., 2010; Gupta & Charles, 1999; Han et al., 2000; Kibet et al., 2013; L'Herroux et al., 1997; Lipoth & Schoenau, 2007; Sheppard & Sanipelli, 2012; Xu et al., 2013; Zhao et al., 2014), research investigating the availability of micronutrients to forage grown in the soil applied with excreta of animal given different supplemental minerals is not available. In the previous chapter, the effect of supplementing different forms of minerals on the excretion and partitioning of micronutrients in urine and faeces was discussed. The different forms of the minerals did not lead to a significant impact on the partitioning of Zn, Cu, Mn and Se between urine and faeces (**Chapter 3.4.1**), nor on the chemical fractionation of Zn, Cu, Mn and Se in the 'plant-available' fraction of faeces (**Chapter 3.4.4**). Other potential factors that might be significant to the micronutrient flux in the system include the application of different types of animal excreta (urine or faeces) and soil properties.

Urine and faeces can go through different processes, such as decomposition and leaching, in pasture systems due to their different chemical and physical natures, which in turn affects micronutrient utilization by forages. However, to date, little is known about how the excreta type (urine or faeces) impacts differently the cycling and fate of micronutrients in grazing pasture systems. Once applied to soil, the excreta go through various biogeochemical reactions before the micronutrients within are able to be taken up by the forage. During this process, the environment of the soil can affect the

biogeochemical reactions enormously (Kao et al., 2020). Factors that have potential impacts on the availability of soil micronutrients to plants include soil pH and Eh, soil OM and oxides, soil microbial activities, element antagonisms and soil fertiliser application (Kao et al., 2020). Among these soil factors, soil OM interacts, chemically or physically, with nearly all soil properties and functions and thus may exert a dominant control on the availability of a micronutrients to plants (**Figure 1.3**). Furthermore, according to the data of North Wyke Farm Platform, OM levels can be variable to a greater extent in soils of the same soil type but under different forage management practices than other soil properties such as soil pH (**Appendix Table B.16**). Therefore, in this chapter soil OM content was included as another potential factor and investigated its influence on the micronutrient flux in the system.

In this chapter, three potential critical factors to flux of micronutrients in the system were proposed: (1) chemical form of the supplemental minerals given to the sheep (2) different excreta type (urine or faeces or urine and faeces) (3) soils of different OM content. The potential influence of the chemical form of the supplemental minerals is still considered because the chemical species of Zn, Cu, Mn and Se in the urine and faeces was not fully determined in **Chapter 3**. The potential difference in chemical species may make a significant difference in micronutrient availability to plants. In housing or in natural pasture, it is unlikely that urine and faeces are excreted completely separately. Therefore, another treatment of urine and faeces mixture is included in this experiment to investigate the interaction effect of urine and faeces on the micronutrient flux.

To investigate the impact of these three factors to the uptake of micronutrients by grass, perennial ryegrass was grown for three cutting cycles in soils of a same soil type were collected from a grassland and a nearby arable. The soils were applied with different

types of sheep excreta (urine or faeces or the combination of urine and faeces) collected from the sheep in Chapter 3 given the organic or inorganic mineral supplements. Samples of grass, soil and leachate were collected for the analysis of total nutrient concentrations in the samples. Soil pH variation was measured and soil wet chemistry analysis was performed to understand the geochemistry alteration in the soils under different treatments.

4.2 Experiment setup

4.2.1 Experimental design

Monoculture perennial ryegrass (*Lolium perenne* cv. Aber Magic), a forage species selected in the recommended list for England and Wales of the Agriculture and Horticulture Development Board (AHDB, 2020), was used in this study. Urine and faeces of sheep given treatments differing in mineral form (IH and OH, **Chapter 3.2**) collected on day 14 of the sheep experiment were applied either separately or in combination to soil collected from an arable land (lower soil OM) or from a grassland (higher soil OM). In total, there were 14 treatment combinations (**Table 4.1**). The experiment followed a Randomized Complete Block Design. There were four blocks, and each block included one replicate of the 14 treatments.

4.2.3 Pot design

Each pot (**Figure 4.1**) was made from a cut PVC water pipe, 13 cm in inner diameter by 22 cm depth. In the middle of the column was a plastic mesh (pore size = 1.5 mm x 1.5 mm) set to separate the two layers of soils but allow the natural flow of the irrigation water. At the bottom of the pot another plastic mesh with the same pore size was set to prevent soil loss but allow leachates to pass through. Underneath the soil column was an acrylic plate with holes in to hold the soil but allow the leachate to filter through to

the collecting container below. A Rhizon soil solution sampler (pore size 0.15 μm, length 10 cm, diameter 2.5 mm, with stainless steel strengthening wire and 10 cm PVC-tube; Rhizosphere Research Products®, Netherlands) was placed diagonally in the top-layer of soil to collect soil solution for pH measurement.

Table 4.1 Experimental treatment codes

Soils	Sheep excreta type		Treatment code
Soil from arable land (**A**)	Control check (**CK**): no excreta applied		**A-CK**
	Application of faeces (**F**)	Excreta of sheep of IH treatment (**I**)	**A-F-I**
		Excreta of sheep of OH treatment (**O**)	**A-F-O**
	Application of urine (**U**)	Excreta of sheep of IH treatment (**I**)	**A-U-I**
		Excreta of sheep of OH treatment (**O**)	**A-U-O**
	Application of mixed urine + faeces (**UF**)	Excreta of sheep of IH treatment (**I**)	**A-UF-I**
		Excreta of sheep of OH treatment (**O**)	**A-UF-O**
Soil from grassland (**G**)	**CK**: no manure applied		**G-CK**
	Application of faeces (**F**)	Excreta of sheep of IH treatment (**I**)	**G-F-I**
		Excreta of sheep of OH treatment (**O**)	**G-F-O**
	Application of urine (**U**)	Excreta of sheep of IH treatment (**I**)	**G-U-I**
		Excreta of sheep of OH treatment (**O**)	**G-U-O**
	Application of mixed urine + faeces (**UF**)	Excreta of sheep of IH treatment (**I**)	**G-UF-I**
		Excreta of sheep of OH treatment (**O**)	**G-UF-O**

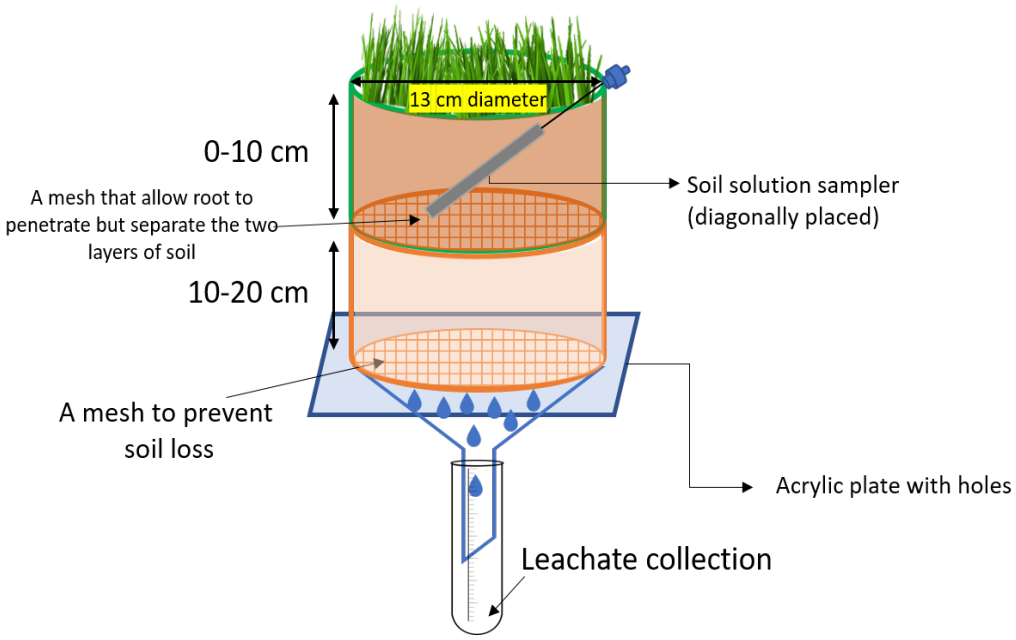


Figure 4.1 The pot used in this study was composed of a soil column with two layers and a leachate collection apparatus.

4.2.2 Experimental soil

The soils used in this study were collected from an arable land (Great Harpenden) and the nearby grassland (Weighbridge Piece) at Rothamsted Research, Harpenden, UK (51.81°N, 0.35°W). The soils from the two fields were Batcombe Series (Clayden & Hollis, 1984) but had significant differences in soil total carbon content (**Table 4.2**). A preliminary test showed that the inorganic carbon content in the arable soil and the grassland soil were ca. 0.01 % and 0.02 %, respectively. Therefore the presented total carbon contents presented were close to total organic carbon contents. The soils of Great Harpenden and of Weighbridge were sampled to 23 cm and 10 cm depth, respectively, to magnify the difference in soil OM content. The soil properties of each soil are shown in **Table 4.2**.

Table 4.2 Soil properties of the experimental soils

Soil properties		Great Harpenden (A)	Weighbridge Piece (G)
Sampling depth		to 23 cm depth	to 10 cm depth
Soil texture	Sand (2.00-0.063 mm)	22%	19%
	Silt (0.063-0.002 mm)	49%	54%
	Clay (<0.002 mm)	29%	27%
	Textural class	Clay loam	Silt clay loam
Active oxides (mg kg^{-1})	Al	1099 ± 8.9	1087 ± 6.7
	Fe	4528 ± 56.6	8200 ± 48.7
	Mn	1506 ± 25.9	1436 ± 23.0
	P	360 ± 7.4	1003 ± 8.7
Bulk density in field (g cm^{-3})		1.4 ± 0.01	1.0 ± 0.06
Soil pH		6.38 ± 0.012	6.31 ± 0.016
Total carbon (%)*		1.56 ± 0.039	3.56 ± 0.024
Total nitrogen (%)		0.15 ± 0.006	0.32 ± 0.005
2M KCl extractible N (mg-N/kg-DM soil)		8.7 ± 0.75	15.9 ± 2.82
Total P (g kg^{-1})		0.81 ± 0.013	1.67 ± 0.058
Total Fe (g kg^{-1})		33.6 ± 0.61	27.3 ± 0.89
Total Mn (g kg^{-1})		1.79 ± 0.060	1.61 ± 0.058
Total Cu (mg kg^{-1})		17.7 ± 0.22	24.1 ± 0.93
Total Zn (mg kg^{-1})		72.1 ± 2.22	101 ± 3.4
Total Se (μg kg^{-1})		782 ± 14.0	865 ± 26.6

*A preliminary test showed that the arable soil and the grassland soil only contained ca. 0.01% and 0.02% inorganic carbon, respectively, which were little in the total carbon content in the soils. Therefore, the presented total carbon contents are close to the content of total organic carbon.

4.2.3 Preparations of soil, the applied sheep excreta and irrigation water

4.2.3.1 Preparation of soil

The collected soils were air-dried in a greenhouse and sieved through a 2 mm stainless-steel mesh. There were two soil layers in a pot, and each layer contained 1.40 kg air-dried soil (bulk density of 0.90 g cm^{-3}). The total DM input of soil is shown in **Table 4.3**. It should be noted that through the procedure of air-drying, the population of microorganism might change. For example, some earthworms in the fresh soils were

filtered through the soil preparation process and, therefore, the total population of earthworms in the experiment soils was lower than that in the fresh soils.

4.2.3.2 Preparation of sheep excreta

Due to the nature of the study, there were no rain drops and less microfaunal activity in the pots than in the field, which would normally help decompose the applied sheep excreta. Therefore, the faeces in this study was crumbled and integrated into the top layer of soil instead of being laid on the soil surface, whereas the urine was applied by spreading on the soil surface on day 0. The fresh faeces (DM%; **Table 4.3**) was crumbled into smaller pieces by passing the faeces through a stainless-steel mesh (hole size = 11 mm). In total, 100 grams of the crumbled moist faeces and 70 mL of urine was applied to each of the allocated treatment pots. The application amount of faeces and urine was determined following a preliminary study (**Appendix A.6**). Due to the high moisture content of the faeces, 100 g moist faeces was applied to make sure that the total DM of faeces was more than the pre-determined application amount (15 g-DM of faeces) (**Table 4.3**).

4.2.3.3 Preparation of irrigation water

The formulation of ARW was based on a mean value of element contents from monthly rainwater samples collected over a ten-year period at Rothamsted Research's North Wyke site (Darch et al., 2019). To make 1 L of a stock solution (1000x), which was used to make 1000 L of artificial rainwater, salts (**Table 4.4**) were added to a 1 L volumetric flask and made up to 1 L with Milli-Q water.

Table 4.3 Total input of soil, faeces and urine and the input of Cu, Zn, Mn and Se from the excreta

Treatments	Soil moisture* content	Soil weight (kg-DM pot^{-1})	Faeces moisture* content	Faeces input (g-DM pot^{-1})	Urine input (mL pot^{-1})	Cu from the excreta† (mg)	Zn from the excreta† (mg)	Mn from the excreta† (mg)	Se from the excreta† (μg)
A-CK			-	-	-	-	-	-	-
A-F-I			78%	22	-	1.049	7.551	8.580	12.70
A-F-O			74%	26	-	1.240	8.579	11.07	15.75
A-U-I	4%	2.68	-	-	70	0.003	0.383	0.008	2.087
A-U-O			-	-	70	0.003	0.376	0.008	1.553
A-UF-I			78%	22	70	1.052	7.934	8.588	14.77
A-UF-O			74%	26	70	1.243	8.955	11.08	17.31
G-CK			-	-	-	-	-	-	-
G-F-I			78%	22	-	1.049	7.551	8.580	12.70
G-F-O			74%	26	-	1.240	8.579	11.07	15.75
G-U-I	6%	2.64	-	-	70	0.003	0.383	0.008	2.087
G-U-O			-	-	70	0.003	0.376	0.008	1.553
G-UF-I			78%	22	70	1.052	7.934	8.588	14.77
G-UF-O			74%	26	70	1.243	8.955	11.08	17.31

*The moisture content of soil and faeces was measured on the day of filling the pot. The values presented was the average of the values of three replicates. The variation of moisture across replicates was less than 1%. †The input of Cu, Zn, Mn and Se were calculated from the concentrations of Day 14 faeces and urine of sheep given OH and IH treatment (data is in Appendix Table B.7 and Table B.8).

Table 4.4 The components of 1 L stock solution used for making artificial rainwater

Salts	Mass (g)
NH_4Cl	1.385
K_2SO_4	1.235
$HNa_2PO_4.2H_2O$	0.016
$FeCl_2.4H_2O$	0.089
NaCl	5.845
CaCl2.2H2O	4.563
NH_4NO_3	0.560
$MgCl_2.6H_2O$	4.057
$(NH_4)_2SO_4$	0.319

4.2.4 Experiment environment

The experiment was carried out in a temperature-controlled room maintained at 20°C during the day and at 16°C at night. An artificial LED light source gave 16 hours of light a day with light illuminance: 330-570 lux (measured from the soil surface using Digital Lux Meter (LX1330B, Dr.meter®)), which mimics the light intensity of direct sunlight.

4.2.5 Experiment timeline and pot management

After the soils (some with faeces incorporated in the upper soil layer) were packed in the pots, 0.5 g seeds of perennial ryegrass was randomly scattered at 1 cm depth from soil surface of each pot. Afterwards, the soils were moistened to water holding capacity (WHC) by immersing the pots in a pool of artificial rainwater (ARW) and allowing the water to be taken up from the bottom of the pot by capillary force.

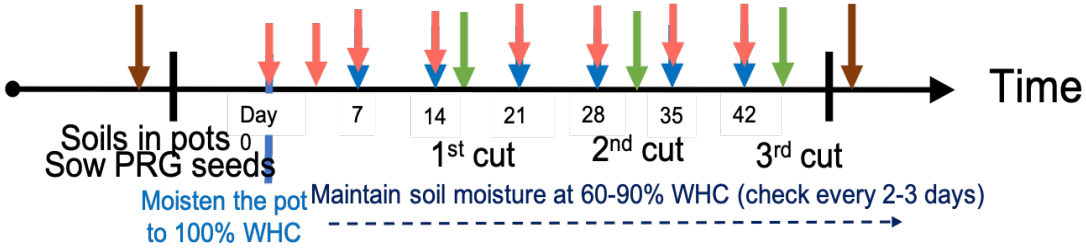


Figure 4.2 Timings for setting up, irrgating and sampling in the pot experiment. The arrows in the colours brown, pink, blue and grass indicate the timings of sampling soil, sampling soil solution, applying the heavy rain event, and harverting the grass, respectively.

On day 0, the pots were removed from the water pool and then placed on the leachate collector. Afterward, the urine was applied to the designated pots at the soil surface. The soil moistures were maintained in the range of 60-90% WHC by weighing the pots and irrigating with ARW every 2-3 d. The 60-90% WHC was the range of moisture that prevents the surface and edge cracking of the soils and does not restrict grass growth. A 'heavy irrigation' was carried out every 7 d (blue arrows in **Figure 4.2**) by irrigating a volume of 300 mL ARW to each pot in a single day to imitate a heavy rain event (equal to 23 mm precipitation d^{-1}). All the leachate coming out from the pot was collected and the total amount measured. The grass was harvested at the 3.0-4.0 age of completely developed leaves (about 2 weeks from sowing the seeds, depending on the growth). The 3.0-4.0 leaf age of perennial ryegrass is recommended by Agriculture and Horticulture Development Board of UK as the best time to graze (AHDB, 2019). In total, three cuts of grass were carried out during the experiment and the time between each harvest was 2 weeks.

4.2.6 Methods of sampling and sample storage

4.2.6.1 Leachate

Any leachate draining from the pot was collected in the tube at the bottom of the pot. The collected leachate was removed from the collection apparatus and stored at -18°C

within 24 h. The total volume of leachate per pot collected on each day was recorded by weight before the sample was stored. Samples collected within two weeks were bulked as one analyte unit to reduce the total number of samples for analysis of total nutrient content (**Chapter 2.1**).

4.2.6.2 Soil solution

A soil solution sample was taken using an embedded soil solution sampler at two hours after a heavy irrigation event (**Figure 4.2**). To sample, the protective cap of the sampler was replaced by a blunt fill needle (1.2 mm x 40 mm, with 5 μm filter, BD®). The needle was then jabbed into a 10 mL vacutainer tube. The negative pressure of the vacuum tube allows the soil solution to be sorbed from soil into the sample tube. Approximately 2 mL soil solution was sampled each time, and normally it took less than 1 h to collect (usually a couple of minutes at soil moisture content greater than 80% WHC). For those that took more than 1 h, a 25 mL syringe was used instead of the vacutainer tube to collect the sample. The pH of the collected samples were measured within 24 h (**Chapter 2.8**).

4.2.6.3 Soil

The treated soils (with or without faeces) collected before the experiment were divided into two parts. One part was kept at -20°C for later analysis, and the other part was packed into the pot. After the experiment finished, the soil taken out from a pot was cut in half vertically using a stainless-steel knife. One half of the cut soil column was stored in -20°C for later use. The soils stored before the experiment and the other half of the soil collected after the experiment were air dried for 1 to 2 months, depending on the moisture content of the soils at the start of drying. Afterwards, samples were prepared and sent for total nutrient content analysis, and TN + TC analysis (**Chapters 2.1** and **2.5**, respectively).

4.2.6.4 Grass

The grass was cut at 2 cm above the soil surface using scissors with stainless-steel blades. This cutting height was chosen because it gave enough grass DM for analysis, allowed the grass to grow after the cutting event, and prevented the grass sample being contaminated by soil when cutting too close to the soil surface. Although the first cut was performed on different days across treatments due to the varying growth rates of different treatments, the time gap between each cut was always 2 weeks. The cut grass was stored in paper bags and the fresh weight measured. These samples were stored at -20°C before freeze drying. Grass samples were also prepared and total nutrient content and TN determined (**Chapters 2.1** and **2.5**, respectively).

4.2.7 Calculations

The micronutrient input contributed from soil and excreta and from irrigation water was calculated using **Equation 4.1** and **Equation 4.2**, respectively. In **Equation 4.1**, the total DM does not include the DM contributed from urine application. The DM input from 70 mL urine of OH and IH treatments was ca. 0.96 ± 0.005 g and 1.28 ± 0.020 g, respectively, which was negligible compared to the DM input from soil and faeces (**Table 4.3**).

$$In_{s+E} = C_{S+E} \times W_{S+E} \quad \textbf{(4.1)}$$

In **Equation 4.1**, In_{S+E}=total input of the element from soil and excreta, C_{S+E}= the concentration of the element in the mixture of soil and excreta (mg kg^{-1}), W_{S+E}= total weight in dry matter of the mixture of soil and excreta in each pot.

$$In_w = C_w \times V_w \quad \textbf{(4.2)}$$

In **Equation 4.2**, In_W= total input of the element from the irrigation water, C_w= the concentration of the element in the irrigation water, V_W= total volume of the irrigation water given to each pot.

To account for time effects, the total accumulations of an element in grass during the three cutting times were summed (**Equation 4.3**).

$$Total\ uptake\ of\ an\ element\ across\ the\ three\ cuts = \frac{C1 \times W1 + C2 \times W2 + C3 \times W3}{(W1 + W2 + W3)} \qquad \textbf{(4.3)}$$

In **Equation 4.3**, C1 = the concentration of an element of the first cut, C2 = the concentration of an element of the second cut, C3 = the concentration of an element of the third cut, W1 = the weight of grass DM of the first cut, W2 = The weight of grass DM of the second cut, W3 = the weight of grass DM of the third cut.

4.2.8 Statistical analysis

A factorial ANOVA model (y~ Block + Excreta type (ET) + Form of supplemental mineral (Form) + Soil + Interactions (ET x Form + ET x Soil + Form x Soil + ET x Form x Soil) was performed to test the influences of the three main factors and their interaction on the response variables including grass DM, nutrient uptake by forage, nutrient leaching, and the pH of soil solution at different sampling time. A principal component analysis (PCA) was performed to analyse the difference of grass harvested at different cutting cycles based on the nutrient components of the grass. Under the circumstances that different cutting cycles can be a significant factor to the nutrient uptake by grass, a modified ANOVA model: 'y~ Time + ET + Form + Soil + ET x Form + ET x Soil + Form x Soil + ET x Form x Soil' was performed to include the potential effect of time. This modified statistical model was used based on the assumption that time effect has no significant interaction with the treatment factors. If significant differences ($P<0.05$) were identified, post hoc comparisons of Fisher's LSD

(α=0.05) were performed. All the statistical analyses were performed in R software (R Core Team, 2018).

4.3 Results

4.3.1 Micronutrient total input and output in the system

There are three sources of the micronutrients in the system: soil, excreta, and irrigation water. The faeces provided 1.0-1.2 mg Cu, 7.6-8.6 mg Zn, 8.6-11 mg Mn, and 13-16 μg Se each pot (**Table 4.3**). The urine provided ca. 0.003 mg Cu, 0.38 mg Zn, 0.008 mg Mn, and 1.6-2.1 μg Se each pot (**Table 4.3**). The untreated arable soil provided 23.7 mg Cu, 97 mg Zn, 2397 mg Mn and 1047 μg Se per layer of soil (**Table 4.5**). The untreated grassland soil provided 31.8 mg Cu, 133 mg Zn, 2021 mg Mn, and 1142 μg Se per layer of soil (**Table 4.5**). Irrigation water was another major input source. The total irrigation volume varied with treatments (**Figure 4.3**), which was due to the variation of the soil moisture content. In order to maintain the soil moisture within the range of 60%-90% WHC, the higher the loss of soil moisture, the more irrigation was given. For pots that had urine applied (with or without faeces), more ARW was given compared to the controls to keep the pots at the required range of WHC. When faeces alone was applied, the ARW requirement was not different from the control treatments. The micronutrient input from the irrigation water depended on the total volume of ARW given. There were about 25-40 μg pot^{-1} of Cu and 76-122 μg pot^{-1} of Zn input from irrigation water across all treatments. The input of Mn and Se from the irrigation water was less than 1 μg pot^{-1} and 0.1 μg pot^{-1}, respectively. The results show that the soil was the dominant source of Cu, Zn, Mn and Se in the system. In the excreta, faeces was the dominant source of the Cu, Zn, Mn and Se compared to urine.

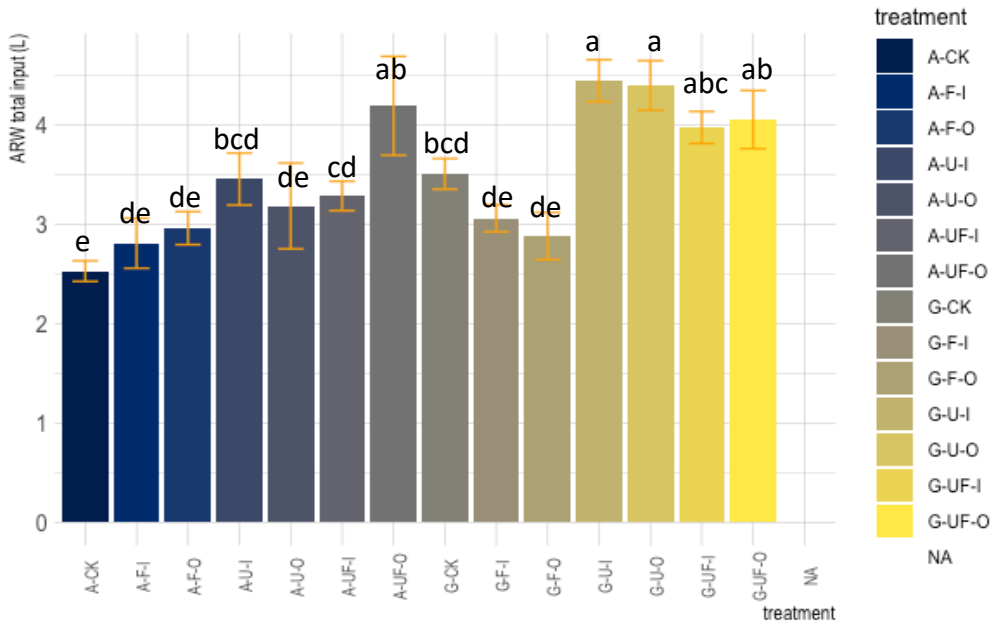


Figure 4.3 Total irrigation water input (L) across treatments during the experiment. The error bars represent the standard error of the result (n=4) and the letters represent the results of Fisher's LSD test across treatments after a significant result of ANOVA.

The output pathways of micronutrients considered in this study were leaching and uptake by grass. The results showed that the total amount of Cu, Zn, Mn and Se in leachate or being removed by plants were all below 1% of their total input (**Table 4.5**). That is, over 99% of the Cu, Zn, Mn and Se input was retained in the soil or in the roots of the grass. Therefore, the movement of elements through the different layers of soil was limited, hence, no separate data for the elements in the two different layers are presented. Depending on different treatments, the grass removed 21.7-94.3 μg Cu, 68.4-360 μg Zn, 357-825 μg Mn, and 0.07-0.24 μg Se from each pot, and there were 1.09-9.10 μg Cu, 2.45-19.1 μg Zn, 3.06-43.2 μg Mn, 0.04-0.14 μg Se leached from each pot. The dominant output pathway for Cu, Zn and Mn was uptake by grass. However, the outputs of Se due to grass uptake and leachate were similar. Different treatments had no significant impact on the amount of Se in leachate, whereas there was significant

difference across treatments in the loss of Zn, Cu and Mn in the leachate (**Table 4.7**). The effect of the treatments was element-dependent. For Cu, the interaction between form and soil was significant. For Zn, the independent effects of excreta type and soil were both significant. The loss of Zn was greater with the urine treatments (with or without faeces) than that with the faeces-only treatment and was greater in the grassland soil than in the arable soil (**Table 4.6**). For Mn, only the excreta type had a significant impact on Mn loss in leachate. The loss of Mn was greater with the urine treatments (with or without faeces) than that with the faeces-only treatment (**Table 4.6**).

4.3.2 Grass growth under the different treatments

Type of excreta applied (urine or faeces) was a significant factor ($P<0.001$) in growth of the first cut of grass, and, for both the second and third cuts, the interaction of soil (from arable land or grassland) and the excreta type was significant ($P<0.001$) (**Table 4.8**). The treatments G-U-I, G-U-O, G-UF-I and G-UF-O had the highest total grass DM (**Table 4.8**). Interestingly, for all the treatments with the addition of urine, the DM of grass was highest at the second cut and then dropped by ca. 40-50% at the third cut. For the control treatments (soil without the addition of urine or faeces), the grass DM was highest at the first cut. For the treatments that only received faeces, the grass DM was highest at the first and second cut for arable soil and grassland soil, respectively.

4.3.3 Total nutrient uptake and concentration in grass

The effect on micronutrient concentration and total uptake in grass of the three treatment factors (soil, excreta type and supplemental mineral form) varied across the three cuts (**Tables B.9, B.10, B.11, B.12, B.13 and B.14**). Grass cut number had a significant influence on both the total uptake and the concentration of all the studied elements in grass (**Table 4.9 and 4.10**; **Figure 4.4**). In terms of total element uptake

Table 4.5 Nutrient input from the soil and the irrigation water.

Treatment	Cu		Zn		Mn		Se	
	Input from 0-10 cm soil* (mg ± SE)	Input from irrigation (μg ± SE)	Input from 0-10 cm soil* (mg ± SE)	Input from irrigation (μg ± SE)	Input from 0-10 cm soil* (mg ± SE)	Input from irrigation (μg ± SE)	Input from 0-10 cm soil* (μg ± SE)	Input from irrigation (μg± SE)
A-CK	23.7±0.30^{de}	25.4±1.05^{e}	96.6±3.0^{e}	76.0±3.13^{e}	2397±80.4^{a}	0.61±0.050^{e}	1047±18.8^{cd}	0.03±0.001^{e}
A-F-I	24.4±0.22^{d}	28.2±2.53^{de}	98.9±1.8^{de}	84.4±7.56^{de}	2146±94.3^{bcd}	0.68±0.122^{de}	990±9.0^{de}	0.03±0.003^{de}
A-F-O	24.6±0.19^{d}	29.7±1.67^{de}	103±0.8^{d}	89.0±4.98^{de}	2333±59.4^{ab}	0.72±0.080^{de}	1003±10.6^{d}	0.03±0.002^{de}
A-U-I	24.0±0.51^{de}	34.7±2.63^{bcd}	96.9±1.8^{e}	104±7.9^{bcd}	2167±55.9^{bcd}	0.84±0.127^{bcd}	1088±54.2^{bc}	0.04±0.003^{bcd}
A-U-O	22.9±0.11^{e}	32.0±4.34^{de}	94.0±2.3^{e}	95.7±12.99^{de}	2199±51.3^{abcd}	0.77±0.209^{de}	994±7.1^{d}	0.04±0.005^{de}
A-UF-I	23.4±0.23^{de}	33.0±1.49^{cd}	98.7±1.0^{de}	98.7±4.44^{cd}	2074±94.3^{cde}	0.80±0.072^{cd}	930±32.9^{e}	0.04±0.002^{cd}
A-UF-O	23.6±0.23^{de}	42.1±5.00^{ab}	97.0±0.5^{e}	126±15.0^{ab}	2264±85.6^{abc}	1.01±0.241^{ab}	927±3.2^{e}	0.06±0.006^{ab}
G-CK	31.8±1.23^{a}	35.2±1.55^{bcd}	133±4.5^{a}	105±4.6b^{cd}	2120±76.7^{bcd}	0.85±0.075^{bcd}	1142±35.0^{a}	0.04±0.002^{bcd}
G-F-I	31.1±0.30^{ab}	30.7±1.37^{de}	131±1.5^{ab}	91.9±4.11^{de}	2001±64.2^{de}	0.74±0.066^{de}	1109±13.5^{ab}	0.03±0.002^{de}
G-F-O	30.9±0.12^{abc}	28.9±2.39^{de}	134±1.3^{a}	86.6±7.15^{de}	2104±106.4^{bcd}	0.70±0.115^{de}	1100±11.9^{ab}	0.03±0.003^{de}
G-U-I	29.8±0.33^{c}	44.7±2.12^{a}	123±2.4^{c}	134±6.48^{a}	2012±124.9^{cde}	1.08±0.102^{a}	1100±17.5^{ab}	0.05±0.05^{a}
G-U-O	30.0±0.47^{bc}	44.2±2.51^{a}	126±1.6^{bc}	132±7.5^{a}	2092±55.7^{bcd}	1.06±0.121^{a}	1101±15.5^{ab}	0.05±0.003^{a}
G-UF-I	30.2±0.28^{bc}	39.9±1.62^{abc}	125±1.4^{bc}	119±4.9^{abc}	1681±43.1^{f}	0.96±0.078^{abc}	1055±7.9^{bc}	0.04±0.002^{bc}
G-UF-O	31.0±0.37^{abc}	40.7±2.94^{ab}	130±0.5^{ab}	122±8.8^{ab}	1874±17.2^{ef}	0.98±0.142^{ab}	1064±10.5^{bc}	0.05±0.003^{ab}

* The data are calculated by multiplying the micronutrient concentrations in the soil and excreta mixture (0-10 cm soil) measured before the experiment by the DM input of soil and faeces. The lower-layer soil (10-20 cm) was assumed to be as the same as the result of the upper-layer soil (0-10 cm soil) with no excreta added. Superscript letters indicate the results of Fisher's LSD test across treatments within one column.

Table 4.6 Nutrient output via grass and leachate.

Treatment	Cu (μg ± SE)*		Zn (μg ± SE)*		Mn (μg ± SE)*		Se (μg ± SE)*	
	Removed by grass	Loss in leachate	Removed by grass	Loss in leachate	Removed by grass	Loss in leachate	Removed by grass	Loss in leachate
A-CK	21.7±0.96^{f}	1.09±0.293^{c}	68.4±5.48^{f}	2.45±0.700^{e}	357±40.0^{e}	3.06±2.686^{e}	0.19±0.012^{abcd}	0.04±0.009
A-F-I	36.1±1.93^{de}	3.53±0.816^{bc}	109±6.8^{f}	4.98±0.840^{bcde}	470±137.0^{de}	9.85±6.782^{de}	0.19±0.026^{abcd}	0.11±0.029
A-F-O	31.8±1.30^{ef}	1.77±0.553^{bc}	100±4.0^{f}	2.96±1.046^{de}	412±105.5^{e}	10.1±13.35^{de}	0.18±0.043^{abcd}	0.06±0.023
A-U-I	38.2±3.35^{c}	1.74±0.556^{bc}	160±6.3d^{e}	7.65±1.666^{bcde}	731±77.0^{abc}	43.2±25.65^{ab}	0.19±0.023^{abcd}	0.09±0.021
A-U-O	47.7±2.00^{c}	2.41±1.723^{bc}	155±9.4^{e}	11.4±4.70^{abcd}	635±113.4^{bc}	20.6±19.43^{abcde}	0.24±0.075^{a}	0.06±0.027
A-UF-I	53.5±3.39^{c}	9.10±3.730^{a}	177±12.3^{de}	12.9±4.84^{ab}	723±89.9^{abc}	46.0±40.35^{a}	0.21±0.018^{ab}	0.13±0.060
A-UF-O	52.8±4.00^{c}	1.28±0.257^{bc}	178±17.7^{de}	4.09±1.454^{cde}	588±88.6^{cd}	33.5±31.16^{abcd}	0.20±0.016^{abc}	0.09±0.036
G-CK	49.3±4.40^{c}	4.98±0.172^{b}	202±21.0^{d}	13.2±1.13^{ab}	612±100.3^{bc}	12.3±11.10^{cde}	0.11±0.010^{cde}	0.14±0.008
G-F-I	46.2±5.82^{cd}	3.35±1.098^{bc}	175±23.4^{de}	6.48±1.572^{bcde}	438±73.0^{e}	7.96±8.822^{de}	0.09±0.017^{de}	0.09±0.028
G-F-O	48.4±3.87^{c}	3.85±1.322^{bc}	192±19.2^{de}	7.88±2.158^{bcde}	360±55.4^{e}	6.64±2.174^{e}	0.07±0.010^{e}	0.10±0.032
G-U-I	77.6±3.36^{b}	4.03±0.988^{bc}	291±15.5^{c}	19.1±4.25^{a}	683±78.8^{abc}	39.1±20.03^{abc}	0.12±0.006^{bcde}	0.09±0.012
G-U-O	93.5±3.83^{a}	4.02±1.649^{bc}	337±14.4^{ab}	18.4±6.52^{a}	825±89.8^{a}	28.8±13.84^{abcde}	0.20±0.038^{abc}	0.12±0.048
G-UF-I	81.1±4.98^{b}	1.64±0.327^{bc}	313±19.5^{bc}	9.45±1.606^{bcde}	677±139.5^{bc}	14.7±2.49^{cde}	0.12±0.008^{bcde}	0.05±0.011
G-UF-O	94.3±3.93^{a}	3.01±0.654^{bc}	360±18.2^{a}	12.2±3.70^{abc}	733±94.5^{ab}	17.8±12.57^{bcde}	0.23±0.059^{a}	0.08±0.019

* Superscript letters indicate the results of Fisher's LSD test across treatments within one column.

Table 4.7 Results of ANOVA analysis on the total loss of an element in leachate

Factors	Elements†								
	Cd (µg)	Cu (µg)	Fe (mg)	Mn (mg)	Mo (µg)	P (mg)	S (mg)	Se (µg)	Zn (µg)
Excreta type (ET)	**<0.001 *** **	0.7581	0.1378	**0.0038 ** **	0.4684	0.3180	**0.0109 * **	0.9933	**0.0023 ** **
Supplemental mineral form (Form)	0.1459	0.1740	0.8416	0.2177	0.3054	0.3215	0.1394	0.6453	0.7390
Soil	0.1177	0.9881	**0.0037 ** **	0.1716	0.0814	**0.0247 * **	**0.0467 * **	0.9427	**0.0106 * **
ET x Form	0.4847	0.2221	0.7256	0.5128	0.7282	0.6112	0.5205	0.9189	0.6110
ET x Soil	0.0739	0.0629	0.1325	0.1731	0.4511	0.5019	0.7195	0.2458	0.2594
Form x Soil	0.2170	**0.0415 * **	0.4783	0.4538	0.0822	**0.0139 * **	0.0582	0.0891	0.3465
ET x Form x Soil	0.3516	0.0638	0.8852	0.8151	0.4260	0.9652	0.2399	0.9325	0.2166

†Symbols '*', '**', '***' indicate statistical significances of the ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

Table 4. 8 Grass dry matter of different cutting times

Treatments	First cut	Second cut	Third cut	Total DM
	(g pot^{-1} ± SE) †			
A-CK	2.12 ± 0.270^{bcde}	1.33 ± 0.135^{e}	0.64 ± 0.092^{e}	4.09 ± 0.164^{e}
A-F-I	2.73 ± 0.470^{abc}	2.07 ± 0.096^{de}	1.41 ± 0.025^{cde}	6.21 ± 0.550^{cd}
A-F-O	2.71 ± 0.352^{abc}	1.85 ± 0.182^{e}	1.28 ± 0.090^{de}	5.84 ± 0.242^{d}
A-U-I	0.95 ± 0.298^{f}	4.15 ± 0.309^{b}	2.16 ± 0.274^{c}	7.26 ± 0.445^{bcd}
A-U-O	1.20 ± 0.388^{ef}	4.04 ± 0.301^{bc}	1.93 ± 0.267^{cd}	7.17 ± 0.230^{bcd}
A-UF-I	1.24 ± 0.063^{ef}	4.09 ± 0.258^{b}	2.19 ± 0.126^{c}	7.52 ± 0.353^{bc}
A-UF-O	1.70 ± 0.466^{cdef}	4.07 ± 0.209^{b}	1.87 ± 0.126^{cd}	7.64 ± 0.545^{bc}
G-CK	3.74 ± 0.381^{a}	3.03 ± 0.282^{cd}	1.68 ± 0.111^{cd}	8.45 ± 0.670^{b}
G-F-I	2.87 ± 0.664^{ab}	3.46 ± 0.766^{bc}	1.73 ± 0.198^{cd}	8.06 ± 0.971^{b}
G-F-O	2.37 ± 0.138^{abc}	3.52 ± 0.256^{bc}	1.38 ± 0.287^{cde}	7.28 ± 0.556$_{bcd}$
G-U-I	1.80 ± 0.114^{cdef}	6.75 ± 0.432^{a}	3.65 ± 0.414^{b}	12.2 ± 0.37$_{a}$
G-U-O	1.92 ± 0.358^{bcdef}	7.53 ± 0.384^{a}	3.94 ± 0.540^{ab}	13.4 ± 0.24^{a}
G-UF-I	1.44 ± 0.141^{def}	6.77 ± 0.480^{a}	4.53 ± 0.544^{c}	12.7 ± 0.62^{a}
G-UF-O	1.47 ± 0.384^{def}	7.17 ± 0.408^{a}	4.46 ± 0.491^{ab}	13.1 ± 0.79^{a}
	P level			
Excreta type (ET)	**<0.001*** **	**<0.001*** **	**<0.001*** **	**<0.001*** **
Supplemental mineral form (Form)	0.7814	0.4929	0.4556	0.8010
Soil	0.3020	**<0.001*** **	**<0.001*** **	**<0.001*** **
ET x Form	0.5708	0.7296	0.7983	0.2443
ET x Soil	0.1818	**0.0112***	**<0.001*** **	**<0.001*** **
Form x Soil	0.4204	0.2214	0.6106	0.5116
ET x Form x Soil	0.9352	0.8260	0.7022	0.4613

†Superscript letters indicate the results of Fisher's LSD test across treatments within one column. Symbols '*'and '***' indicate statistical significances of the ANOVA test at p-value<0.05 and <0.001, respectively.

(**Figure 4.4a**), the interaction of excreta type and soil were the dominant factors for Cd, Cu, Fe, P, S, Zn. The excreta type and soil were both major factors with no interaction for Mo and Se. For Mn, the effect of excreta type and the interaction between mineral form and soil were significant. On the other hand, in terms of element concentration in

grass (**Figure 4.4b**), the interaction of excreta type and soil were dominant factors for Cd, Fe, Mn, Mo, P, S, Zn. For Mn and Se, the excreta type and soil were both major factors with no interaction. For Cu, the effect of excreta type, soil and the interaction between mineral form to animal and soil were significant. Although, treatment effects presented in **Figures 4.4a** and **4.4b** are not exactly the same, they both show a clear pattern: the soil and the excreta type were major factors, either it was the independent effect of soil or excreta type or their interaction, with only a few exceptions, i.e. the total uptake of Mn and the concentration of Cu were influenced by the interaction of soil and the form of supplemental minerals. The excreta collected from sheep fed with different forms of supplemental mineral had a subtle independent effect compared to the influence of different soil and excreta type.

Cutting time was a significant influential factor when considering either the total uptake of nutrients or the concentration of nutrients in grass (**Tables 4.9** and **4.10**). The PCA analysis showed an effect of cutting time on both the total uptake of nutrients and the nutrient concentrations in grass (**Figure 4.5**). The grass of the second and the third cuts were of the same component group in the PCA analysis according to both the total uptake of nutrients and the nutrient concentration in grass. Conversely, grass from the first cut differed from that of the third as shown by different component groups.

The concentrations of micronutrients in grass varied with time. The requirement level for a growing lamb of micronutrients in grass varied with the body weight of sheep (**Appendix Table B.17**). The concentrations of Cu in grass grown in the untreated and treated soils were all above the Cu requirement level for a 70 kg growing lamb (3.92 mg-Cu kg-DM^{-1}) across the three cutting times. However, only the grass of the first cut grown in the grassland soil or grown in the arable soil with urine application (with or without faeces application) had Cu concentrations higher than the Cu requirement level

for a growing lamb that requires a higher level of Cu (6.10 mg-Cu kg-DM^{-1} for a 30 kg growing lamb). The application of urine raised the Cu concentrations in grass to a greater degree than the application of faeces. The impact of the supplemental mineral form on the Cu concentration level in grass was not as significant as the impact of cutting time, excreta treatment and soil. Although the treatments of G-U-O and G-UF-O raised the Cu concentrations in grass of the second cut, 6.49 and 7.19 mg-Cu kg-DM^{-1}, respectively, up to the level higher than the Cu requirement level of 6.10 mg-Cu kg-DM^{-1} yet the Cu concentrations in the second cut of grass of G-U-I and G-UF-I, 5.45 and 6.02 mg-Cu kg-DM^{-1}, respectively, were below the Cu requirement level. In contrast, the effect of mineral form in the arable soil was the opposite from the effect in the grassland soil. The treatments of A-U-I and A-UF-I had Cu concentrations higher than the requirement level, 6.35 and 6.17 mg-Cu kg-DM^{-1}, respectively, whereas the Cu concentrations of the treatments of A-U-O and A-UF-O were 5.84 and 5.87 mg-Cu kg-DM^{-1}, respectively.

For Zn, the grass of the first cut had Zn concentrations higher than the Zn requirement level for a 20 kg growing lamb (20.63 mg-Zn kg-DM^{-1}). However, only the grass of the first cut grown in the grassland with urine application had Zn concentrations higher than the level for a growing lamb that requires a higher level of Zn (38.93 mg-Zn kg-DM^{-1} for a 60 kg growing lamb). The grass of the second and third cuts all had Zn concentrations below the recommended level for a growing lamb, except for G-U-O, G-UF-O and G-UF-I. Similar to the results of Cu concentrations, the urine application raised the Zn concentrations in grass to a greater degree than the faeces application. The impact of the supplemental mineral form on the Zn concentrations in grass was not as significant as the impact on soil and excreta.

The concentrations of Mn in grass were all higher than the requirement levels for growing lambs of different body weights (15.05-22.86 mg-Mn kg-DM^{-1}) across the three cuts (**Figure 4.8**). The concentrations of Mn in grass at the third cut was higher than the previous two cuts. At the second and the third cuts, the concentrations of grass grown in the arable soil were higher than those grown in the grassland soil. Interestingly, the excreta application lowered the Mn concentration in grass at the second and third cuts. The effect of the supplemental mineral form on the Mn concentrations was not statistically significant across the three cutting times.

The Se concentrations in grass of different treatments were all below the requirement levels of growing lambs of different body weights (0.16-0.48 mg-Se kg-DM^{-1}) across the three cutting-times (**Figure 4.9**). Additionally, the application of urine or faeces did not increase the Se concentrations. The Se concentrations under the excreta treatments were either similar to or below the concentrations of the control treatments. The overall concentrations of Se in grass were highest at the third cut, and the concentrations were higher in the arable soil than in the grassland soil. The effect of the supplemental mineral form was not statistically significant.

To investigate the treatment effects on micronutrient removal by grass, the total accumulation of an element in grass across the three harvests was summed (**Equation 4.3**). The elements were grouped according to the result of total accumulation in grass under different treatments (**Figure 4.10**). Elements of the first group, including Cu, Zn, Fe, Cd, S, P and N had the highest uptake in grass in the urine treatments (U-I, U-O,

Table 4.9 The result of ANOVA analysis of micronutrient total uptakes in grass across three cuts (includes time effect)

Total uptake across three cuts	Elements									
	Cd	Cu	Fe	Mn	Mo	P	S	Se	Zn	N
Time	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****
Excreta type (ET)	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****	**0.0306 ***	**<0.001 *****	**<0.001 *****
Supplemental mineral form (Form)	0.5419	0.3413	0.5520	0.3452	0.2739	0.3442	0.3805	0.0985	0.3149	0.3712
Soil	**<0.001 *****	**<0.001 *****	**<0.001 *****	0.3704	**<0.001 *****	**<0.001 *****	**<0.001 *****	**0.0031 ****	**<0.001 *****	**<0.001 *****
ET x Form	0.9789	0.6534	0.6237	0.4274	0.3588	0.4993	0.5443	0.2718	0.8321	0.7258
ET x Soil	**0.0105 ***	**0.0226 ***	**0.0064 ****	0.2486	0.2589	**<0.001 *****	**<0.001 *****	0.2813	**0.0382 ***	**0.0049 ****
Form x Soil	0.1428	0.0731	0.5871	**0.0254 ***	0.1585	0.2381	0.0978	0.2906	0.1426	0.1980
ET x Form x Soil	0.8330	0.7611	0.7307	0.1780	0.7309	0.5669	0.8090	0.4239	0.9118	0.8472

Symbols '*', '**', '***' indicate statistical significances of ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

Table 4.10 The result of ANOVA analysis of micronutrient concentrations in grass of the three cuts (includes time effect)

Total uptake across three cuts	Elements									
	Cd	Cu	Fe	Mn	Mo	P	S	Se	Zn	N
Time	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***
Excreta type (ET)	**<0.001** ***	**<0.001** ***	0.3896	**0.0155** *	**<0.001** ***	**<0.001** ***	0.7023	0.7719	**<0.001** ***	**<0.001** ***
Supplemental mineral form (Form)	0.8176	0.2905	0.9127	0.2689	0.1051	0.1040	0.4308	0.1257	0.2861	0.7046
Soil	**<0.001** ***	**0.0393** *	**<0.001** ***	**<0.001** ***	0.0891	0.9094	0.3385	**<0.001** ***	**<0.001** ***	**0.0021** **
ET x Form	0.5070	0.9902	0.6995	0.5894	0.9208	0.9057	0.9758	0.6138	0.7809	0.7831
ET x Soil	0.1541	0.7609	0.9942	**0.0342** *	**<0.001** ***	**<0.001** ***	**0.0035** **	0.2711	0.4259	0.4803
Form x Soil	0.1035	**0.0145** *	0.7083	0.1436	0.3494	0.2560	0.2301	0.9121	0.0694	0.0765
ET x Form x Soil	0.5068	0.8183	0.6417	0.3766	0.9501	0.6688	0.8626	0.5171	0.6148	0.9768

Symbols '*', '**', '***' indicate statistical significances of ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

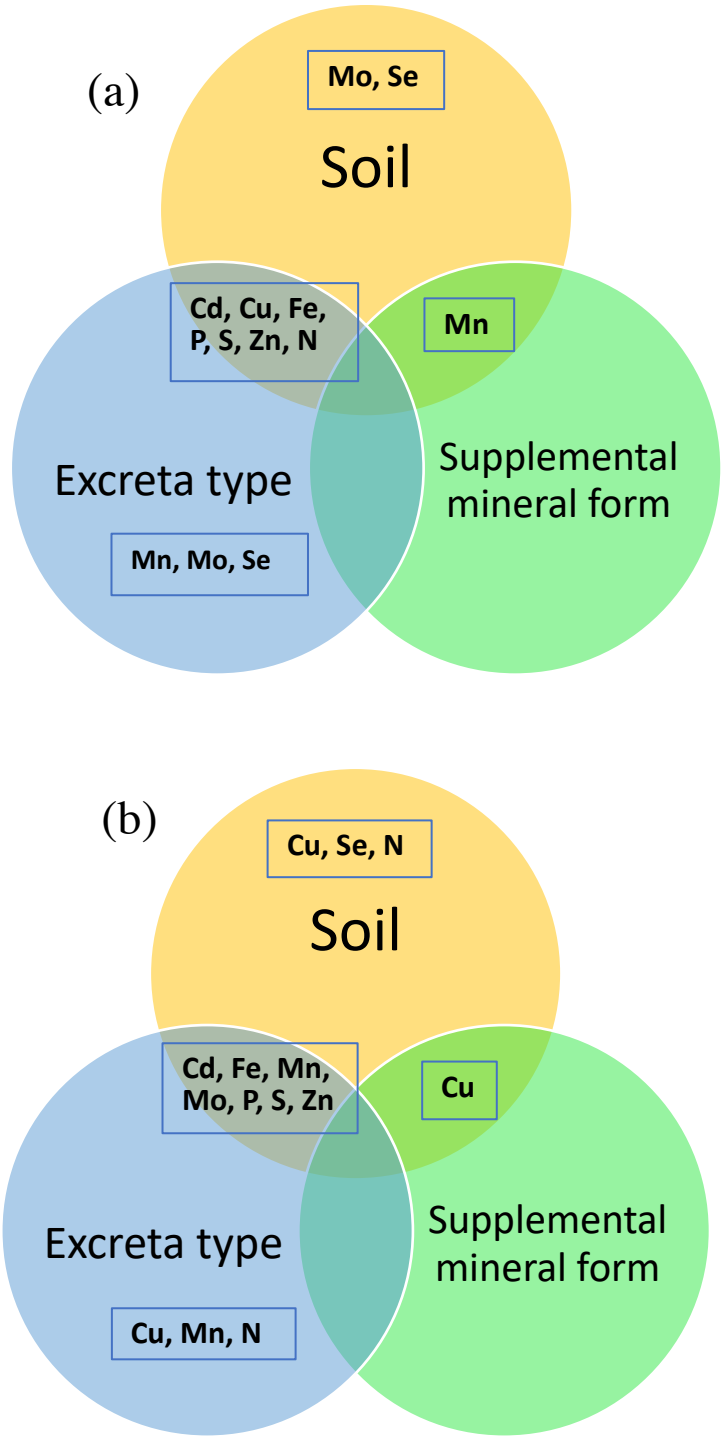


Figure 4.4 Summary Venn diagram of the ANOVA analysis of the treatment effects on either (a) total element uptakes in grass or (b) element concentrations in grass of the three cuts based on the results from Table 4.9 and Table 4.10, respectively. The overlapping areas represent the interaction between the factors in ANOVA.

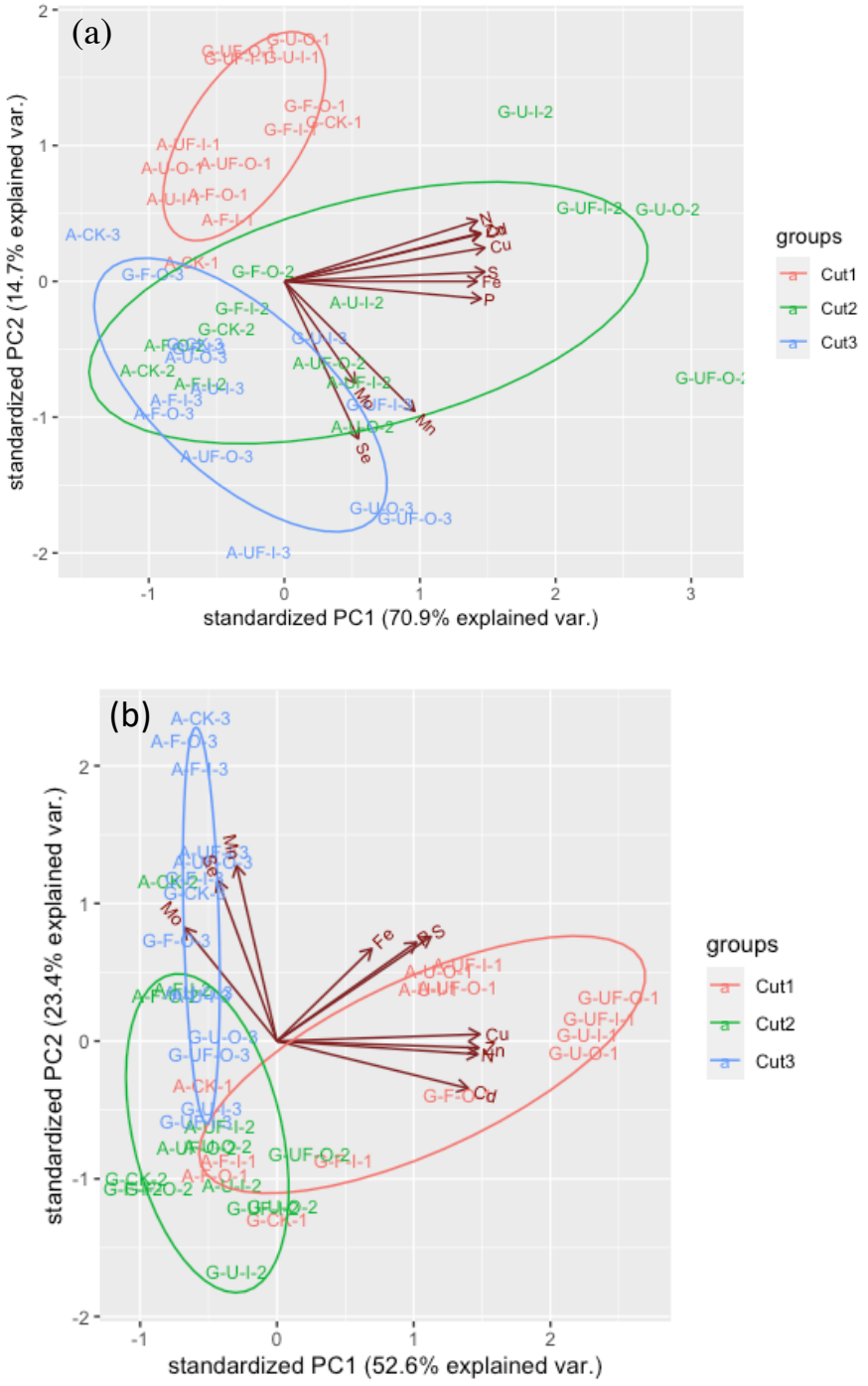


Figure 4.5 PCA analysis according to either (a) total contents of the elements in grass or (b) concentrations of the elements in grass, grouped by the different cutting time. Sample codes are explained in Table 4.1.

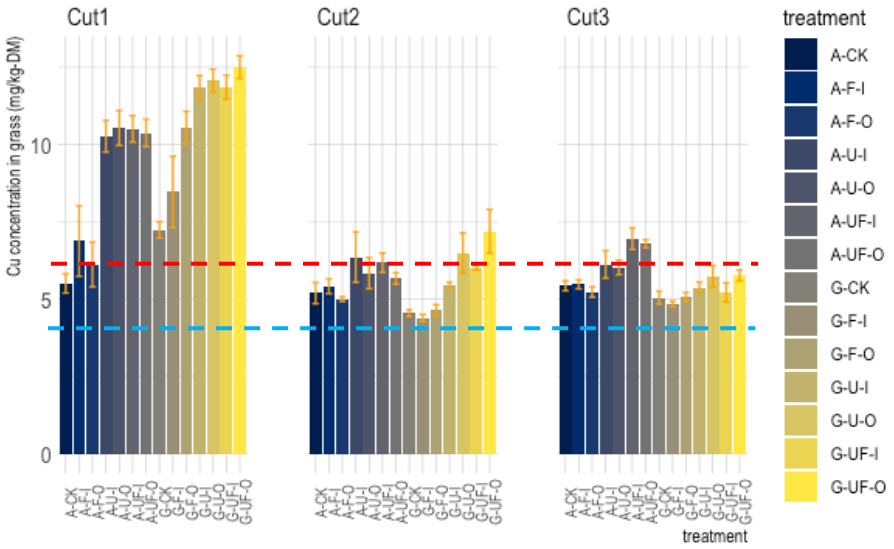


Figure 4.6 Cu concentrations (mg kg-DM^{-1}) in grass at different cutting time. The error bars are standard errors of the result. The dotted lines are the dietary requirement levels of growing lambs of different body weights (Appendix Table B.17). The blue line is a lower requirement level: 3.92 mg kg-DM^{-1} for a 70 kg lamb and red line is a higher requirement level: 6.10 mg kg-DM^{-1} for a 30 kg lamb.

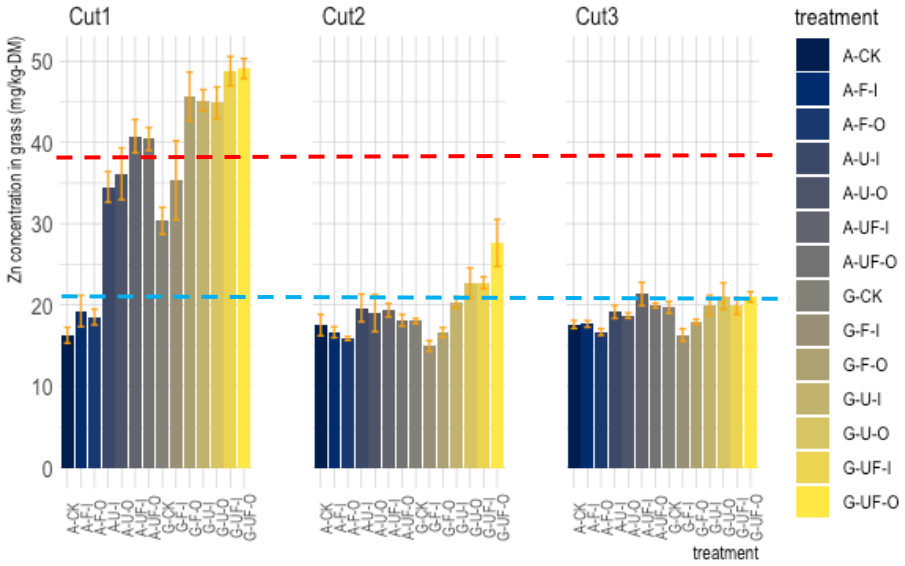


Figure 4.7 Zn concentrations (mg kg-DM^{-1}) in grass at different cutting time. The error bars are standard errors of the result. The dotted lines are the dietary requirement level of growing lamb of different body weights (Appendix B Table B.17). The blue line is a lower requirement level: 20.63 mg kg-DM^{-1} for a 20 kg lamb and red line is a higher requirement level: 38.93 mg kg-DM^{-1} for a 60 kg lamb.

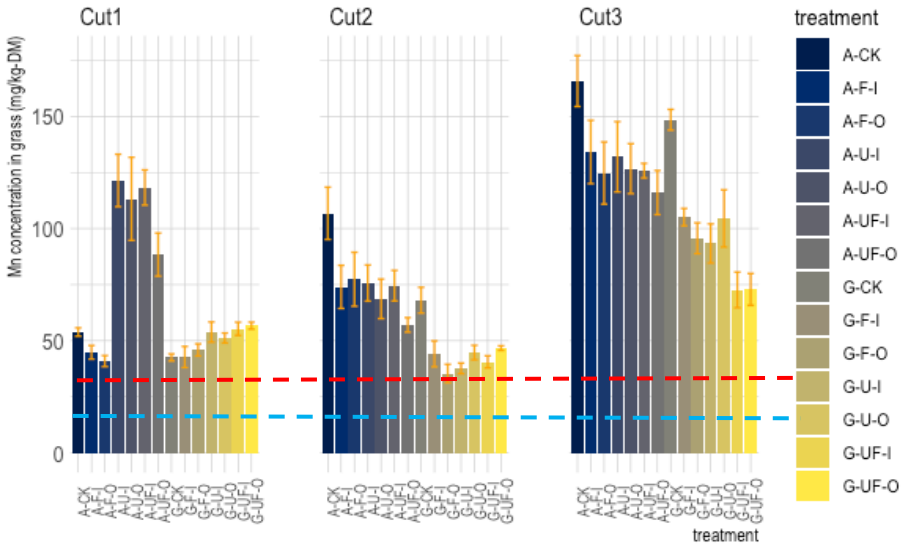


Figure 4.8 Mn concentrations (mg kg-DM^{-1}) in grass at different cutting time. The error bars are standard errors of the result. The dotted lines are the dietary requirement level of growing lamb of different body weights (Appendix B Table B.17). The blue line is a lower requirement level: 15.05 mg kg-DM^{-1} for a 70 kg lamb and red line is a higher requirement level: 22.86 mg kg-DM^{-1} for a 30 kg lamb.

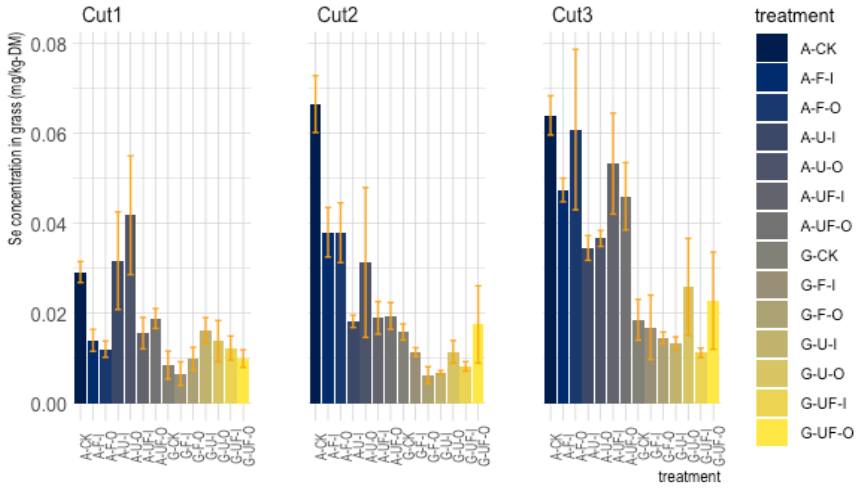


Figure 4.9 Se concentrations (mg kg-DM^{-1}) in grass at different cutting time. The error bars are standard errors of the result. The lines representing the dietary requirement level of animal are not presented because they are out of the scale of y-axis. A low and a high requirement level for a 80 kg and a 20 kg growing lamb are 0.16 mg kg-DM^{-1} and 0.48 mg kg-DM^{-1}, respectively (Appendix B Table B.17).

UF-I and UF-O treatment) in grassland soil, and had the lowest uptake in the untreated arable soil followed by the faeces-only treatment in the arable soil, similar to **Figure 4.4a**. The total uptake of the elements in this group was significantly influenced by the interaction of soil and excreta application. In the arable soil, the total uptake of elements under the treatment of faeces or urine or the combination were either equal to or higher than untreated groups. In the grassland soil, however, the treatments with urine always significantly increased the total uptake of elements in grass. The faeces-only treatments in grassland soil did not increase the total uptake of the elements in soil compared to the untreated grassland soil. The total uptake of the elements was higher in the grassland soil than in the arable soil.

For Mn, the different excreta treatments (the type or the form of the supplemental form) had more influence than the different soils (**Figure 4.10d**), which was aligned with the results shown in **Figure 4.4a**. The treatments with urine increased the total uptake of Mn in both the arable soil and the grassland soil. In the grassland soil, the Mn uptake by grass was higher in experiments treated with urine from sheep given the organic form supplements than in the equivalent experiments with inorganic form supplements, but the results were opposite in the arable soil.

The total uptake of Se in grass was not significantly different across all the treatments in the arable soil (**Figure 4.10i**). However, in the grassland soil, all of the treatments, except for the urine treatment from animals that were supplemented with organic minerals, had lower Se total uptake than those in the arable soil, although not significant at all time points.

The uptake of Mo in grass was higher in all treatments of the grassland soil than the arable soil. In addition, the effect of different excreta treatments was consistent across

the two different soils, where faeces-containing treatments (F-O, F-I, UF-O, UF-I) had the highest total uptake of Mo in grass over the controls and those with urine-only.

4.3.5 pH variation in soil solution

Variations in pH affect the chemical environment in soil solution, which can in turn influence the availability of a nutrient to grass. The soil and excreta type (urine/faeces) had significant impact on the pH of the soil solutions with no significant interaction between these two factors (**Table 4.11**). The pH values of different excreta applications followed the order: F (faeces application only) > UF (urine + faeces application) > U (urine application only). The pH of the original urine and faeces (in water) were pH = 9.5 and 8.10, respectively. However, the pH values of soil solution of urine treatments were lower than those of faecal treatments (**Figure 4.11**). The soil pH (in water) pre-measured before the experiment was similar, 6.38 and 6.31, in the arable and the grassland soil, respectively. The soil solution pH of arable soil was higher than the grassland soil (**Figure 4.12**), which can be attributed to the difference in pH of the soil *per se*. The difference in soil solution pH between the two soils was wider during the first two weeks and was narrower after week 3 (**Figure 4.12**).

Table 4.11 ANOVA test results of soil solution pH values throughout the experiment

	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
Excreta type (ET)	0.6212	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****	**<0.001 *****
Supplemental mineral form (Form)	0.8467	0.5013	0.4083	0.8638	0.9085	0.2642
Soil	0.1123	**<0.001 *****	**<0.001 *****	**0.0314 ***	**0.0141 ***	**0.0062 ****
ET x Form	0.5451	0.0872	0.0950	0.4781	0.1366	0.8014
ET x Soil	0.6077	**0.0297 ***	0.4671	0.5272	0.3464	0.6510
Form x Soil	0.1443	0.2043	0.9727	0.3201	0.0680	0.4004
ET x Form x Soil	0.7441	0.4079	0.5096	0.4100	0.2245	0.7884

Symbols '*', '**', '***' indicate statistical significances at p-value<0.05, <0.01, <0.001, respectively.

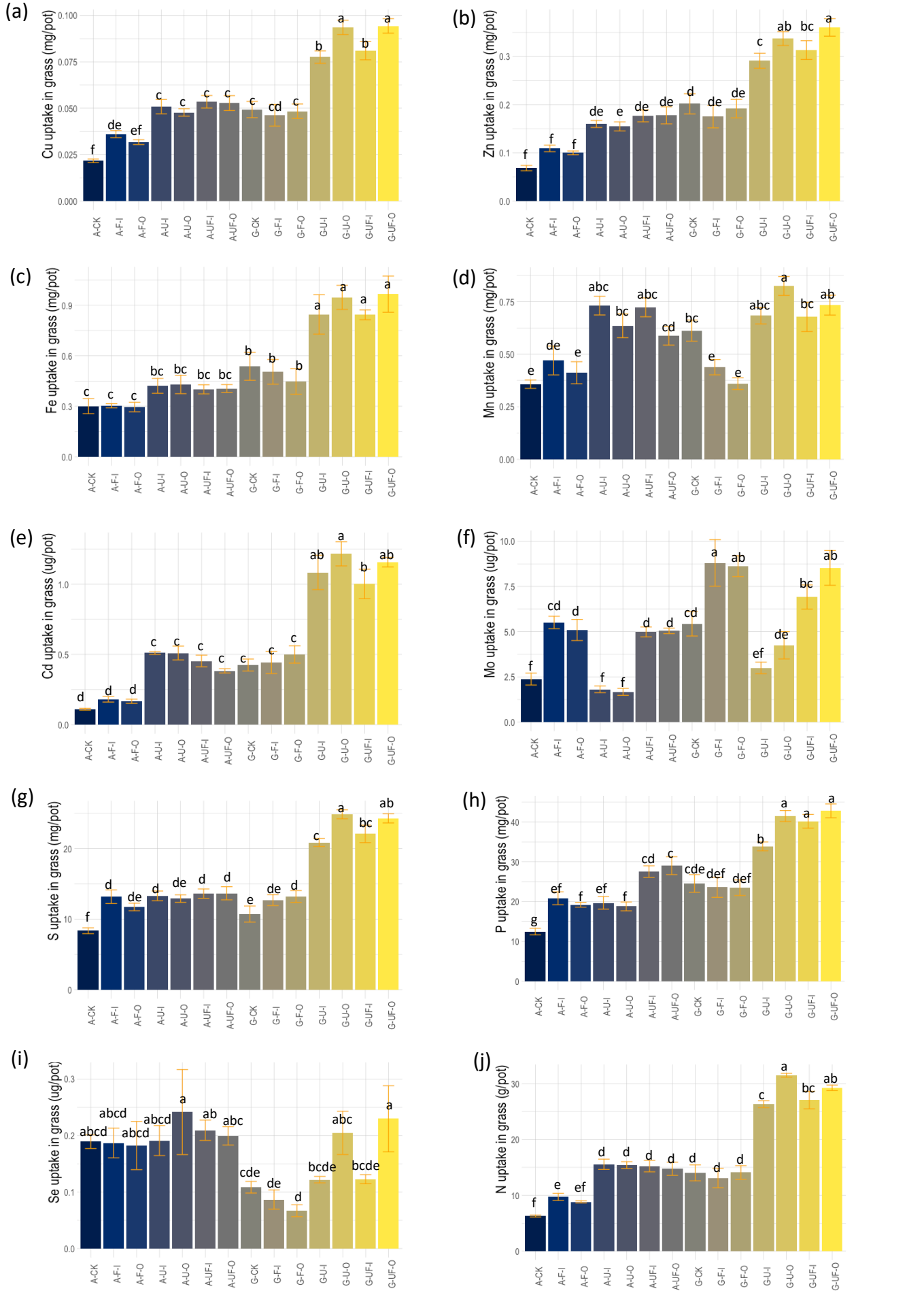


Figure 4.10 Total uptake of (a) Cu, (b) Zn, (c) Fe, (d) Mn, (e) Cd, (f) Mo, (g) S, (h) P, (i) Se, (j) N in grass across the three cuts. The labelled letters indicate the results of Fisher LSD test (α=0.05) after the ANOVA tests.

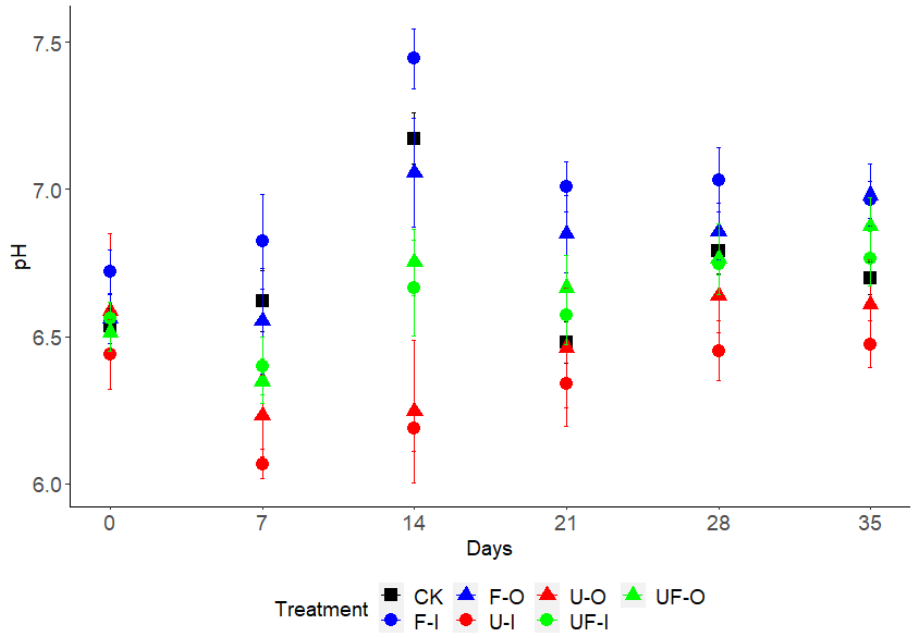


Figure 4.11 Soil solution pH of different excreta treatment at different sampling times. The mean of each data point is the average for excreta treatment across soil types. The error bars are the standard errors of the data (n=8).

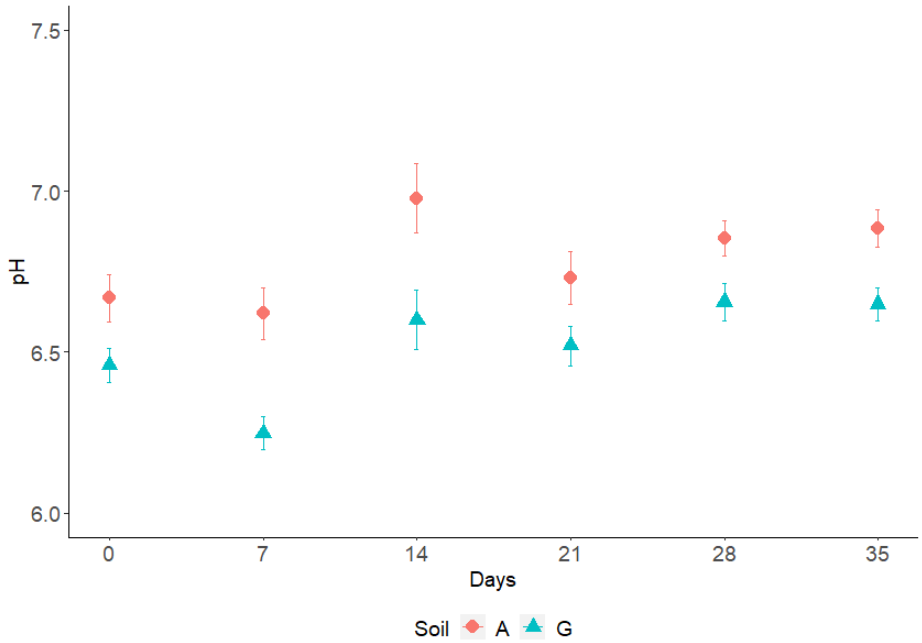


Figure 4.12 Soil solution pH of arable (A) or grassland (G) soil at different sampling times. The mean of each data point is the average for soil type across different excreta treatments. The error bars are the standard errors of the data (n=28).

Table 4.12 Soil extractable Cu, Zn, Mn, N, K and P

Treatments	Extractable Cu (mg kg^{-1})	Extractable Zn (mg kg^{-1})	Extractable Mn (mg kg^{-1})	Extractable $NO_2^-+NO_3^-+NH_4^+$ (g-N $kg\text{-}DM^{-1}$ soil)	Extractable K (mg kg^{-1})	Extractable PO_4^{3-} (mg kg^{-1})
	(mean ± SE)†					
A-CK	0.00±0.001^{j}	0.33±0.004d	14.2±0.28^{b}	8.74±0.752^{e}	277±1.4^{h}	29.5±0.47^{f}
A-F-I	0.04±0.004^{f}	0.11±0.004h	6.93±0.239^{e}	39.4±1.78^{de}	385±0.7^{g}	63.9±1.85^{e}
A-F-O	0.07±0.006^{c}	0.18±0.008f	24.9±0.56^{a}	22.2±2.31^{e}	377±3.0^{g}	89.9±1.84^{c}
A-U-I	0.03±0.001^{g}	0.02±0.004jk	4.11±0.058^{g}	268±25.3^{c}	964±3.9^{b}	32.3±0.14^{f}
A-U-O	0.02±0.001^{hi}	0.02±0.001k	3.47±0.042^{h}	287±11.0^{bc}	869±2.3^{d}	31.9±0.54^{f}
A-UF-I	0.08±0.001^{bc}	0.04±0.001j	5.08±0.014^{f}	282±6.79^{bc}	1070±9.0^{a}	73.3±2.27^{d}
A-UF-O	0.05±0.002^{e}	0.06±0.001i	9.88±0.056^{d}	313±23.5^{b}	936±7.4^{c}	66.8±1.66^{de}
G-CK	0.02±0.001^{i}	1.39±0.005a	12.0±0.05^{c}	15.9±2.82^{e}	147±0.4^{k}	64.8±0.79^{e}
G-F-I	0.05±0.001^{ef}	0.63±0.009c	3.84±0.018^{gh}	62.3±4.89^{d}	228±1.1^{i}	95.0±4.03^{c}
G-F-O	0.06±0.001^{d}	0.69±0.005b	4.13±0.124^{g}	65.0±5.02^{d}	208±3.1^{j}	117±3.3^{a}
G-U-I	0.02±0.001^{hi}	0.21±0.002e	0.65±0.010^{i}	349±14.8^{a}	807±4.1^{e}	60.9±1.79^{e}
G-U-O	0.03±0.000^{gh}	0.18±0.002f	0.68±0.003^{i}	299±6.0^{bc}	757±4.1^{f}	64.9±1.15^{e}
G-UF-I	0.08±0.001^{b}	0.18±0.003f	1.07±0.024^{i}	358±12.9^{a}	873±1.0^{d}	107±4.7^{b}
G-UF-O	0.10±0.007^{a}	0.15±0.005g	1.05±0.090^{i}	377±10.6^{a}	808±7.2^{e}	123±4.7^{a}
	P level					
Excreta type (ET)	**<0.001*****	**<0.001*****	**<0.001*****	**<0.001*****	**<0.001*****	**<0.001*****
Supplemental mineral form (Form)	**0.004****	**<0.001*****	**<0.001*****	0.9403	**<0.001*****	**<0.001*****
Soil	**<0.001*****	**<0.001*****	**<0.001*****	**<0.001*****	**<0.001*****	**<0.001*****
ET x Form	**<0.001*****	**<0.001*****	**<0.001*****	0.088	**<0.001*****	**<0.001*****
ET x Soil	**<0.001*****	**<0.001*****	**<0.001*****	0.1428	**<0.001*****	**<0.001*****
Form x Soil	**<0.001*****	**<0.001*****	**<0.001*****	0.1848	**<0.001*****	**0.025***
ET x Form x Soil	**<0.001*****	**<0.001*****	**<0.001*****	0.0602	**<0.001*****	**0.007****

†Superscript letters indicate the results of Fisher's LSD test across treatments within one column. Symbols '*', '**', '***' indicate statistical significances of the ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

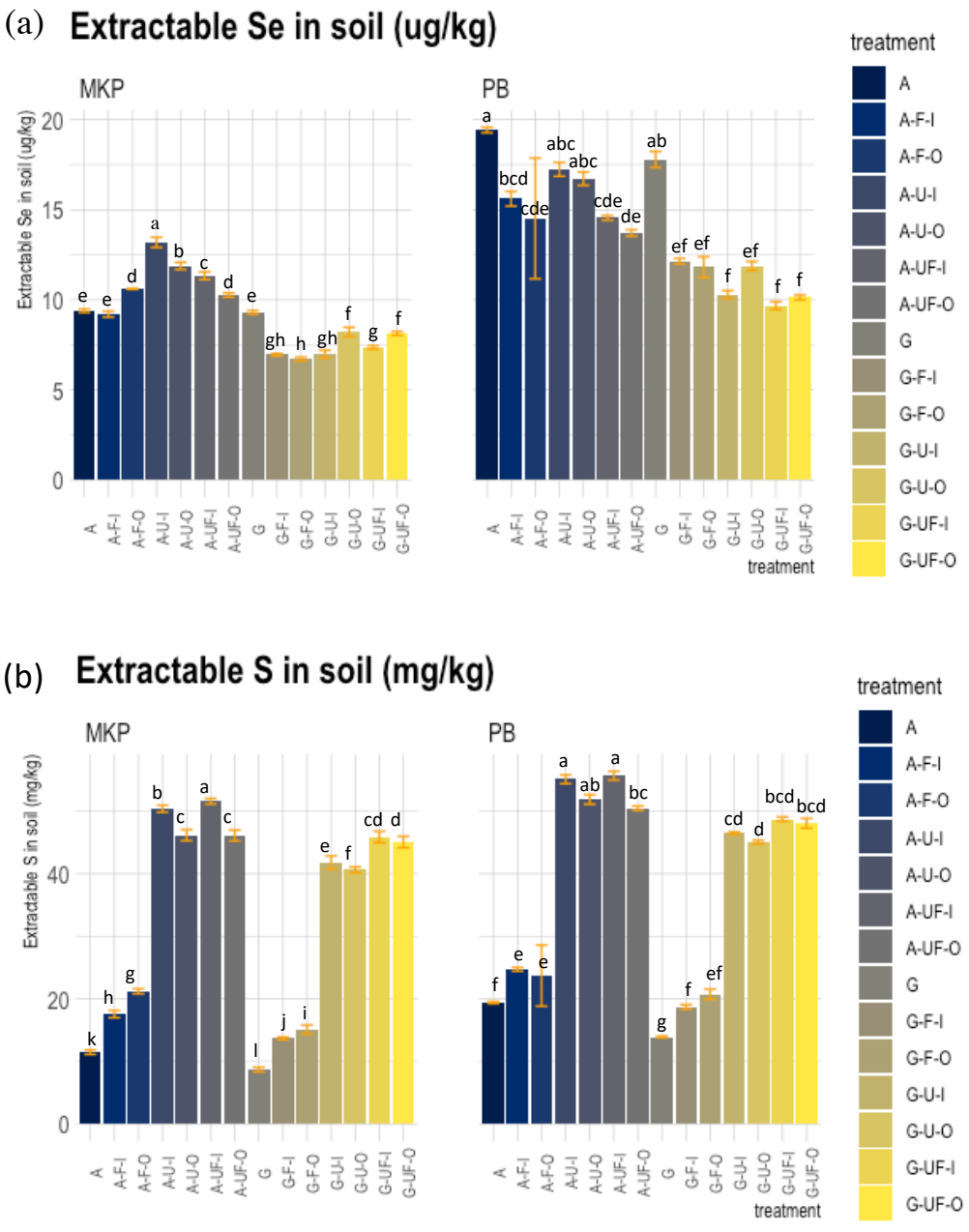


Figure 4.13 The extractable (a) Se (b) S of soil before the experiment in KH_2PO_4 solution (MKP, pH=4.8) or in P-buffer solution (PB, pH=7.5). The error bars are the standard errors of the extraction results (n=3).

4.3.6 Soil extractions

The pool of plant-available nutrients in soil was estimated by chemical extractions of the soil (with faeces or urine incorporated) sampled at the start of the experiment. The soil extractable Cu, Zn and Mn was significantly influenced by the interaction of the three main factors (P<0.001, **Table 4.12**). The extractable Cu in both soils was higher after the application of sheep excreta. The treatments with faeces (F-I, F-O, UF-I, UF-O) had higher extractable Cu than the urine-only treatment. On the other hand, the application of sheep excreta resulted in less extractable Zn and Mn. The treatments with urine (U-I, U-O, UF-I, UF-O) had the lowest extractable Zn and Mn among the treatments of the same soil.

The soil-extractable N in the grassland soil was significantly higher than that in the arable soil (P<0.001). The application of sheep excreta significantly raised the extractable N in soil (P<0.001, **Table 4.12**). The application of urine resulted in more than 20 times higher soil-extractable N than the untreated soils, whereas the application of faeces resulted in ca. 4 times higher soil-extractable N than the untreated soils. The soil-extractable K in the grassland soil was, however, lower than that in the arable soil. Soil-extractable K was higher under the urine treatments followed by faeces treatments and the untreated soils (**Table 4.12**). The soil-extractable P in the grassland soil was higher than that in the arable soil. Application of faeces significantly increased the concentrations of extractable P, whereas the treatments of only urine had no significant effect on raising the concentrations of soil extractable P (**Table 4.12**).

The soil-extractable Se and S was analysed using two P solutions of different pH. In both PB and MKP extraction, the extractable Se of the untreated arable soil was not significantly different from that of the untreated grassland soil. The extractable Se under different treatments was not consistent at different extraction pH. The MKP extraction

(pH 4.8) gave lower extractable Se than that of the PB extraction (pH 7.5) (**Figure 4.13a**). In the PB extraction, the application of sheep excreta lowered the extractable Se. In the MKP extraction, the treatment of sheep excreta in the grassland soil had lower extractable Se than the untreated grassland soil. However, the MKP-extractable Se of the treated arable soil was not significantly lower than that of the untreated arable soil. On the other hand, the concentrations of S were consistent across the extractions at different pH. The application of sheep excreta raised the extractable S (**Figure 4.13b**). The treatments with urine resulted in much higher extractable S than the treatments with faeces only. The extractable S in the arable soil was higher than that in the grassland soil.

4.4 Discussion

4.4.1 The effect of the form (organic or inorganic) of the supplemental trace minerals to sheep on the total uptake and the concentration of nutrients in grass

The form of the supplemental mineral given to sheep showed significant impact, and an interaction with soil, on the total uptake of Mn and the concentration of Cu in grass (**Figure 4.4 a and 4.4b, respectively**). For other elements, the effect of the form of supplemental minerals on their uptake in grass was not significant. The effect of the mineral form at each cutting date and on individual elements (**Tables B.9** to **Table B.14**) was variable and not consistent. The significant interaction of the soil and the mineral form on the total uptake of Mn in grass became nonsignificant when considering the individual cuts (**Tables B.12** to **Table B.14**). For Cu, although the interaction of the soil and the mineral form on the concentration in grass remained significant at the second cut, the effect of mineral form was not significant *per se* (**Table B.13**). The total uptake of P and S in the second cut was significantly influenced by both the mineral form and the interaction of mineral form and soil. However, the effect of the mineral

form on total uptake of P and S in the second cut became nonsignificant when the effect of cutting date was removed (**Table 4.9**).

Accordingly, the influence of the supplemental mineral form on the uptake of the studied elements can be seen as variable and not consistent, and is therefore relatively unimportant as compared to other factors, e.g., soil and excreta, which show consistently significant effects after removing the time effect. This implies that, in a pasture system, the chemical form of supplemental minerals to sheep is less influential to nutrient uptake in grass compared with factors such as soil properties and the type of applied excreta.

4.4.2 The effect of excreta and soil on the concentration and total uptake of the studied elements in grass

4.4.2.1 Copper and zinc

The impact of excreta and soil, either dependent or independently, on the concentrations and total uptake of Cu and Zn in grass was significant (**Figure 4.4**). The responses of both concentrations in grass and total uptake of Cu and Zn in the different treatments showed similar trends. Among the three cuts of grass, the concentrations of Cu and Zn in grass of the first cut were higher than those of the second and third cuts (**Figures 4.6** and **4.7**). At first glance, the lower concentrations of Cu and Zn at the second and third cut compared to the first cut might be due to the dilution effect of grass growth because the grass DM was the highest at the second cut followed by the third and the first cut (**Table 4.8**). However, in the untreated soils (A-CK and G-CK), the concentrations of Cu and Zn remained consistent across the three cuts albeit the variable grass DM (**Figures 4.6** and **4.7**). This implies that the decreasing trend of Cu and Zn concentrations under the urine treatments through time cannot be attributed to the dilution effect causing by the variable grass DM.

The higher Cu and Zn concentrations under urine treatments at the first cut might be taken to suggest higher input of readily available Cu and Zn from urine compared to faeces. However, the extractable Cu and Zn (**Table 4.12)** did not follow the results of the concentrations of Cu and Zn in the grass of the first cut. For Cu, the faeces treatments contributed significantly more extractable Cu than the urine treatments. For Zn, both the urine and the faeces treatments decreased the extractable Zn in soils. Therefore, the increased Cu and Zn concentrations under the urine treatments did not result from a greater input of available Cu and Zn.

Although the Cu and Zn concentrations in grass at the first cut were higher than the controls and were above the requirement level, the grass growth under the urine treatments was suppressed (**Table 4.8**), which may be attributed to a high input of salts from the urine. Nizam (2011) reported that perennial ryegrass DM (root and shoot) decreased significantly when the salinity level was higher than 8 dS m^{-1} during the germination period. The salinities of the liquid urine applied to the U-O and U-I treatments were 15.8 ± 0.10 and 20.2 ± 0.06 dS m^{-1}, respectively. However, after the urine was applied into the soils, the electrical conductivity (EC) in the soil solutions of treatments with urine was only about 1.50 dS m^{-1}, which was still about twice the value of the untreated soils (**Appendix B Figure B.4**). The supressed growth of grass by the salinity stress might lead to a 'concentration' effect, hence the higher concentrations of Cu and Zn. Therefore, simply analysing the micronutrient concentration without a proper evaluation of grass growth can be misleading, especially when the grass is under a stress, such as salinity.

The treatments with urine (U-I, U-O, UF-I, UF-O) led to higher total uptake of Cu and Zn in grass in both soils, regardless of the addition of faeces or the supplemental mineral form (**Figures 4.10a** and **4.10b**). This effect was much more significant in the grassland

soil than in the arable soil. Faeces only, on the other hand, did not significantly increase the total uptake of Cu and Zn in grass, despite the faeces having higher density of micronutrients than the urine. Hypothetically, the urine treatment which lowered the pH of the soil environment (**Figure 4.12**) could lead to more mobile Cu^{2+} and Zn^{2+}. However, according to the pH-Eh diagram of Zn (**Figure 1.4a)**, within the pH range of the current pot experiment (pH = 6.0 to 7.5), the inorganic form of Zn should be mostly in the form of Zn^{2+} across the different treatments. Additionally, the significantly lower extractable Zn measured in the urine treatments than the controls in both the arable and the grassland soils (**Table 4.12**) does not explain the consistently higher total Zn uptake by grass under the urine treatments. Furthermore, the soil-extractable Cu and Zn was both higher in the faeces-only treatments than the urine treatments (with or without faeces). Therefore, the higher total Cu and Zn uptake in grass was not associated with their extractable concentrations in soil that were altered by the application of urine and/or faeces.

The ARW input varied across the treatments and could affect the total Cu and Zn uptake results (**Figure 4.3**). The amount of Cu and Zn input from the ARW (**Table 4.5**) were at a similar quantitative level as the amount of their accumulation in the grass. Therefore, it is possible that the variable amount of ARW input could have affected the results. The irrigation amount of ARW was varied according to the soil moisture taken up by the grass, which directly resulted from the growth of grass: the greater the grass growth, the more moisture (ARW) was needed and subsequently Cu and Zn applied via ARW. Furthermore, the trend of total uptake of Cu and Zn across treatments was aligned with the grass DM (**Table 4.8**). This implied that the pool of plant-available Cu and Zn in soil was sufficient for grass to maintain adequate concentrations for plant growth, which

explains the consistent Cu and Zn concentrations in grass after the first cut and also explains the higher uptake of Cu and Zn with grass growth.

Despite the relatively low input of total micronutrients compared to faeces (**Figure 3.3**), the available concentrations of N and K were significantly higher in the urine treatments (**Table 4.12**). Joblin and Keogh (1979) also found that grass grown in urine patches had higher N and K. The perennial ryegrass grown in urine patches by (Keogh, 1973) had better growth and was grazed more intensively by sheep than the grass grown in areas not receiving urine. Therefore, it was possible that the higher input of available N and K from urine drove the higher growth of grass and hence increased the total uptake of Cu and Zn.

It is noticeable that the extractable P was higher in the grassland soil than in the arable soil (**Table 4.12**). The additive effect of P in addition to other macro-nutrients might be able to explain the greater grass growth, and hence more nutrient uptake in the grassland soil than in the arable soil. However, if the available P was the major factor that drove the significant difference in grass growth, and hence difference in the total Cu and Zn uptake, the treatments of the combination of faeces and urine should have resulted in a significantly higher grass growth over urine-only treatment due to the additive effect of both high N and P input from urine and faeces, respectively. However, the grass yield under the treatments with or without faeces were similar (**Table 4.8**). This indicates that the major factor that drove the difference between the two soils was not the amount of available P *per se*.

The reason that the increased soil P under faeces treatments did not make a significant difference on grass growth was possibly because the original soil P level was above the level at which the grass growth plateaus. Waddell et al. (2016) reported that no more response of growth of perennial ryegrass was observed when the external P fertiliser

(KH_2PO_4) was applied to soil (with 9 mg-P kg^{-1}) greater than 20.6 mg-P pot^{-1}, equal to ca. 15.8 mg-P kg^{-1} soil. Even though this critical value of 15.8 mg-P kg-DM^{-1} relates to the 'additional P' but not to the total extractable P in soil, the critical value of extractable P for the growth response of perennial ryegrass is not greater than 24.8 mg-P kg^{-1} soil (9 + 15.8 mg-P kg^{-1} soil), assuming that the original and the additional P in (Waddell et al., 2016) were 100% extractable. In the current study, the lowest concentration of extractable P across the treatments was 29.5 mg kg^{-1} soil (untreated arable soil). The extractable P concentrations of other treatments were all above 30 mg kg^{-1} soil, which was higher than the concentration at which the grass has growth response to additional P input.

From the soil health perspective, a healthy soil for a plant to grow in requires adequate nutrients, water and air. The higher OM content in the grassland tends to provide better soil aggregate structure, which contributes to micropores for retaining nutrients and also macropores for water flow and root growth. From the photos of the soils before grinding (**Appendix B Figure B.5**), the soil collected from the arable land was sticker and had fewer small aggregates than the soil collected from the grassland. This difference in structure may explain the higher growth of grass and hence higher nutrient uptakes from a holistic perspective. Within the same soil, it is interesting that urine, which contained minimal micronutrients, could have produced higher total Cu and Zn uptakes than the faeces treatments which contributed much more micronutrients than urine to the soils. It is also interesting that the application of faeces did not significantly improve the growth of grass within the three cuttings of grass. Presumably, in the short term, the applied fresh faeces had not been decomposed to the form that can contribute to both available nutrient input and better physical properties of a soil, and therefore was not helpful for the uptake of Cu and Zn in grass. This again indicates that the interaction

among nutrients and soil physiochemical properties can be much more important than the exact content of nutrient input in terms of plant uptake.

4.4.2.2 Manganese

The concentrations of Mn in grass, unlike Cu and Zn, increased across cutting time (**Figure 4.8**). The application of urine and faeces decreased the concentrations of Mn in grass, except those under the urine treatments at the first cut, which was believed to be due to a 'concentration effect' resulting from the reduced growth of grass under salinity stress. Despite the decreased concentrations under the excreta treatments, the overall Mn concentrations increased across cutting times, including the control treatments. Furthermore, the overall concentrations were still higher than the requirement levels for growing lambs. It was unlikely that applying excreta would lower the grass Mn to a level of inadequacy. This result is aligned with the reported Mn concentrations of UK pastures, which tends to be higher than the required concentration (**Table 1.1**).

The total Mn accumulation in grass across the three cuts was higher than the controls under the treatment of urine application and either lower or similar to the controls under the faeces application (**Figure 4.10d**). This result was not related to the extractable Mn in soil directly (**Table 4.12**), which showed decreased extractable Mn under urine application. Like Cu and Zn, Mn accumulation is more likely to be related to the 'grass growth effect' discussed in section **4.4.2.1**, in which nutrient removal from soil increased with the growth of grass. This, again, implies that the pool of plant-available Mn was sufficient, so that the total uptake of Mn increased with subsequent grass growth.

4.4.2.3. Selenium

The concentrations of Se in grass under different treatments across the three cutting times were all below the requirement level for a growing lamb (**Figure 4.9**). The Se concentrations in grass grown in the arable soil were higher than grass grown in the grassland soil. Under the treatments in the arable soil, the application of urine and/or faeces lowered the Se concentrations in grass, whereas this trend was not significant in the grassland soil, possibly because the concentrations of grass grown in the untreated grassland soil without the excreta application were already low and the Se concentrations of the analytes were close to the detection limit of ICP-MS.

The trend of decreasing Se concentrations in grass under the treatments of excreta in the arable soil and low Se grass concentrations in the grassland soil could be due to a dilution effect driven by grass growth. However, Se was still lower even when viewed as the total Se accumulation, which would include the effect of approximately two times greater grass DM in the grassland soil than in the arable soil (**Figure 4.10i**). This implies that the low Se concentration in grass was not solely due to a dilution effect but also resulted from a limited pool of available Se in the grassland soil.

The possible reasons for the low available Se in the grassland soil are: (1) low available Se in soil *per se;* (2) antagonism by other elements in soil; (3) soil properties resulting in Se species alteration reducing availability of applied Se. The first mechanism can be further attributed to the different amount of Se sorption onto clay minerals, oxides or OM. Soil extractions using P solutions were carried out to differentiate the first and the second mechanism. Monopotassium phosphate (KH_2PO_4, MKP) is known as a good reagent for extracting plant-available S (Zhao & McGrath, 1994). The chemical similarity between SO_4^{2-} and SeO_4^{2-} led to the use of the reagent to extract Se from soil in a previous study (Stroud et al., 2010). However, Se adsorption to clay minerals, OM

and oxides is highly pH-dependent. The pH value of the MKP solution was 4.8, which was much lower than the pH value of the soil solution measured (**Figure 4.11**). Therefore, a P-buffer (NaH_2PO_4/Na_2HPO_4, PB) solution with a pH value of 7.5 was also adopted for the Se extraction to compare the results. The result showed that in both the MKP and PB extractions, the extractable Se was not significantly higher in the arable soil than in the grassland soil (**Figure 4.13a**), despite the higher total Se concentration in the grassland soil than the arable soil (**Table 4.2**). Yet the difference in the extractable Se between the two soils became significant after excreta treatments. The concentrations of the extractable Se under the excreta treatments were significantly lower in the grassland soil compared to those in the arable soil. This result implies that there was an interaction between soil and excreta application in terms of extractable Se.

The extractability of an element is directly related to the sorption of the element in a soil, which is influenced by the content and composition of clay minerals, oxides and OM (**Chapters 1.6.2 and 1.6.3**). The analysis of soil properties (**Table 4.2**) showed that there was little difference in clay content between the two soils. The contents of active Fe oxides and total carbon were twice as high in the grassland soil than in the arable soil. The higher amounts of the Fe oxides and total carbon can significantly increase the sorption of Se and, hence, lower the extractable Se in the grassland. However, the result of extractable Se for both extractions was not significantly lower in the grassland soil (**Figure 4.13a**). From this perspective, the different sorption effect of the two soils was not the major driver of the different grass Se uptake.

The fact that there was no significant difference in soil extractable Se between the arable soil and the grassland soil (**Figure 4.13a**), yet the uptake of Se was significantly lower in the grassland soil, leads to other possibilities: antagonism by other elements in

Se uptake by grass, or Se species alteration resulting in different Se availability to plants. The uptake of SeO_4^{2-} and SeO_3^{2-} by grass are subject to competition with SO_4^{2-} and PO_4^{3-}, respectively, due to the similar electron configuration of the outermost electron shells (Hopper & Parker, 1999), and the fact that SeO_4^{2-} and SO_4^{2-} uptake is thought to take place through the same transporters in plants, and the uptake of SeO_3^{2-} through passive diffusion can be inhibited by PO_4^{3-} (Sors et al., 2005).

The extractable S in the arable soil, using two P solutions of different pH, was either higher or similar to the extractable S in the grassland soil (**Figure 4.13b**). Therefore the SO_4^{2-} antagonism might not be the reason why the total uptake of Se in grass was higher in arable soil. If SO_4^{2-} antagonism were the major driver, the arable soil, which had more extractable S, should result in lower Se uptake by grass than the grassland soil. However, the Se uptake by grass grown in the arable soil was higher than that grown in the grassland soil. Although, as the analysis of total extractable S by ICP-OES does not differentiate the amount of inorganic S from organic S in the extracts, which makes it too arbitrary to deny the S-Se antagonism as the main driver, it is reasonable to believe that the amount of P-extractable S was highly related to the inorganic S content in a soil. Furthermore, although urine treatments had much higher extractable S than others, which was attributed to the high inorganic S content in urine, the Se uptake in grass of the urine treatments was not lower. On the contrary, the Se uptake in grass of the urine treatments in the arable soil had equal Se uptake to the control, and in the grassland soil the treatment of G-U-O and G-UF-O had even higher Se total uptake than the control soil. Therefore, the Se-S antagonism does not appear to be the major driver of the different Se uptake in the two different soils.

According to the pH and Eh environment of the studied soils (**Figure 1.4b**), the inorganic Se in the soil solution was most likely to be in the form of $HSeO_3^-$. SeO_3^{2-} has

similar electron configuration to PO_4^{3-} and, therefore, they may undergo similar chemical reactions, including surface adsorption and transportation by plant roots. The correlation analysis results showed that the soil extractable PO_4^{3-} was negatively correlated with the Se uptake and Se concentration in grass ($P< 0.001$) in the first cut (**Figure B.3**). The concentration of PO_4^{3-} in the grassland soil was significantly higher than that in the arable soil (**Table 4.12**). The application of excreta increased the extractable P, especially under the application of faeces. This result is unsurprising because the P concentration in faeces was about 13 g kg-DM^{-1} and (**Figure 3.4c**), yet the concentration of P in the sheep urine was only about 5 mg L^{-1} (**Figure 3.4d**). However, if the soil PO_4^{3-} concentration were the sole driver of Se uptake in grass, the total uptake of Se in the faeces treatments of the arable soil should be significantly lower than the control treatment of the arable soil, which was not the case (**Figure 4.10i**). Therefore, although it is possible that the PO_4^{3-} in soil could compete with SeO_3^{2-} for uptake by grass, it does not appear to be the sole determinative factor to explain the Se uptake by grass response between the two soils.

Although total Se uptake by grass was not significantly different between the control and the excreta treatments in the arable soil, all of the excreta treatments in both soils showed lower PB-extractable Se concentrations than the control treatments (**Figure 4.13a**). In the P extraction, PO_4^{3-}, which has similar election configuration to SeO_3^{2-}, had higher adsorption affinity to oxides over SeO_3^{2-}, SeO_4^{2-}, and SO_4^{2-} (Balistrieri & Chao, 1987), which is why P solutions are used to extract available Se and S. However, this also suggests that Se not in the form of SeO_3^{2-} or SeO_4^{2-} might not be extractable by the P solutions. Furthermore, all the concentrations of the extractable Se in the MKP extraction were lower than those in the PB extraction, but only the control treatments had significant decreases in the concentrations over the excreta treatments, which might

be due to the different dominant Se species in the control treatments from the excreta treatments.

The speciation, mobility and bioavailability of Se in the environment are highly affected by microbial activities (Fernández-Martínez & Charlet, 2009). Alemi et al. (1991) reported that in an aerobic C-enriched soil environment, the microbe-driven reduction of SeO_4^{2-} into more immobile forms, such as SeO_3^{2-} and elemental Se, was the predominant transformation process of the added SeO_4^{2-}. Under the excreta treatments, the urine and/or faeces provided the carbon or nutrients needed for microbial activities, which could, therefore, drive a greater extent of microbial reduction reactions. Fernández-Martínez and Charlet (2009) indicated that there are two types of Se reduction that alter the Se species in the environment: dissimilatory reduction and assimilatory reduction, and both are driven by microorganisms. In dissimilatory reduction, microorganisms use the oxidized SeO_4^{2-} and SeO_3^{2-} for respiration as the terminal electron acceptors outside the cells during oxidation of organic carbon, or reduced S (produced by sulphate-reducing bacteria), and produce reduced forms of Se, such as Se (0) or Se (-II), as the end product (Fernández-Martínez & Charlet, 2009). In assimilatory reduction, the microorganisms incorporate inorganic Se in cells into organic compounds, such as SeMet and SeCys, and this is generally assumed to be similar to the pathways of S incorporation in microorganisms due to the chemical similarities between S and Se (Fernández-Martínez & Charlet, 2009).

Of the different Se speciation alteration pathways, assimilatory microbial reduction is unlikely to be the dominant pathway in the current study because the end products, SeMet and SeCys are known to be plant-available and can be taken up by plants more efficiently than inorganic Se (Kikkert & Berkelaar, 2013). Additionally, there is no study reporting a high adsorption affinity of Se in the form of amino-acids over Se in

the form of SeO_3^{2-} onto clay minerals, OM nor oxides. That is, if the lower extractable Se in PB solution was due to microbial assimilatory reduction, which transforms SeO_3^{2-} into SeMet and SeCys, the Se uptake in grass under excreta treatments should be higher than the control treatments, which was not the case. On the other hand, the reduced Se (0) is known to be less available to plants than SeO_3^{2-} and SeO_4^{2-} (Mayland et al., 1991), and is of low solubility (Fernández-Martínez & Charlet, 2009). Therefore, microbial dissimilatory reduction of Se could explain the less extractable Se in PB extraction under excreta treatment.

This hypothesis explains similar findings in previous studies that also reported reduced Se uptake by plants after animal excreta application. Fan et al. (2008) found decreased Se concentrations in wheat grains after FYM application. Qingyun et al. (2016) also showed that a 20-year soil application of organic compost led to lower Se accumulation in wheat and maize compared to all other applications including control, inorganic N, P and K plus organic compost, and inorganic N, P and K application. In the Qingyun et al. (2016) study, despite having the highest soil Se concentration, the application of organic compost did not bring about correspondingly higher Se in the exchangeable fraction. Instead, higher oxidizable Se was reported compared to the other treatments, resulting in lower Se availability from the soil. The organically bound Se was also found to be the dominant Se in all the studied soils reported by Gustafsson and Johnsson (1992).

In the grassland soil, which had more OM and Fe oxides than the arable soil, the reduced Se could be more likely to be 'fixed' onto soil OM and/or to co-precipitate with oxides and, therefore, become less available to the grass. This could be the reason why the Se total uptake was significantly lower in the grassland soil. The OM, which is more abundant in the grassland, can act as both the sorption sites of the reduced Se and the

carbon source for microorganisms. In the arable soil, there were fewer OM sorption sites to 'fix' the reduced Se, hence higher Se uptake than the that of the grassland soil. Unfortunately, in this study it is difficult to differentiate the dominant sorption sites of the reduced Se between OM and Fe oxides, because both the OM and Fe oxides, the two major sorbents of Se, were higher in the grassland soil than in the arable soil. There are very few studies investigating sorption of the microbially reduced Se onto those sorbents, and this will need to be further studied.

The theory that the interaction between high Se sorbents and microbial reduction driven by excreta application might also explain an apparent difference in Se uptake under treatments using different supplement forms. The treatments G-U-O and G-UF-O had higher Se total uptake than the treatments G-U-I and G-UF-I, respectively (**Figure 4.10i**), and had a similar level of total Se uptake as the treatments in the arable soil, although the ANOVA test showed insignificant impact of supplemental mineral form on total Se uptake. The Se species in the urine samples may have had different chemical forms resulting from the different supplementation types, which affected the extent of Se reduction and fixation in the soil. Unfortunately, it was not possible to define the Se species directly using HPLC-HG-AFS due to the low Se concentration in urine. Previously, using HPLC-ICP-MS, Shiobara et al. (1998) indicated that under a diet of either SeO_3^{2-} or SeMet at different levels, the Se species in the urine of rats were dominated by monomethylselenol (MMSe) with no difference between the dietary form of Se. However, there has been no similar study in ruminants. It is likely that the metabolic mechanism of a ruminant, with rumen microbially derived reduction of Se species (Lee et al., 2019a), will be different from a monogastric animal and, therefore, might produce different Se species in urine under different Se supplementation treatments. Some examples of the differences of Se metabolism between monogastric

animals and ruminants are discussed in Mehdi et al. (2013). The efficiency of intestinal absorption of Se is much lower in ruminants than in monogastric animals, and the absorption varies with Se species. Furthermore, urine is the dominant route of Se excretion in monogastric animals, whereas in ruminants, the urinary excretion of Se is generally low. Further study is needed to test whether form of supplemental minerals affects the Se speciation in sheep urine and whether the difference in Se uptake by grass results from a difference in Se species in urine.

Overall, in the current study it was hypothesized that the dominant Se species in the control treatments was SeO_3^{2-}, which adsorbs onto sorbents in the soil and was extractable by P solutions. SeO_3^{2-} was susceptible to the antagonism by PO_4^{3-} for plant uptake, hence the lower Se uptake in the control grassland soil. However, the dominant Se species under the excreta treatments was not SeO_3^{2-}, and was mostly likely to be a reduced form of Se, which was less soluble and plant-available and could be 'fixed' onto the soil sorbents. The interaction of Se sorption and the microbial reduction of Se could be the main reasons for the generally lower Se uptake in grass under the excreta treatments in the grassland soil.

For both the arable soil and the grassland soil, the Se concentrations in grass did not reach the required levels of Se density in grass for growing lambs and the excreta applications did not raise the Se total uptake by grass and might lower the Se uptake further due to the interaction of Se sorption in soil and microbial reduction. Therefore, in a grazing livestock system, which normally has a high soil OM content and regular animal excreta input, it is possible to have grass of consistently low Se concentration. To increase animal Se intake, it is therefore easier to supplement the animal directly rather than use soil fertilizer, especially not in the form of animal excreta which can

drive Se reduction and decrease Se uptake by grass. The hypothesised mechanism of Se microbial reduction and soil fixation needs further study.

4.5 Conclusions

Soil OM and the type of applied excreta had more significant and consistent influence on micronutrient uptake by perennial ryegrass than the form of the supplemental minerals given to sheep. The form of the supplemental minerals had no consistent impact on the uptake of Cu, Zn, Mn and Se by perennial ryegrass and no significant impact on the leaching of the Cu, Zn, Mn and Se in the system. In contrast, the application of urine raised to a greater extent, the concentrations of Cu and Zn in grass, particularly at the first cut. The effect was attributed to potential salinity stress caused by urine application and did not last to the second cut. Even without the application of excreta, the concentration of Mn in the grass was already well above the recommended requirement level of Mn for a growing lamb. For Se the application of excreta was not enough to raise the concentration of Se in grass to the recommended requirement level of Se for a growing lamb.

The cycling of Cu, Zn and Mn was highly related to grass growth. Although the faeces contained a higher density of nutrients than urine, the urine contributed more available N and K, which, in turn, improved the grass growth, which led to significantly higher uptake of Zn, Cu and Mn by perennial ryegrass. Therefore, in this study, urine played a more important role than faeces in the cycling of Zn, Cu and Mn in grazing pasture systems due to the synergies between the macronutrients and micronutrients. On the other hand, the grass uptake of Se, a non-essential element to perennial ryegrass, was not related to plant growth but rather to the availability of Se to grass. High soil PO_4^{3-} can lead to antagonism with SeO_3^{2-} which makes SeO_3^{2-} less available to grass.

Furthermore, although soil OM can help improve grass growth, it can cause more Se fixation, especially when treated with excreta, which drives microbial reduction and reduces Se uptake.

Chapter 5

Summary, applications and future work

5.1 Summary

Forage from pasture is the major feed source in a sustainable ruminant livestock system because of its low unit cost and reduced competition for human-edible feed (Wilkinson & Lee, 2018). However, the levels of micronutrients in forages, which may be sufficient for optimum crop yields are not always adequate to meet the needs of livestock (Gupta et al., 2008; Lee et al., 2018). Inadequate micronutrients in pasture can affect animal health and production. To prevent micronutrient deficiency, mineral supplementation to animals is usually adopted prophylactically and routinely as part of the standard practice. However, it was unclear how the practice of mineral supplementation to grazing animals could make an impact on the flux of micronutrients in pasture systems and what the major factors that would have significant impact are (**Chapter 1.7**). Understanding this can improve our knowledge of how to maintain high quality of forage which will, in turn, benefit the health of grazing animals.

To understand the how mineral supplementation would influence the flux of micronutrients in pasture systems, I began with asking the following research questions: (1) Does the chemical form of supplemental minerals (organic or inorganic) have a significant impact on the flux of micronutrients (particularly Cu, Zn, Mn and Se) in pasture systems? (2) If the form of the supplemental minerals has a significant impact, what is the mechanism? (3) If the supplemental mineral form does not make a significant impact, what are the other most influential factors?

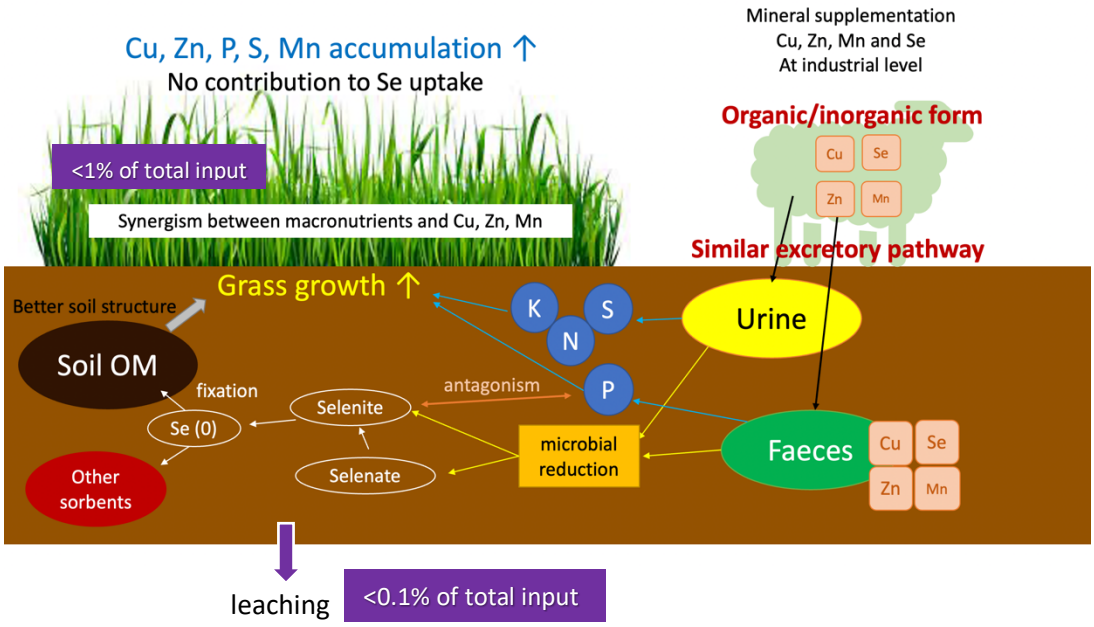


Figure 5.1 Summary of the micronutrient processes and pathways investigated in this study, including excretion, interactions in soil and uptake by grass following Cu, Zn, Mn and Se supplementation of sheep.

Figure 5.1 summarises the findings in the experiments. In order to answer the above research questions, I firstly investigated the potential impact of the forms (organic or inorganic) of minerals supplemented at typical industrial levels on nutrient excretion and partitioning in sheep urine and faeces (**Chapter 3**). In the sheep experiment, over 90% of the consumed Zn, Cu and Se, and over 60% of the consumed Mn were excreted, and among the excreted micronutrients, over 90% of the Cu, Zn and Mn, and over 80% of the Se were excreted via faeces (**Figure 3.5**). The mineral supplement treatments did not significantly influence the partitioning of micronutrient excretion between urine and faeces. Different forms of supplemental minerals did not show significant impact on the total excretion of Cu, Zn, Mn and Se. However, the chemical species of Zn, Cu, Mn and Se in the urine and faeces was not fully determined, and the difference in chemical species may make a significant difference in micronutrient availability to plants. Therefore, the form of the supplemental minerals could still have an impact on

micronutrient cycling by altering the chemical species of Zn, Cu, Mn and Se in the excreta, and requires elucidation via speciation in future studies.

To further study the influence of mineral form on micronutrient cycling in a pasture system, a lysimeter pot experiment (**Chapter 4**) was then carried out using soils of different OM content and applied with the excreta collected from the sheep experiment. The results of the pot experiment indicate that the form of the supplemental minerals had no consistent influence on the grass uptake and leaching of Zn, Cu, Mn and Se. There was also no significant interaction between the mineral form and the excreta type (urine, faeces or the combination) on micronutrient uptake or leaching. Compared to the form of the supplemented minerals, the soil OM and excreta type had more significant and consistent impact on the uptake of Zn, Cu, Mn and Se by perennial ryegrass.

The uptake of Cu, Zn and Mn by perennial ryegrass was strongly related to grass growth. Although the faeces contained a higher density of nutrients than urine, urine contributed more available N and K, which, in turn, improved the grass growth, leading to a significantly higher uptake of Zn, Cu and Mn by perennial ryegrass. Therefore, urine plays a more important role than faeces in the cycling of Zn, Cu and Mn in grazing pasture systems due to the synergisms between the macronutrients and micronutrients. The uptake of Se, on the other hand, was not related to plant growth but was determined by the availability of Se in the soil for the plant. High soil PO_4^{3-} can lead to antagonism with SeO_3^{2-} which makes SeO_3^{2-} less available to grass. Furthermore, although soil OM can help improve grass growth, it can cause more Se fixation, especially under the treatment of excreta, which drives microbial reduction and reduces Se uptake.

5.2 Applications

Findings from this study can be useful for the management of a sustainable grazing pasture system. The application of excreta onto pastures is commonly and increasingly used as a sustainable soil fertiliser for providing nutrients to grass for grazing animals (**Chapter 1.4**). However, this research showed that the micronutrient level in grass is not necessarily increased to the level required by sheep by simply applying sheep excreta (**Chapter 4.3.3**). More important than the total input of nutrients in a soil from animal excreta is the balance between the input of macronutrients, such as N in this study, and micronutrients. Although faeces contribute substantially more micronutrients to soils by mass than urine, applying urine is more efficient than applying faeces for increasing the total uptake of Cu, Zn and Mn by perennial ryegrass, due to the high input of available N from urine (**Chapter 4.4.2**). Therefore, understanding the synergism of micronutrients and macronutrients is critical when choosing a soil fertiliser that is effective and efficient.

For Se, the application of animal excreta to soil does not help to increase the uptake of Se by grass, as the input Se can be chemically reduced and then largely fixed in the soil during the decomposition process of excreta driven by microorganisms (**Chapter 4.4.2.3**). In a pasture system with soil rich in OM, the soil fixation of Se can occur to a greater extent than lower OM arable soil. Therefore, for farmers who wish to increase the inclusion level of Se of a grazing animal, it is better to supplement the animal directly than to apply animal excreta containing Se to soil.

Soil fertility maintenance is essential to the development of sustainable food production systems (Power & Prasad, 1997). To a large degree, the sustainability is dependent on the increased efficiency of output by recycling the inputs into a production system, (Power & Prasad, 1997). In this study, the mass of micronutrients taken up by the

perennial ryegrass and the amount lost to the leachate were respectively less than 1% and less than 0.1% of the overall input (**Chapter 4.3.1**). Most of the input micronutrients were retained in the soils. Although not all the nutrients retained in the soils are readily available to plants, this result highlights the importance of soil as a reservoir for micronutrients. As most of the micronutrients were retained in the topsoil, the protection of surface soil from erosion is therefore critical for storing the precious nutrients within the system and reducing pollution of water courses.

5.3 Future work

(1) Using isotope technique to evaluate the flux of micronutrients in the system: In **Chapter 3.3.2**, the data showed that the basal diet in the animal feed contributed more than 70% of the Mn, and ca. 50% of Cu and Zn. The drinking water was also found as a significant Zn intake source. The high background concentrations of Zn, Cu and Mn in the basal diet might have caused a masking effect that made the evaluation of the micronutrient return form the supplemental minerals of different treatments more difficult. In **Chapter 3.4.2.2**, it was indicated that using isotope technique might help with the evaluation of the flux of Se at farms adopting the industrial dose level of supplemental minerals, where the concentration of Se is high in the basal diet and is relatively low in the given supplemental minerals. Therefore, using isotope technique to evaluate the impact of supplemental minerals on the flux of micronutrients at typical farming scenarios is suggested.

(2) Confirming the impact of supplementing minerals on the retention of P by collecting excreta samples at time points prior to Day 0: In **Chapter 3.4.3**, the findings of decreased P in the urine and the increased S in the urine during the mineral supplementation were discussed. To confirm that the observations were truly resulted

from introducing the supplemental minerals, and not just a random data variation, a 'stable' data baseline, verified by more than two data points prior to Day 0, is required. However, it was a shame that no data of the P and S concentrations in the excreta was collected prior to Day 0. For the future work to evaluate the impact of supplemental minerals on the retention of P and S through time, sampling at days prior to Day 0 is required to show a stable baseline.

(3) Applying urine and/or faeces at different amounts and at different times in soils: In the pot experiment, the urine and/or faeces were applied at fixed amounts, which were determined using the methods proposed in **Appendix A.6**. Although the purpose of the evaluation methods proposed **(Appendix A.6)** were to replicate the on-farm situation, the amount of the urine and faeces received by soils in real situations can be quite variable. Furthermore, in the pot experiment, the urine and faeces were applied to the soils at the same time, whereas the timing of that a soil receiving urine and faeces in a natural environment is also variable. Therefore, the inclusion of different application amounts and timings of urine and/or faeces will be needed to evaluate the impact of urine and faeces on micronutrient uptake by grass in a natural grazing pasture system.

(4) Method development to consider the impact of microfauna in a pot experiment: In the current pot experiment, some of the microfauna were removed from the soils during the procedure of drying and sieving soils. Although the current procedure of soil preparation was standard to maintain the consistency of the soil environment, it omitted the potential impact of microfauna. The development of a methodology that is able to consider the impact of microfauna in a pot experiment whereas do not sacrifice the consistency of the system is needed.

(5) Including the evaluation of the impact of mixed swards in the system: In the pot experiment, only one species of pasture forages (perennial ryegrass) was included. To improve the sustainability of a grazing livestock system, it is aware that the discussion on introducing mixed swards in the system has been increasing. Therefore, it will be worth considering the inclusion of mixed swards in the evaluation of micronutrient flux in the system.

(6) Evaluating the correlation between Se concentrations in the UK forages and soil OM and/or metal oxides: In **Chapter 4.4.2.3**, it was hypothesised that the decreased Se total uptake in grassland soil and the decreased extractable Se in soil after excreta application were attributed to the microbial reduction of Se driven by the application of animal excreta, and subsequent sorption of Se onto soil OM and metal oxides. In a grazing pasture system, the soil typically contains higher organic matter than the soil of an arable land, and the pasture soil constantly receives manure from ruminants during the grazing seasons. Therefore, the issue of Se deficiency in pasture forages is expected to be more serious than in arable lands. However, an evaluation on the Se concentrations in forages across various UK pasture systems, of which the soils contain different amount of OM and/or oxides and were applied with various organic fertilizers, is lacking. Therefore, a further on-farm investigation of the impact of soil OM and metal oxides and the application of organic fertilizers on the uptake of Se by pasture forages is needed. The investigation can bridge the current findings and the practical farm management practices in terms of increasing Se recycling efficiency.

5.4 Conclusions

Through scientific research, we aim to find the best way of running a sustainable grazing livestock system where the grazing animal can live healthy and happy with the

external resource input and the resource loss reduced to the least. To reach this goal, understanding factors that influence the input, output and flux of micronutrients in the system is extremely critical.

The three major findings in this thesis are (1) the impact of the different form (organic versus inorganic) of mineral supplements (Se, Zn, Cu and Mn) on the excretion and upcycling of Se, Zn, Cu and Mn in the system is insignificant. Soil and the type of the applied excreta play are more significant. (2) The upcycling of Zn, Cu and Mn by perennial ryegrass is highly associated with the synergism effect between macronutrients (N, K and S in the current study) and micronutrients. Urine, although contains much less micronutrients compared to faeces, has greater impact to the total uptake of micronutrients due to improved growth of grass because of the high input of macronutrients from urine. (3) The concentration of Se in perennial ryegrass can be lower in soils applied with the sheep excreta. The total uptake of Se by perennial ryegrass can be significantly lower in soils with high OM or high active Fe oxides. This finding implies that the Se in perennial ryegrass grown in a grazing pasture can have

To improve the forage uptake of Zn, Cu and Mn from the soil, managing the nutrient balance between macronutrients and micronutrients in the soil is most important. To improve the forage uptake of Se from the soil, further investigation on the interaction between Se sorption in soil and microbial reduction of Se is critical.

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List of Terminology and Abbreviations

Terminology / Abbreviation	Meaning / Full name
Thesis Introduction	
Cu	Copper
Zn	Zinc
Mn	Manganese
Se	Selenium
I	Iodine
OM	Organic matter
Chapter 1	
Co	Cobalt
Fe	Iron
Mo	Molybdenum
S	Sulphur
DM	Dry Matter
Ti	Titanium
RRes-NW	Rothamsted Research, North Wyke
w/w	Weight/weight
Cys	Cysteine
His	Histidine
N	Nitrogen
P	Phosphorus
K	Potassium
FAOSTAT	Global statistics from the Food and Agricultural Organization of the United Nations
SeMet	Selenomethionine
$MnSO_4$	Manganese sulphate
MnO	Manganese (II) oxide
$MnCO_3$	Manganese carbonate
$ZnSO_4$	Zinc sulphate
Zn-Met	Zinc-methionine
$CuSO_4$	Copper sulphate
CuO	Copper (II) oxide
CuS	Copper monosulphide
SEP	Sequential extraction procedure
BCR	Bureau Community of Reference
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
R^2	Coefficients of determination
SeCys	Selenocysteine
SeO_4^{2-}	Selenate
SeO_3^{2-}	Selenite
Eh	Redox potential
S^{2-}	Sulphide
LMWOAs	Low-molecular-weight organic acids
FA	Fulvic acid
kDa	Kilodalton; 1 Da = 1 g/mol
*K*d	Adsorption coefficient

XANES	X-ray absorption near edge structure
micro-XRF	Micro x-ray fluorescence
Fe_2O_3	Hematite
$CaCO_3$	Calcite
$CaMg(CO_3)_2$	Dolomite
MnO_2	Manganese dioxide
DMSe	Dimethyl selenide
DMDSe	Dimethyl diselenide
DMSeS	Selenenyl sulphide
CH_3I	Methyl iodide
PO_4^{3-}	Phosphate
SO_4^{2-}	Sulphate
FYM	Farmyard manure
SOC	Soil organic carbon
DOC	Dissolved organic carbon
TMAH	Tetramethylammoniumhydroxide
Chapter 2	
ACU	Analytical Chemistry Unit
ICP-OES	Inductively coupled plasma- optical emission spectrometry
ICP-MS	Inductively coupled plasma - mass spectrometry
RF	Radio frequency
KED	Kinetic energy discrimination
SCD	Segmented-array charged-coupled detector
QA	Quality Assurance
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide
WEPAL	Wageningen Evaluating Programs for Analytical Laboratories
QC	Quality control
HCl	Hydrogen chloride
Cd	Cadmium
v/v	Volume/volume
HPLC-HG-AFS	High-performance liquid chromatography - hydride generator - atomic fluorescence spectrometry
Na_2SeO_3	Sodium selenite
PB	P-buffer (NaH_2PO_4/Na_2HPO_4)
LOD	Limit of detection
mADF	Modified acid detergent fibre
ADF	Acid detergent fibre
NDF	Neutral detergent fibre
ADL	Acid detergent lignin
VFAs	Volatile fatty acids
DAD	Diode array detector
TN	Total nitrogen
TC	Total carbon
KCl	Potassium chloride
Chapter 3	

IND	Industrial levels of inclusion
OH	Treatment of organic mineral supplements at the high dose
OL	Treatment of organic mineral supplements at the low dose
IH	Treatment of inorganic mineral supplements at the high dose
IL	Treatment of inorganic mineral supplements at the low dose
BCS	Body condition score
DUP	Digestible undegraded protein
ERDP	Effective rumen degradable protein
ANOVA	Analysis of variance
DNA	Deoxyribonucleic acid
$SePO_3^{3-}$	Selenophosphate
ATP	Adenosine triphosphate
$TMSe^+$	Trimethylselenonium ion
PGR	Perennial ryegrass
Chapter 4	
WHC	Water holding capacity
ARW	Artificial rainwater
PVC	Polyvinyl chloride
Fisher's LSD	Fisher's Least Significant Difference
EC	Electrical conductivity
MKP	Monopotassium phosphate
MMSe	Monomethylselenol
Appendix A	
NRC	National Research Council
LOQ	Limit of quantification
rpm	Revolutions per minute

Appendix A

Preliminary tests

A.1 Quality assurance of Se analysis using HPLC-HG-AFS

A.1.1 Introduction

HPLC-HG-AFS is a useful analytical instrument for total and speciation analysis of Se. However, at the time of carrying out this research, it had not been applied to analyse Se in faecal extracts. Various reagents were used for extracting Se from sheep faeces. Testing for quality assurance (QA) is the very first task to do before running the samples collected from the main experiment. **Figure A.1** shows some characteristics that are commonly looked at for evaluating the quality assurance (QA) of an analysis. In this test, the limit of detection (LOD) of Se analysis and the potential matrix effects of different background reagents on Se detection were evaluated.

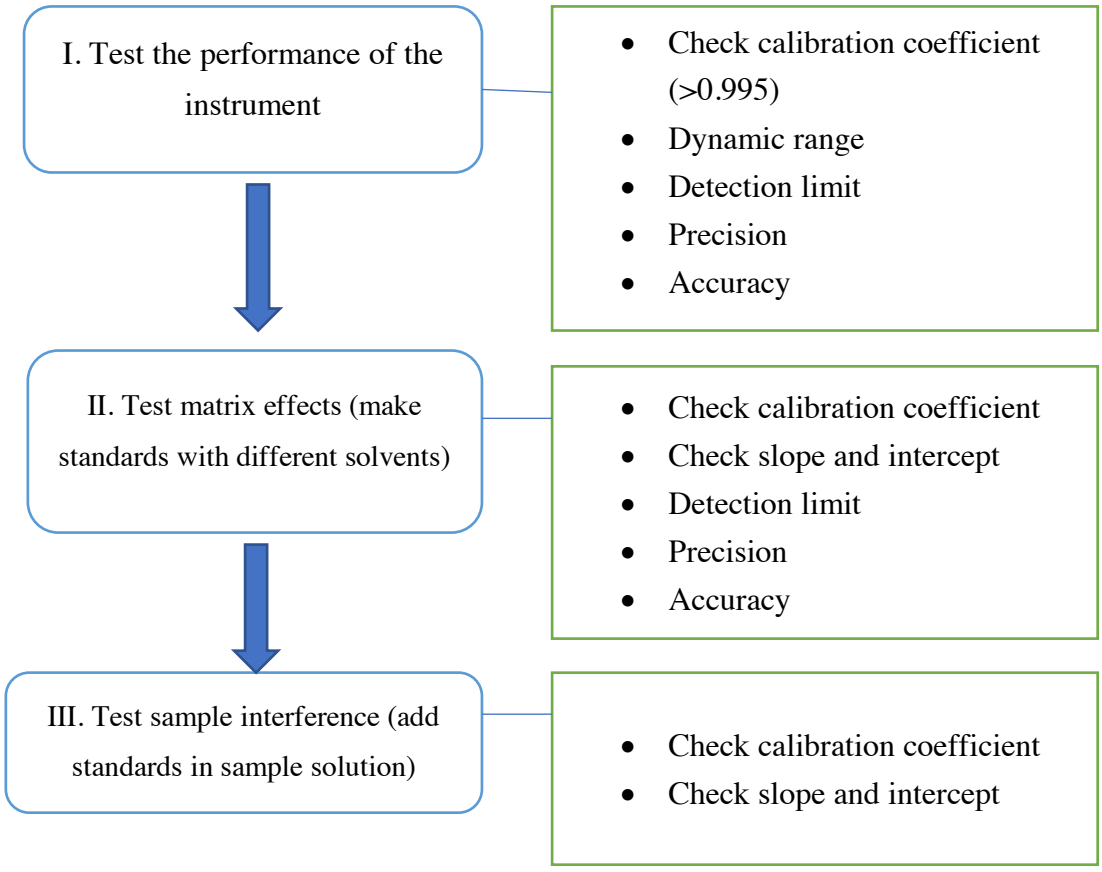

Figure A.1 Schematic diagram of QA of Se analysis using HPLC-HG-AFS.

A.1.2 Materials and methods

The Se stocks were formulated with Na_2SeO_3 (≧ 98%, Sigma®) and ultra-pure water (18 MΩ) following the formulation detailed in **Table A.1**. Two calibration curves at different ranges of Se concentrations of standard solution: 0-5 μg L^{-1} and 0-40 μg L^{-1} were made. In total, 24 blanks were run and the LOD were calculated according to the two different calibration curves. To evaluate the matrix effects of the reagents used in Se sequential extraction (**Chapter 2.2.2**), calibration curves of 0, 10, 20, 40 μg L^{-1} of Se solutions formulated using different background reagents: ultra-pure water (18 MΩ), 0.01 M KNO_3, 0.016 M KH_2PO_4, 0.008M KH_2PO_4, 2.5% TMAH, 5% TMAH, 10% TMAH or 5% HNO_3, were made.

Table A.1 Formulation of calibration samples of different Se concentrations

Concentration made to (μg L^{-1})	**Volume of Se standard solution used**	**Finished volume (mL)**
0	-	-
1.0	2.5 mL 10 μg L^{-1} Se solution	25
2.0	5 mL 10 μg L^{-1} Se solution	25
5	12.5 mL 10 μg L^{-1} Se solution	25
10	0.5 mL 1 mg L^{-1} Se stock	50
20	1 mL 1 mg L^{-1} Se stock	50
40	2 mL 1 mg L^{-1} Se stock	50

A.1.3 Results and discussion

According to the results of analysis (**Table A.2**), the detection limit of Se analysis using the HPLC-HG-AFS was 2 μg L^{-1}. The current dynamic range of analysis was about 7 to 40 μg L^{-1} according to **Table A.2**. However, the upper limit of analysis was known to be ca. 250 μg L^{-1}. In conclusion, the Se of concentrations less than 2 μg L^{-1} in a sample is defined undetectable and the analysis of Se of concentrations less than 7 μg L^{-1} would be out of the robust dynamic range of analysis.

Table A.2 Calibrations of different ranges of Se concentrations

	0-5 μg L^{-1} calibration curve	0-40 μg L^{-1} calibration curve
Number of standards	4	7
Calibration R square	0.993	0.999
Linear regression line	y = 227.05x - 8.8154	y = 196.23x + 14.765
Limit of detection	1.8 μg L^{-1}	2.1 μg L^{-1}
Limit of quantitation	6.0 μg L^{-1}	6.8 μg L^{-1}

To test the matrix effects, Se solutions made in different background reagents were analysed. The results showed that except for 0.016 M KH_2PO_4, all the reagents deliver 'good' calibration result with the value of R^2 greater than 0.995 (**Table A.3**). However, compared to the slope of linear regression line of ultra-pure water, the slopes of calibration curves made of various chemical reagents were significantly lower. This gives the conclusion that the chemical reagents led to lower sensitivity of analysis. However, since the R square values of calibration were still greater than 0.995, the analysis is still proceedable, only the calibration solutions should be made using the same background solution of sample.

Table A.3 Calibration curve of Se standards in different background solutions

Background reagents	Calibration R square	Linear regression line
Ultra-pure water	0.9994	y=197.894-45.702
0.01 M KNO_3	0.9965	y=59.094x-160.26
0.016 M KH_2PO_4	0.9722	y=50.768x-122.57
0.008 M KH_2PO_4	0.9980	y=59.867x-52.135
2.5% TMAH	0.9986	y=51.420x+33.185
5.0% TMAH	0.9996	y=52.338x+15.681
10% TMAH	0.9982	y=54.909x+32.211
5% HNO_3	0.9993	y=56.775x-57.032

A.2 Test of the faecal sample drying method

A.2.1 Introduction

In the sheep experiment, the collected faecal samples go through a sample preparation process, including drying, grinding, sieving and storage, before sample analysis. According to literature, Se loss may occur through volatilization, and this could happen during the drying process during which the moisture content of the sample can drive chemical reactions. To ensure that no element loss during the drying process occurred, different drying methods were trialled: air-drying, oven-drying (at 80°C) and freeze-drying. The dried samples then proceeded through the preparation procedures (grinding, sieving and storage) before analysis of total element content, Co, Fe, Cu, Mn, Zn and Se, using ICP-MS or ICP-OES or the analysis of sequential extractions.

A.2.2 Materials and Methods

Sheep manure samples (approx. 250 g fresh weight) were collected from sheep offered 28g/head/day of a mineral blend on top of a basal diet of grass silage for 7 days. The ingredients of the blend are shown in **Table A.4** below. The collected faeces were well mixed as one bulked sample and then divided into three subsamples for the three different drying methods: air-drying, oven-drying at 80°C, and freeze-drying. Each drying method had three technical replicates drying in individual containers. The drying was considered complete when the sample weight remained constant (< 0.1% DM change) with at least 6 hours additional drying time (Peters et al., 2003). Grinding, sieving and storage followed the methods commonly used in soil samples (Stroud et al., 2012). The total element analysis and sequential extractions followed the methods of **Chapters 2.1** and **2.2**, respectively.

Table A.4 Nutrient contents of the mineral blend given to the sheep

Ingredient	Concentration
Calcium	23.50 %
Magnesium	5.00 %
Sodium	8.65 %
Phosphorus	2.00 %
Vitamin A	300000 iu kg^{-1}
Vitamin D3	800000 iu kg^{-1}
Vitamin E	2500 iu kg^{-1}
Vitamin B12	1000 mcg kg^{-1}
Iodine (Calcium iodate, anhydrous)	200 mg kg^{-1}
Manganese (Mn (II)-oxide)	2000 mg kg^{-1}
Zinc (Zinc oxide)	6000 mg kg^{-1}
Selenium (Sodium selenite)	30 mg kg^{-1}
Iron (Iron sulphate monohydrate)	1000 mg kg^{-1}
Cobalt (Coated granulated carbonate)	50 g kg^{-1}

A.2.3 Results and discussion

The results showed no significant difference between different drying methods regarding total micronutrients concentrations in faeces, and the microwave-assisted HNO_3/H_2O_2 digestion method is suitable for the targeted elements according to the digestion recovery rates of the elements ranging within 100% ± 10% (**Table A.5**).

Table A.5 Total concentrations of micronutrient in faeces of different drying methods

Drying methods	Co (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})	Se (µg kg^{-1})
Air drying	3.139 ± 0.094	38.05 ± 1.37	1348 ± 19	718.8 ± 11.7	324.1 ± 7.2	1164 ± 33
Oven drying (80°C)	3.427 ± 0.119	40.23 ± 2.50	1476 ± 84	742.6 ± 8.0	341.8 ± 2.5	1218 ± 24
Freeze drying	3.770 ± 0.298	37.94 ± 0.87	1454 ± 43	749.1 ± 11.7	345.7 ± 7.2	1307 ± 53
P level	0.1097	0.5838	0.2605	0.1485	0.0595	0.0800
Digestion recovery %*	94.6	91.6	94.7	105.0	95.1	103.0

*The recovery rate was calculated in reference to the certified now manure sample (MARSEP275) presented in Appendix Table B.1

The results of sequential extractions show that, the revised-BRC SEPs all had good extraction recovery rates (100 ± 10%) for the metallic elements, including Co, Cu, Fe, Mn and Zn, across the three different sample drying methods **(Table A.6)**. This confirms that different faecal sample drying methods would not affect the recovery rates of the elements in the revised-BCR extraction. For Se, since there was no available data for step 2 and step 3 due to the low detectable peak signals in HPLC-HG-AFS, so no recovery rate was generated. Later for the samples collected in the main experiment, samples were analysed using ICP-MS and the extraction recovery rate was evaluated. Despite there being no difference in the extraction recovery rate, significant differences in element concentrations of extracts in some fractions were observed across different drying methods. However, since there was no certified standard samples to compare the differences with, there was no way to determine which drying method was 'the best' based on the results we had. What is noticeable is that more of the Co, Fe, Mn, Se extracted from the freeze-dried samples tended to stay in the first fraction and less of the Co, Cu, Fe tended to stay in the third fraction than air-dried or oven-dried samples. In the main experiment, we used oven-drying method to dry the faecal samples because it is faster and easier to deal with than the air-drying or freeze-drying method.

Table A.6 The concentrations of elements in the extracts of the SEPs and extraction recovery rates

		Co (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})	Se (μg kg^{-1})
Step 1	Air drying	0.872 ± 0.060^{b}	1.943 ± 0.041	27.59 ± 0.438^{b}	467.3 ± 12.00^{b}	99.907 ± 2.796^{c}	150.3 ± 17.27^{ab}
	Oven drying	0.955 ± 0.041^{b}	2.718 ± 0.086	33.19 ± 0.220^{b}	516.9 ± 4.859^{a}	119.9 ± 3.974^{a}	103.1 ± 19.21^{b}
	Freeze drying	1.456 ± 0.161^{a}	2.517 ± 0.323	56.71 ± 5.600^{a}	511.1 ± 12.83^{a}	109.9 ± 0.453^{b}	175.9 ± 7.879^{a}
	p-values (>F)	**0.014 ***	0.070	**0.002 ****	**0.030 ***	**0.007 ****	**0.042 ***
Step 2	Air drying	0.624 ± 0.058	7.833 ± 0.126^{c}	464.9 ± 7.896	134.5 ± 5.993	179.7 ± 4.363	N.A.
	Oven drying	0.635 ± 0.012	8.714 ± 0.109^{b}	461.7 ± 13.41	132.3 ± 3.272	175.3 ± 2.982	N.A.
	Freeze drying	0.748 ± 0.063	10.74 ± 0.232^{a}	483.6 ± 9.663	123.0 ± 2.903	167.9 ± 6.450	N.A.
	p-values (>F)	0.230	**<0.001 *****	0.3534	0.211	0.290	N.A.
Step 3	Air drying	0.931 ± 0.011^{b}	19.93 ± 0.214^{a}	388.6 ± 18.05^{a}	16.67 ± 1.224	16.447 ± 1.121	N.A.
	Oven drying	1.155 ± 0.044^{a}	20.15 ± 0.411^{a}	373.6 ± 13.92^{a}	17.01 ± 0.653	19.23 ± 0.657	N.A.
	Freeze drying	0.594 ± 0.053^{c}	15.56 ± 0.575^{b}	289.1 ± 24.18^{b}	16.20 ± 1.493	23.09 ± 2.669	N.A.
	p-values (>F)	**<0.001 *****	**<0.001 *****	**0.021 ***	0.890	0.086	N.A.
Step 4	Air drying	0.175 ± 0.003^{ab}	2.790 ± 0.926	296.8 ± 24.51	5.398 ± 0.505	3.604 ± 0.485	235.4 ± 88.86
	Oven drying	0.229 ± 0.027^{a}	2.325 ± 0.083	327.0 ± 16.63	6.483 ± 0.329	9.526 ± 5.417	258.3 ± 72.62
	Freeze drying	0.134 ± 0.010^{b}	1.818 ± 0.098	307.2 ± 51.46	5.702 ± 0.310	4.432 ± 0.771	124.7 ± 30.02
	p-values (>F)	**0.0199 ***	0.4876	0.8231	0.2102	0.4147	0.3952
Recovery rate	Air drying	98.1%	101%	103%	103%	110%	N.A.
	Oven drying	101%	98.1%	94%	105%	110%	N.A.
	Freeze drying	91.3%	94.7%	91.7%	103%	104%	N.A.

N.A.: The data is not available.

A.3 Test of the mineral contents in drinking water provided from the sheep facility

A.3.1 Introduction

Drinking water is a potential source of minerals for livestock. Before the beginning of the sheep experiment, the contents of micronutrients in the drinking water provided in the small ruminant facility were analysed to test whether drinking water might be an important variable to consider.

A.3.2 Materials and methods

The water supplied for the studied site was classified as soft (total hardness level = 15 mg-calcium L^{-1}) and was at pH = 7.86. There were 24 sheep pens with one galvanised drinking trough in each pen in the small ruminant facility. The water drinking troughs were cleaned using a nylon-brush before sampling. Each water sample consisted of a 1-2 mL subsample from each drinking trough (bulked from 24 individual samples). The 24 subsamples were taken using a clean syringe and mixed together in a clean polypropylene tube as one sample. In total, three replicates were taken. The samples were filtered through Whatman No.42 filter paper before analysis by ICP-MS or ICP-OES.

A.3.3 Results and discussion

The concentrations of Co, Se and Fe in the drinking water were close to or below the detection limits of analysis (**Table A.7**). All the minerals had low concentrations in the drinking water, except for zinc (Zn) (1.82 ± 0.02 mg L^{-1}). Assuming a sheep drinks 2-6 L of water daily, the sheep could consume 3.6-10.9 mg Zn d^{-1} from water alone. In the sheep experiment, the sheep were fed a concentrate containing Zn in different forms at a requirement level (104 mg Zn kg^{-1}). Extrapolating the daily intake of the concentrate (approx. 600 g d^{-1}), the Zn provision from the drinking water contributed circa 6 – 18 % of the daily Zn intake. Therefore, Zn content in

drinking water should be taken into consideration within animal nutrition studies which use galvanized troughs to fully determine Zn daily intake.

Table A.7 The concentrations of the micronutrients in the drinking water samples

	Co ($\mu g\ L^{-1}$)	**Cu** ($\mu g\ L^{-1}$)	**Fe** ($mg\ L^{-1}$)	**Mn** ($\mu g\ L^{-1}$)	**Se** ($\mu g\ L^{-1}$)	**Zn** ($mg\ L^{-1}$)
Sample 1	0.01	2.33	0.010	3.88	0.05	1.810
Sample 2	0.04	3.36	0.011	4.35	0.03	1.851
Sample 3	0.01	1.91	0.012	3.92	0.03	1.811
Mean ± SD	0.02 ± 0.02	2.53 ± 0.75	0.011 ± 0.001	4.05 ± 0.26	0.04 ± 0.02	1.820 ± 0.020
Detection limits of the ICP-MS or ICP-OES	0.05	0.03	0.005	0.02	0.04	0.006

A.4 Test of Se content in sheep exhalation using a bag collection technique

A.4.1 Introduction

Selenium, when dosed to sheep at high levels (>2 mg kg^{-1} body weight), was found to be significantly lost through exhalation within four hours after dosing (Davis *et al*., 2013; Tiwary *et al*., 2005) quantified using a bag collection technique. Significantly higher Se was found from the sheep supplemented with sodium selenite (Na_2SeO_3) or selenomethionine (Se-Met) than from control sheep with no supplement. However, data on Se exhalation from sheep fed with Se at requirement level (0.23 mg kg^{-1} diet) regulated by (NRC, 2007) using the same analysing technique is lacking. We therefore performed a preliminary trial to determine whether this technique is suitable for detecting Se exhalation from sheep supplemented Se at the NRC level, to help inform future full Se-balance trials comparing organic and inorganic Se.

A.4.2 Material and methods

Following the methods proposed by Tiwary *et al*. (2005), sheep exhalate was collected by a hand-made breath collection apparatus (**Figure A.2**), encompassing a breathing mask connected to a non-rebreathing valve allowing the sheep to breath in normally from one side of the valve and to exhale out on the other side of the valve into a 3 L collection bag. Two sheep were housed together in the small ruminant facility for five days. During housing, the sheep had their diet individually titrated from a 100% forage diet (grass silage) to a 60:40 forage: concentrate (Control) diet. On the last housing day, sheep were fed a concentrate containing 0.6 mg-Se kg^{-1} (OH), in the form of Se-yeast (with Se-Met as the dominant form) to provide 0.23 mg kg^{-1} of the total diet as recommended by NRC. The nutrient composition of the feed concentrates are referred to the 'Control' and the 'OH' concentrates, respectively, in **Table 3.4**. A 2 L exhalate sample was collected from each sheep at the following time points on the last day: before feeding, and 4 h and 8 h after the feeding of Se-containing concentrate. The collected exhalate sample was passed through an activated charcoal tube (containing two

compartments) at 1 L min^{-1} flow rate for 2 min. The sampling timepoints and the associated sampling and analytical methodologies are according to Tiwary *et al*. (2005) as to when significant Se exhalations were observed in their study. To analyse the Se sorbed on the charcoal, the charcoal was extracted with 3 mL 50:50 ratio of ethanol and water on a rotary shaker (at 200 rpm) for 2 h. After the extraction, the extracts were centrifuged at 500 *g* for 10 min. The ethanol extracts were mixed with distilled water at a volume ratio of extract: final analyte = 1:10 or 1:5 to evaluate potential matrix effects in ICP-MS analysis. The analytes were kept at 4°C and analysed within two days from sample extraction.

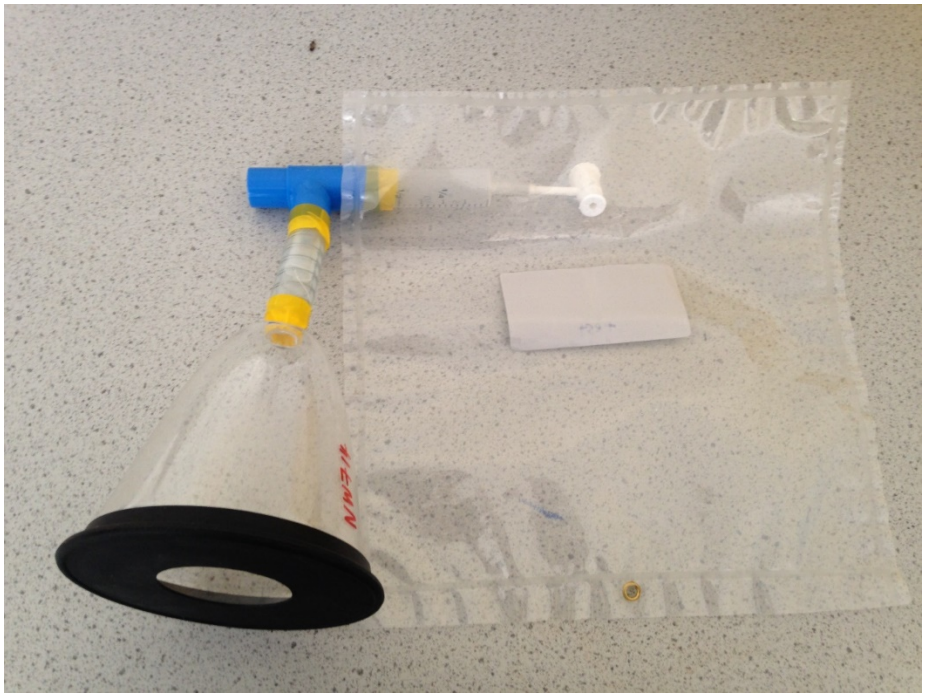

Figure A.2 Apparatus for collecting sheep exhalate.

A.4.3 Results and discussion

The results showed that none of the exhalates collected after 4 or 8 h were different to the Se content of the controls (samples before Se-supplementation), and all the Se concentrations in the exhalate extracts were close to the detection limits of ICP-MS (0.05-0.1 µg L^{-1}) (**Table A.8**). To measure the total exhalation of Se from standard diets more precisely, a chamber system that enables collecting exhalate for a longer period may be more suitable for the detection of low-level Se exhalation than the mask-collection method used in this preliminary trial to

determine if exhalated Se should be included in future trials. According to the results of this preliminary trial, I did not collect sheep breath samples in the main sheep experiment as this would result in stress for the animals for no scientific gain, as the levels observed were close to detection limits.

Table A.8 ICP-MS analysis of Se concentrations in the exhalate extracts and the calculated total Se content in the 2L exhalate samples

Sample collection time	Sheep number	Se concentrations in the ICP-MS analyte ($\mu g\ L^{-1}$)	Volume ratio of extract in the final analyte	Calculated Se in the 2L-exhalate sample (μg)
Before Se-feeding	Sheep 1	0.044	1:10	0.001
		0.091	1:5	0.001
	Sheep 2	0.043	1:10	0.001
		0.094	1:5	0.001
4 hours after Se-feeding	Sheep 1	0.037	1:10	0.001
		0.085	1:5	0.001
	Sheep 2	0.047	1:10	0.001
		0.071	1:5	0.001
8 hours after Se-feeding	Sheep 1	0.045	1:10	0.001
		0.112	1:5	0.002
	Sheep 2	0.045	1:10	0.001
		0.062	1:5	0.001

A.5 Test of the extraction method for Se speciation analysis of faeces

A.5.1 Introduction

Although there are studies in the literature investigating Se species in soil (Stroud et al., 2012), there is no study investigating Se species in ruminant faecal sample to the author's knowledge. The aim of this preliminary study was to determine a method for extracting plant-available Se from faeces for Se speciation analysis on an HPLC-HG-AFS (LOD = 2.0 μg L^{-1}; LOQ = 7.0 μg L^{-1}). It is known that the inorganic forms of Se, i.e. SeO_3^{2-} and SeO_4^{2-}, are available to plants, whereas Se in amino acids, e.g., SeMet and SeCys are also plant available. Since there is no established method for plant-available Se extraction in faecal sample, the extraction methods adopted in this study refer to the methods that are commonly used in Se extraction in soil (Stroud et al., 2012). Referring to the methods that were adopted to extract soil samples, two different P solutions (KH_2PO_4 solution and P-buffer (NaH_2PO_4/Na_2HPO_4) solution) at two different concentrations (0.06 M or 0.016 M) were used to extract faecal samples collected from the sheep experiment (IH and OH groups were used for this preliminary test). Secondly, oven-dried/freeze-dried faecal samples of the IH and OH group were extracted and the results compared for potential differences.

A.5.2 Material and methods

Faecal samples before dehydration are not suitable for this analysis because the moisture content in faeces dilute the analyte. If it is necessary to use fresh faecal samples, the moisture content needs to be measured and the sample weight needs to be changed accordingly to maintain the suitable extraction ratio of faeces:extractant. The faecal samples used in this preliminary test were collected from the OH and IH groups of the sheep experiment. Samples were either oven dried at 80°C or freeze dried.

An aliquot of 5 g of dried faeces was weighed into a 50 mL polypropylene tube, and then 30 mL of the extractant (0.06 M KH_2PO_4 or P-buffer) was added into the tube to make a ratio of faeces and extractant= 1:6 (w/v). Stroud et al. (2012a) found that a one-hour extraction of KH_2PO_4 solution was sufficient for soil samples, with no additional SeO_3^{2-} extracted after 3, 6, 24 h of shaking. One hour extraction was also used in other studies of Se species analysis in soils (Martens & Suarez, 1996, 1997). Therefore, in this study, the one-hour approach was adopted. Samples were extracted by mechanical shaking at room temperature for 1 h. After shaking, the samples were centrifuged at rpm= 2500 for 5 min. The supernatant was filtered through a 0.45 μm syringe filter into an analyte tube and analysed immediately in HPLC-HG-AFS.

A.5.3 Results and discussion

Both the faecal extracts of IH and OH group showed the peak of SeO_3^{2-}, and no difference was found across extracts of different P solutions (**Figure A.3**). The pH of KH_2PO_4 solution is about 4.5, and the pH of the P buffer is 7.4. The results, accordingly, suggest that the pH of the P solutions does not cause difference in the extractable Se species. The ratio of faeces:P solution = 1:6 (w/v) was suitable to produce a viable signal peak. It was also found that in order to get enough analyte, the amount of the dried faeces for each extraction should not be less than 2 g.

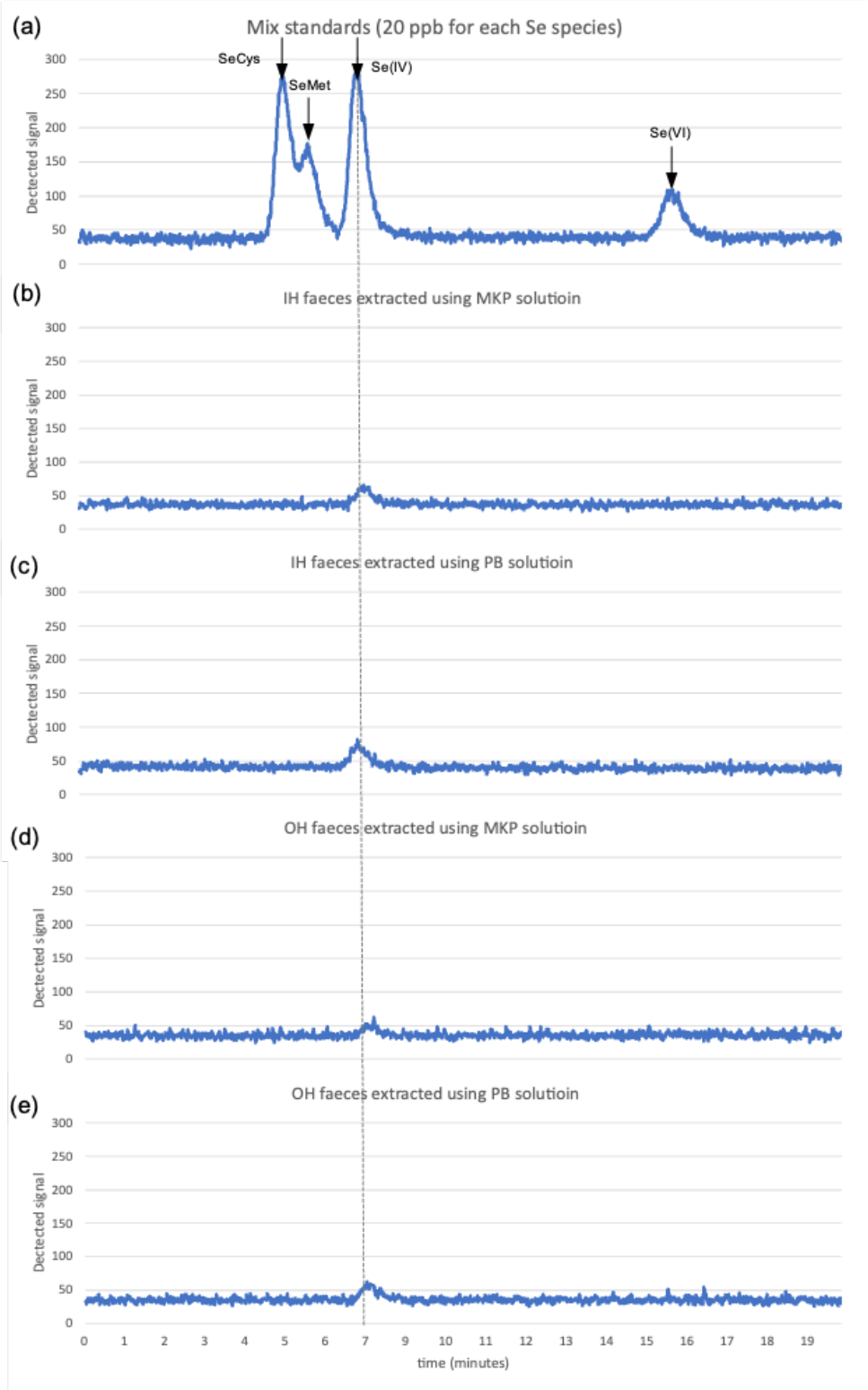

Figure A.3 HPLC chromatography of Se speciation analysis on faeces extracted by different P solutions. The results presented are the chromatographic fingerprints of (a) mixed standard solutions of selenium, and extracted faeces of (b) IH treatment using MKP (KH_2PO_4) solution (c) IH treatment using P-buffer (NaH_2PO_4/Na_2HPO_4) (d) OH treatment using MKP solution (e) IH treatment using P-buffer .

A.6 Calculation of urine and faeces densities in field patches

A.6.1 Introduction

To determine the appropriate application rate of faeces and urine in the pot experiment, two approaches were trialled to determine the best approach for the main experiment: method 1 – to assess the size of excretion events from grazing fields at North Wyke; method 2 – to rely on the literature from previous grazing experiments. In this preliminary test, both methods were applied to compare the difference and the application rate of faeces and urine for the pot experiment were decided accordingly.

A.6.2 Materials and methods

Field faecal sample collection and density calculation

Photos (n=30) of fresh sheep faecal patches from the farm field at North Wyke were taken. The area of the sheep faeces in the same photo were then measured using the software 'SketchAndCalc™ ' developed by iCalc®. The scale of a grid was calibrated by drawing several straight lines on the scale meter and changing the scale of the canvas accordingly. After calibrating the scale, lines were circled around the edge of the faecal patch (**Figure A.4**).

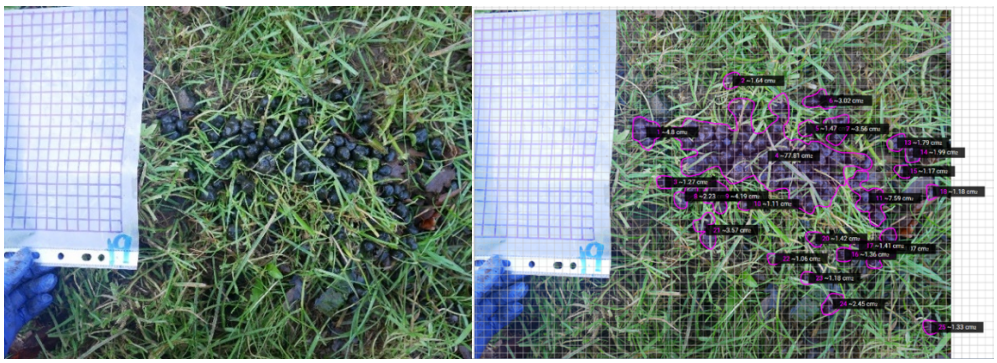

Figure A.4 Sheep faeces patch photo taking and area calculation

The area circled was calculated accordingly. At the end, the areas of all the circled faecal patches were summed to give the total area of a faecal patch. Afterwards, the faeces of the sample patch were collected by gloved hands into an aluminium-foil box and put in an oven set at 105°C for 48 h. This gave the DM content of the faeces of the same patch. The density of faeces in field (g cm^{-2}) was then calculated accordingly using **Equation A.1**:

$$D_F(faecal\ patch) = \frac{In_F\ (faecal\ patch)}{A\ (faecal\ patch)} \quad \textbf{(A.1)}$$

$$D_F(field) = \frac{In_F\ (field)}{A(field)} \quad \textbf{(A.2)}$$

In **Equation A.1 and A.2**, D_F = faecal density in either a faecal patch or a field, In_F = the input of faeces in dry matter weight, A= the area included in the measurement (the area of a faecal patch or the area of a field.) It should be remembered that the faecal density calculated using **Equation A.1** is higher than the faecal density calculated using **Equation A.2**, because in the later calculation, the area of a field includes both areas that are and are not covered by faeces.

Urine density calculation

Method 1: The urine density (mL cm^{-2}) was calculated according to the daily excretion ratio of urine and faeces (**Equation A.3**). However, this calculation assumes that the daily frequency of urinating and defecating of a sheep are equal, which might overestimate urine density. According to initial statistical results, the data of ratios of the excretion of urine:faeces was right skewed (n=23). Therefore, the data was logarithmically transformed. The transformed mean of the ratios was 0.678, which gave the final ratio = 4.764 mL g-DM^{-1} after inversing the data transformation by exponential function.

$$D_U = D_F \times R_{U:F} = D_F \times 4.764 \quad \textbf{(A.3)}$$

In **Equation A.3**, D_U= urine density in a patch (mL cm^{-2}), D_F= faecal density in either a faecal patch (g-DM cm^{-2}), $R_{U:F}$ = the ratio of the excretory amount of urine and faeces (mL g-DM^{-1}).

Method 2: Calculated the urine density (mL cm^{-2}) according to the observed areas of urine patches in field reported by Doak (1952) and the average volume of each urination of sheep reported by Sears et al. (1942).

A.6.3 Results and discussion

The investigated faecal densities were averaged after confirming the normality of the data points (**Figure A.5**). The calculated mean via Method 1 of the faecal density of a patch was 0.6 g cm^{-2} (**Table A.9**). The average urine density, calculated using **Equation A.3** was 2.8 mL cm^{-2} according to the calculated faecal density (**Table A.10**). However, it should be remembered that the faeces and urine distribution in field calculated using the densities of Method 1 tend to be overestimated. This is because, **Equation A.1** excludes the area not covered by faeces but still around or within the same faecal patch. If calculated via **Equation A.2**, which is used to estimate the average faeces input across a field, the faecal density is much lower. For example, White (1960) reported a sheep faecal density in a grassland field of 119 cm^3 m^{-2} with a sheep density of 157 heads per 100 ha. This faecal density is less than 0.05 g cm^{-2}, considering the various moisture content of faeces samples.

Method 2 used urine density data reported from the literature. The surface area of soil covered by faeces and urine patches at grazing pasture tend to be small and not evenly distributed (Williams & Haynes, 1990). The average covered surface areas reviewed by (Williams & Haynes, 1990) were 30 and 10 cm^2 for sheep urine and faeces, respectively, which were much lowered than the urine covered area (= 290 cm^2) reported by (Doak, 1952) and the area covered by faeces observed via Method 1 in the current study (range = 14 to 134 cm^2, mean= 43 cm^2). The observed urine patch area observed by Doak (1952) ranged from 25 to 54 in^2 (= 161 to 348 cm^2) and with an average of 45 in^2 (= 290 cm^2). Doak (1952) reported a daily volume of urine from sheep varied between 1700 and 3800 mL, with an average of 2900 mL. The volume observed from the sheep experiment (**Chapter 3**) was 1760 mL averaged across 24 housed

sheep. Doak (1952) used a value of 150 mL as the average volume of each urination event. This number was referred from Sears and Goodall (1942) in which an electrical counting device was operated to record the volume of urination. Using the volume of 150 mL and the mean urinated area 290 cm^2, it gives a urination density = 0.52 mL cm^{-2}, which is five times lower than the density calculated via Method 1.

The calculated faeces and urine densities in field can vary widely depending on the method of calculation. In the pot experiment (**Chapter 4**), if using the faeces and urine densities calculated via Method 1, 80 g-DM and 370 mL of faeces and urine, respectively, would be applied to each pot. For faeces, the 80 g-DM equals to ca. 320 g moist faeces at the moisture = 75%. Mixing 320 g moist faeces into the soil layer could change the soil structure to a great extent. The change of soil physical structure could become a significant covariate and, therefore, should be prevented as much as possible. Furthermore, the pot experiment was originally designed to mimic the environment of grassland fields as much as possible, the overestimated faeces and urine densities could mislead the interpretation of the result. Therefore, in the pot experiment (**Chapter 4**), the lower urine density = 0.52 mL cm^{-2} calculated via Method 2 was applied, which gives the application amount of 70 mL urine/pot. Calculated using the urine density of 0.52 mL cm^{-2} and the excretion ratio of urine and faeces of 4.764 mL g-DM^{-1} (Method 1), a faeces density of 0.11 g-DM cm^{-2} (15 g-DM faeces pot^{-1}) was adopted as the reference of the application amount of faeces. However, since the faeces was applied 'into' the soil instead of being applied on the soil surface, the eventual application amount was adjusted according to the volume of the lysimeter pot.

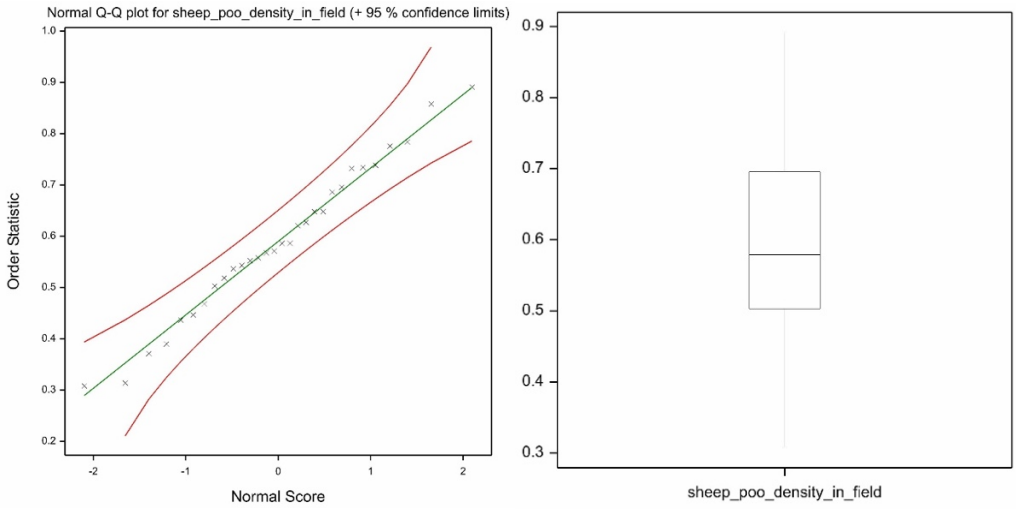

Figure A.5 Normal Q-Q plot (left) and box plot (right) of data of the investigated faeces densities (g-DM cm^{-2}).

Table A. 9 Statistical results of the investigated faeces densities (g-DM cm^{-2})

Number of observations =	30
Mean =	**0.590**
Median =	0.579
Minimum =	0.308
Maximum =	0.891
Lower quartile =	0.503
Upper quartile =	0.696

Table A.10 Results of calculated urine densities via Method 1

Sample number	Faeces density (g-DM cm^{-2})	Calculated urine density (mL cm^{-2})
1	0.3	1.5
2	0.5	2.6
3	0.5	2.6
4	0.4	1.9
5	0.6	2.7
6	0.6	2.8
7	0.4	2.1
8	0.6	2.6
9	0.5	2.5
10	0.7	3.3
11	0.6	2.7
12	0.6	3.0
13	0.4	2.1
14	0.6	2.7
15	0.5	2.4
16	0.7	3.5
17	0.7	3.5
18	0.6	2.8
19	0.3	1.5
20	0.5	2.2
21	0.9	4.2
22	0.7	3.3
23	0.6	3.1
24	0.6	3.1
25	0.4	1.8
26	0.9	4.1
27	0.6	3.0
28	0.8	3.7
29	0.8	3.7
30	0.7	3.5
Average	**0.6**	**2.8**

Appendix B

Supplementary data

Table B.1 Total concentrations of elements in the certified standard samples

Element	MARSEP 275 (cow manure)	IPE 154 (grass)	ISE 962 (Soil)
Cadmium	0.307 mg kg^{-1}	0.006 mg kg^{-1}	0.237 mg kg^{-1}
Cobalt	2.86 mg kg^{-1}	0.085 mg kg^{-1}	9.67 mg kg^{-1}
Copper	85.5 mg kg^{-1}	7.16 mg kg^{-1}	13 mg kg^{-1}
Iron	0.937 g kg^{-1}	331 mg kg^{-1}	30700 mg kg^{-1}
Magnesium	401 mg kg^{-1}	3879 mg kg^{-1}	9420 mg kg^{-1}
Molybdenum	4.85 mg kg^{-1}	0.977 mg kg^{-1}	0.438 mg kg^{-1}
Phosphorus	19.5 g kg^{-1}	5139 mg kg^{-1}	754 mg kg^{-1}
Sulphur	5530 mg kg^{-1}	3460 mg kg^{-1}	1880 mg kg^{-1}
Selenium	852 μg kg^{-1}	0.032 mg kg^{-1}	0.421 mg kg^{-1}
Zinc	396 mg kg^{-1}	39.4 mg kg^{-1}	85.8 mg kg^{-1}

Table B.2 Summary table of sheep liveweights by allocation block

Block	Minimum weight (kg)	Maximum weight (kg)
1	50.0	52.0
2	52.5	54.0
3	55.5	56.5
4	57.0	58.0
5	58.5	60.0
6	60.5	61.5

Table B.3 Summary table of sheep liveweight by treatment

Treatment	Minimum weight (kg)	Maximum weight (kg)
OH	50.5	61.0
OL	50.0	61.5
IH	51.5	60.5
IL	52.0	61.5

Table B.4 Silage dry matter intake of the sheep under different supplementary treatments on different days

Treatment	Day0	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10	Day11	Day12	Day13	Temporal effect p-value (>F)
IL	741±38.2	768±41.5	772±39.0	764±39.8	775±41.7	780±42.8	793±44.4	724±45.2	781±54.6	749±47.0	752±48.4	751±47.8	761±47.9	760±50.1	0.9891
IH	809±43.2	762±69.9	769±70.8	765±71.9	759±77.1	779±61.9	781±61.8	699±50.0	730±51.5	700±59.6	695±58.7	704±58.9	690±52.1	709±58.2	0.9378
OL	590±76.1	583±95.5	635±68.6	605±78.9	622±74.7	651±55.8	635±51.5	689±47.4	755±67.2	736±58.8	737±59.7	739±58.2	754±57.9	746±65.5	0.0696
OH	731±37.0	767±38.2	774±39.6	765±35.5	809±46.1	778±42.4	784±40.5	735±32.1	827±41.6	781±34.5	779±35.8	786±36.4	809±41.2	805±41.4	0.8385
Treatment effect p-value (>F)	0.08148	0.1590	0.2580	0.1870	0.2333	0.2321	0.1155	0.8856	0.7153	0.7890	0.7653	0.7883	0.5016	0.7298	

The ANOVA model for the analysis of temporal effect was: y~Block + Day. The ANOVA model for the analysis of treatment effect was: y~Block + Treatment.

Table B.5 Concentrate intake of the sheep under different supplementary treatments on different days

	Total amount of the intake concentrates (g day^{-1} $animal^{-1}$ ± SE)*														
Treatment	Day0	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10	Day11	Day12	Day13	Temporal effect p-value (>F)
IL	604±32.0	604±32.0	604±32.0	604±32.0	604±32.0	604±32.0	604±32.0	604±32.0	604±32.0	604±32.0	604±32.0	604±32.0	604±32.0	604±32.0	0.4175
IH	613±45.3	613±45.3	613±45.3	613±45.3	597±54.4	597±54.4	597±54.4	597±54.4	597±54.4	597±54.4	597±54.4	597±54.4	597±54.4	597±54.4	0.4618
OL	555±40.1	540±49.2	540±49.2	540±49.2	514±55.7	517±40.5	517±40.5	517±40.5	501±40.5	501±45.5	501±45.5	501±45.5	501±45.5	501±45.5	0.5581
OH	613±33.9	613±33.9	613±33.9	613±33.9	613±33.9	613±33.9	613±33.9	613±33.9	613±33.9	613±33.9	613±33.9	613±33.9	613±33.9	613±33.9	0.0633
Treatment effect p-value (>F)	0.7588	0.7205	0.7205	0.7205	0.8512	0.7472	0.7472	0.7472	0.6187	0.6187	0.6187	0.6187	0.6187	0.6187	

*The amount of the concrete was weighed before given to the animal and was checked that the given amount was all consumed by the designated animal to maintain a 60:40 ratio of silage: concentrate intake. The ANOVA model for the analysis of temporal effect was: y~Block + Day. The ANOVA model for the analysis of treatment effect was: y~Block + Treatment.

Table B.6 Faecal and urinary excretion across different treatment groups throughout the experimental period and ANOVA test results

Faecal excretion (g-DM day^{-1} $animal^{-1}$ ± SE)					
	Day 1	Day 3	Day 7	Day 10	Day 14
IL	297 ± 33.3	365 ± 42.4	353 ± 25.9	381 ± 21.0	360 ± 31.7
IH	325 ± 14.4	394 ± 42.2	359 ± 27.3	396 ± 37.4	379 ± 36.5
OL	263 ± 49.4	323 ± 76.3	310 ± 38.3	314 ± 40.1	327 ± 28.1
OH	348 ± 14.3	330 ± 29.9	378 ± 25.7	383 ± 24.4	354 ± 38.3
P level					
F_{form}	0.6630	0.4053	0.9486	0.8008	03206
F_{dose}	0.8708	0.7727	0.1392	0.6286	0.7285
F_{form} x F_{dose}	0.0753	**0.0410***	0.0609	0.1627	0.8881
Urine excretion (L day^{-1} $animal^{-1}$ ± SE)					
	Day 1	Day 3	Day 7	Day 10	Day 14
IL	1.20 ± 0.249	0.96 ± 0.200	1.43 ± 0.293	1.46 ± 0.219	1.61 ± 0.302
IH	1.65 ± 0.321	1.60 ± 0.396	2.43 ± 0.425	1.96 ± 0.279	1.71 ± 0.266
OL	1.79 ± 0.173	1.70 ± 0.150	2.00 ± 0.285	1.90 ± 0.339	1.67 ± 0.252
OH	1.27 ± 0.207	1.25 ± 0.262	1.85 ± 0.162	1.65 ± 0.270	1.88 ± 0.328
P level					
F_{form}	0.3528	0.4960	0.5467	0.5617	0.5431
F_{dose}	0.1801	0.5194	0.8822	0.8546	0.3245
F_{form} x F_{dose}	0.5867	0.6216	0.1807	0.2500	0.1550

'*' indicates statistical significances at p-value< 0.05.

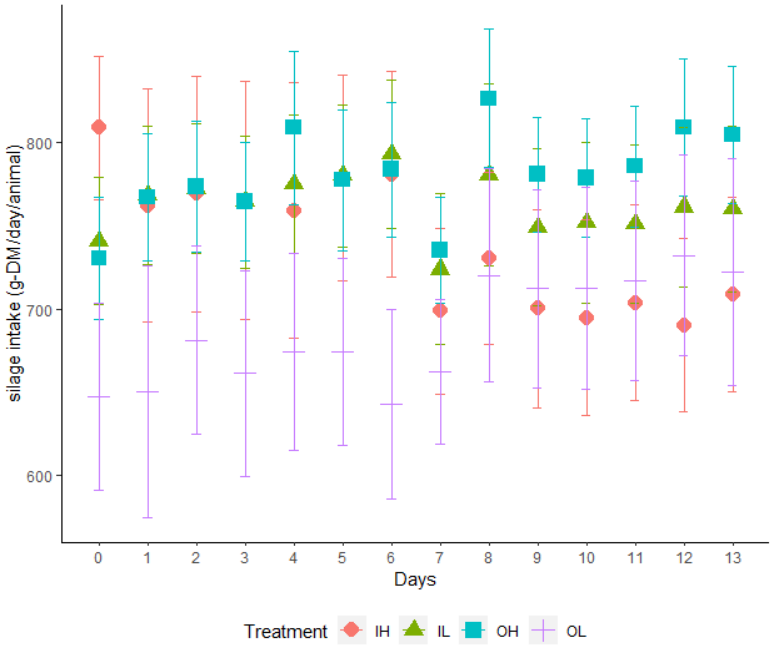

Figure B.1 Daily silage intake (g-DM day^{-1} animal^{-1}) by treatments. The error bars are the calculated standard errors.

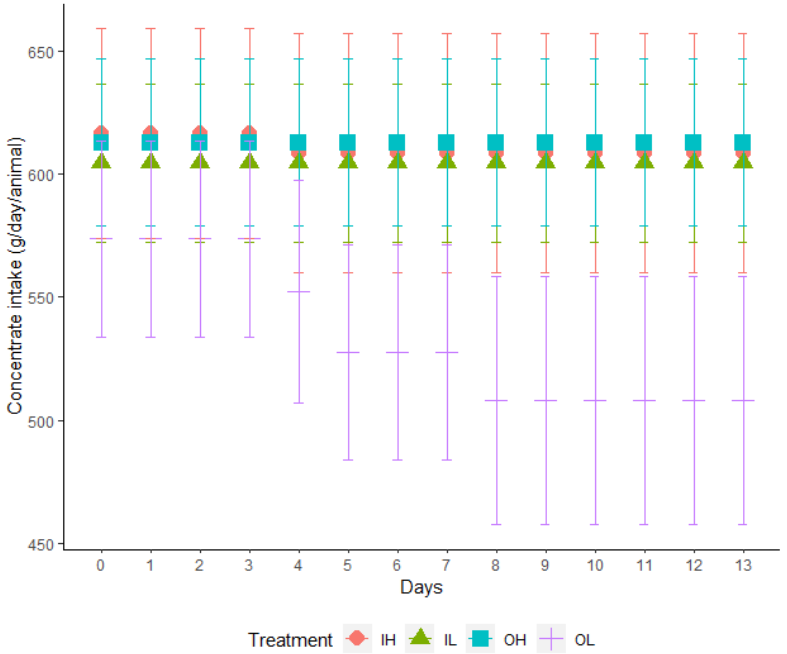

Figure B.2 Daily concentrate intake (g-DM day^{-1} animal^{-1}) by treatments. The error bars are the calculated standard errors.

Table B.7 Micronutrient concentrations ± SE in faecal samples on different days during the supplementary period and ANOVA test results

Element	Treatment	Day 0	Day 1	Day 3	Day 7	Day 10	Day 14	Temporal effect Pr (>F)
Cu (mg kg^{-1})	IL	25.1 ± 0.50	28.6 ± 0.63	47.4 ± 2.96	41.6 ± 1.28	54.2 ± 2.38	50.0 ± 1.51	**<0.001*** **
	IH	23.4 ± 0.62	28.5 ± 0.87	38.9 ± 3.04	42.1 ± 1.62	55.3 ± 1.18	47.7 ± 1.71	
	OL	25.4 ± 1.93	31.5 ± 3.23	50.6 ± 2.51	49.1 ± 5.58	55.2 ± 6.54	45.7 ± 2.99	
	OH	23.6 ± 0.82	28.5 ± 1.20	45.5 ± 2.08	43.7 ± 2.14	53.7 ± 2.14	47.7 ± 2.71	
	F_{form} (Pr>F)	0.2636	0.4641	0.4909	0.1272	0.9290	0.3308	
	F_{dose} (Pr>F)	0.8252	0.4347	0.1576	0.4025	0.9500	0.9393	
	F_{form} x F_{dose} (Pr>F)	0.9566	0.3443	0.4972	0.3144	0.7216	0.3313	
	RSD	2.5982	4.3619	6.0610	6.8986	8.6405	5.3252	
Mn (mg kg^{-1})	IL	344 ± 12.1	337 ± 11.8	394 ± 7.6	422 ± 16.1	429 ± 13.5	431 ± 20.2	**<0.001*** **
	IH	339 ± 13.8	315 ± 4.5	415 ± 12.7	408 ± 13.1	390 ± 10.5	337 ± 13.7	
	OL	337 ± 9.0	339 ± 9.6	415 ± 16.5	451 ± 20.3	455 ± 35.8	405 ± 14.9	
	OH	320 ± 10.9	345 ± 5.7	375 ± 5.9	428 ± 14.2	430 ± 10.9	426 ± 16.1	
	F_{form} (Pr>F)	0.3204	0.7776	0.2377	0.2128	0.2445	0.7748	
	F_{dose} (Pr>F)	0.4106	0.9033	**0.0130***	0.3510	0.2691	0.5532	
	F_{form} x F_{dose} (Pr>F)	0.6352	0.9670	0.5730	0.6370	0.9395	0.0820	
	RSD	30.705	11.497	28.644	39.475	48.398	40.858	
Zn (mg kg^{-1})	IL	199 ± 10.2	200 ± 12.3	305 ± 20.8	326 ± 39.6	309 ± 10.7	329 ± 21.1	**<0.001*** **
	IH	200 ± 34.2	215 ± 32.7	266 ± 40.6	302 ± 25.8	321 ± 38.2	343 ± 47.0	
	OL	197 ± 30.9	184 ± 7.8	320 ± 9.2	329 ± 26.1	343 ± 41.3	316 ± 31.9	
	OH	165 ± 10.1	181 ± 9.4	291 ± 15.2	321 ± 13.9	330 ± 15.4	330 ± 12.7	
	F_{form} (Pr>F)	0.4794	0.1882	0.9212	0.6836	0.4450	0.6313	
	F_{dose} (Pr>F)	0.5413	0.8585	0.6130	0.5398	0.9764	0.6100	
	F_{form} x F_{dose} (Pr>F)	0.5292	0.5466	0.4652	0.7670	0.6626	0.9989	
	RSD	62.961	48.562	55.671	65.079	66.529	66.388	
Se (µg kg^{-1})	IL	147 ± 3.1	164 ± 6.3	340 ± 23.2	386 ± 28.8	358 ± 15.4	366 ± 31.2	**<0.001*** **
	IH	141 ± 9.3	215 ± 18.9	460 ± 37.9	541 ± 18.0	547 ± 21.3	576 ± 23.4	
	OL	150 ± 10.3	175 ± 7.0	337 ± 8.6	378 ± 37.7	344 ± 31.4	327 ± 18.9	
	OH	141 ± 4.5	216 ± 9.2	550 ± 40.1	517 ± 42.9	588 ± 26.1	606 ± 21.2	
	F_{form} (Pr>F)	0.8411	0.7380	0.8697	0.6720	0.5826	0.8343	
	F_{dose} (Pr>F)	0.3924	**<0.001 *** **	**<0.001*** **	**0.0012** **	**<0.001*** **	**<0.001*** **	
	F_{form} x F_{dose} (Pr>F)	0.8583	0.7326	0.8071	0.8232	0.2694	0.1037	
	RSD	18.642	26.292	79.754	90.057	58.831	48.468	

Fe (mg kg^{-1})	IL	1107 ± 63.9	876 ± 54.4	840 ± 19.1	1086 ± 63.9	1175 ± 38.1	1156 ± 54.2	**<0.001*** **
	IH	977 ± 18.3b	836 ± 35.3c	697 ± 40.1c	1020 ± 12.9b	1154 ± 22.7a	1044 ± 39.6b	
	OL	1053 ± 41.1	903 ± 34.2	967 ± 80.3	1112 ± 52.4	1283 ± 82.2	1177 ± 56.2	
	OH	1065 ± 49.4	896 ± 40.4	801 ± 38.5	1024 ± 38.3	1138 ± 37.5	1115 ± 78.4	
	F_{form} (Pr>F)	0.7360	0.2623	0.2220	0.7615	0.3695	0.4797	
	F_{dose} (Pr>F)	0.2611	0.6019	**0.0498***	0.1315	0.1162	0.1909	
	F_{form} x F_{dose} (Pr>F)	0.1790	0.6550	0.1418	0.8237	0.2354	0.7001	
	RSD	122.79	92.885	110.20	117.90	122.09	155.53	
Cd (µg kg^{-1})	IL	241 ± 4.3	235 ± 4.7	222 ± 8.1	207 ± 10.7	214 ± 6.1	227 ± 10.1	**0.0201***
	IH	230 ± 8.3	219 ± 3.7	181 ± 6.1	194 ± 5.1	197 ± 4.6	208 ± 8.5	
	OL	224 ± 5.4	242 ± 7.1	286 ± 16.0	243 ± 18.8	240 ± 22.4	241 ± 5.2	
	OH	228 ± 6.9	226 ± 7.5	194 ± 7.1	190 ± 7.4	196 ± 7.8	207 ± 7.6	
	F_{form} (Pr>F)	0.1340	0.2388	**0.0457***	0.2163	0.3472	0.4503	
	F_{dose} (Pr>F)	0.5735	**0.0058****	**<0.001*****	**0.0187***	**0.0316***	**0.0051****	
	F_{form} x F_{dose} (Pr>F)	0.2401	0.6838	**0.0020****	0.1246	0.3005	0.4145	
	RSD	14.946	11.801	26.553	30.691	31.831	19.805	
Mo (mg kg^{-1})	IL	2.87 ± 0.116	2.79 ± 0.098	2.84 ± 0.108	2.63 ± 0.108	2.80 ± 0.109	2.95 ± 0.118	**0.0314***
	IH	2.77 ± 0.142	2.72 ± 0.053	2.40 ± 0.101	2.67 ± 0.093	2.81 ± 0.082	2.90 ± 0.091	
	OL	2.76 ± 0.044	2.85 ± 0.045	2.92 ± 0.114	2.63 ± 0.160	2.82 ± 0.335	2.76 ± 0.120	
	OH	2.72 ± 0.120	2.81 ± 0.127	2.76 ± 0.136	2.59 ± 0.093	2.76 ± 0.083	2.91 ± 0.092	
	F_{form} (Pr>F)	0.4833	0.1991	0.8615	0.7193	0.9364	0.4235	
	F_{dose} (Pr>F)	0.5352	0.4228	0.4238	0.9524	0.8945	0.6250	
	F_{form} x F_{dose} (Pr>F)	0.7624	0.6821	0.6666	0.7319	0.8220	0.3293	
	RSD	273.38	161.63	298.16	272.30	433.86	242.36	
P (g kg^{-1})	IL	14.2 ± 0.73	13.0 ± 0.83	13.5 ± 0.91	12.8 ± 0.55	13.3 ± 0.61	13.6 ± 0.47	0.6054
	IH	12.9 ± 0.82	12.6 ± 0.42	10.3 ± 0.22	12.1 ± 0.68	12.6 ± 0.76	12.3 ± 0.78	
	OL	13.7 ± 0.61	14.6 ± 0.84	16.4 ± 1.35	14.5 ± 1.26	15.4 ± 0.95	13.0 ± 0.51	
	OH	13.2 ± 0.58	13.9 ± 0.74	12.2 ± 0.34	12.9 ± 0.38	12.8 ± 0.73	12.2 ± 0.33	
	F_{form} (Pr>F)	0.8293	**0.0223***	0.1217	0.1358	0.1348	0.5102	
	F_{dose} (Pr>F)	0.2086	0.4866	**0.0074****	0.1862	**0.0446***	0.0855	
	F_{form} x F_{dose} (Pr>F)	0.5909	0.6803	0.1597	0.5746	0.2269	0.6906	
	RSD	1653.0	1391.4	2258.7	1990.7	1856.6	1389.6	
S (g kg^{-1})	IL	3.99 ± 0.068	3.72 ± 0.107	3.71 ± 0.128	3.70 ± 0.155	3.79 ± 0.121	3.85 ± 0.171	0.0992
	IH	3.85 ± 0.147a	3.54 ± 0.047 b	2.95 ± 0.062 b	3.55 ± 0.054b	3.55 ± 0.064b	3.52 ± 0.077b	
	OL	3.86 ± 0.085	3.82 ± 0.065	3.98 ± 0.098	3.85 ± 0.133	4.00 ± 0.356	3.74 ± 0.088	
	OH	3.83 ± 0.120	3.64 ± 0.086	3.46 ± 0.074	3.55 ± 0.067	3.61 ± 0.092	3.70 ± 0.096	
	F_{form} (Pr>F)	0.4738	0.3541	0.1304	0.5526	0.4742	0.7898	

	F_{dose} (Pr>F)	0.4149	**0.0215***	**<0.001*****	0.0612	0.1135	0.1434
	F_{form} x F_{dose} (Pr>F)	0.6131	0.5241	0.1755	0.5011	0.7050	0.2517
	RSD	254.93	166.57	222.83	273.23	457.34	292.16

The 'Pr>F' means the p-value in F-test of the factorial ANOVA test. Symbols '*', '**', '***' indicate statistical significances of the ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

Table B.8 Micronutrient concentrations ± SE in urine samples on different days during supplementary period and ANOVA test results

Element	Treatment	Day 0	Day 1	Day 3	Day 7	Day 10	Day 14	Temporal effect Pr (>F)
Cu (µg L^{-1})	IL	33.2 ± 2.66	57.0 ± 8.00	59.4 ± 4.45	44.5 ± 9.69	40.5 ± 19.40	56.6 ± 19.40	**0.0157***
	IH	28.5 ± 4.34	36.7 ± 6.38	49.3 ± 8.86	33.5 ± 6.10	28.9 ± 3.04	39.8 ± 5.81	
	OL	30.1 ± 6.23	30.0 ± 2.82	35.3 ± 3.97	33.9 ± 3.93	36.1 ± 1.97	40.5 ± 7.31	
	OH	33.9 ± 4.19	41.7 ± 6.13	46.1 ± 8.65	32.4 ± 3.42	36.9 ± 4.60	40.1 ± 3.72	
	F_{form} (Pr>F)	0.7768	0.0531	**0.0473***	0.3519	0.6997	0.4513	
	F_{dose} (Pr>F)	0.9197	0.3733	0.9601	0.3221	0.2601	0.4117	
	F_{form} x F_{dose} (Pr>F)	0.2995	**0.0257***	0.1203	0.4527	0.2004	0.4338	
	RSD	9.654	14.265	15.502	14.987	11.318	25.067	
Mn (µg L^{-1})	IL	140 ± 42.4	221 ± 87.8	171 ± 42.8	111 ± 19.4	120 ± 24.9	130 ± 29.9	**0.0319***
	IH	111 ± 12.9	150 ± 25.0	123 ± 28.6	83.8 ± 15.85	81.0 ± 9.82	114 ± 16.0	
	OL	99.5 ± 18.51	110 ± 21.3	102 ± 21.7	73.8 ± 15.37	109 ± 33.0	113 ± 32.9	
	OH	147 ± 33.7	183 ± 48.7	186 ± 49.3	110 ± 26.9	149 ± 52.7	117 ± 29.2	
	F_{form} (Pr>F)	0.9472	0.4739	0.9233	0.7260	0.3175	0.7569	
	F_{dose} (Pr>F)	0.7368	0.9638	0.6119	0.7935	0.9839	0.7989	
	F_{form} x F_{dose} (Pr>F)	0.2034	0.1599	0.0782	0.0674	0.1780	0.6638	
	RSD	70.197	124.56	85.376	39.408	68.239	53.449	
Zn (mg L^{-1})	IL	1.54 ± 0.248	2.22 ± 0.696	4.23 ± 1.303	3.71 ± 0.720	2.81 ± 0.474	4.48 ± 1.218	**<0.001*****
	IH	1.17 ± 0.189	2.09 ± 0.255	3.71 ± 0.684	3.19 ± 0.419	2.89 ± 0.500	5.47 ± 0.768	
	OL	1.13 ± 0.202	1.89 ± 0.412	2.60 ± 0.575	2.65 ± 0.381	2.94 ± 0.575	4.92 ± 1.705	
	OH	2.04 ± 0.449	2.66 ± 0.641	5.96 ± 1.635	3.69 ± 0.591	3.19 ± 0.418	5.38 ± 1.192	
	F_{form} (Pr>F)	0.4709	0.7853	0.7538	0.6045	0.6712	0.8764	
	F_{dose} (Pr>F)	0.4040	0.4857	0.1693	0.6314	0.7371	0.5086	
	F_{form} x F_{dose} (Pr>F)	0.0606	0.2989	0.0666	0.1636	0.8622	08050	
	RSD	0.7731	1.1502	2.4065	1.3115	1.9738	2.6148	

Se (µg L^{-1})	IL	19.9 ± 4.65	28.8 ± 5.28	30.5 ± 4.08	29.7 ± 4.41	19.8 ± 3.95	21.0 ± 4.95	**0.0120***
	IH	16.7 ± 2.31	21.2 ± 2.51	27.8 ± 6.11	25.5 ± 5.61	20.9 ± 2.95	29.8 ± 5.46	
	OL	15.7 ± 2.64	17.3 ± 1.72	18.0 ± 2.50	19.1 ± 3.02	14.5 ± 3.00	16.4 ± 2.76	
	OH	23.0 ± 2.32	28.8 ± 4.31	32.8 ± 3.60	28.1 ± 3.12	22.5 ± 2.45	22.2 ± 3.65	
	F_{form} (Pr>F)	0.7660	0.5052	0.4263	0.3525	0.5782	0.1073	
	F_{dose} (Pr>F)	0.5511	0.6515	0.2067	0.5783	0.1740	0.0580	
	F_{form} x F_{dose} (Pr>F)	0.1388	**0.0233***	0.0748	0.1311	0.2985	0.6761	
	RSD	8.2596	9.0096	11.168	10.153	7.8067	9.7409	
Fe (µg L^{-1})	IL	540 ± 89.3	921 ± 257.2	1095 ± 189.6	693 ± 79.9	637 ± 93.2	689 ± 135.8	**<0.001*****
	IH	480 ± 46.0	629 ± 98.1	771 ± 137.2	507 ± 78.2	420 ± 42.2	524 ± 61.0	
	OL	457 ± 81.0	534 ± 97.9	665 ± 102.0	525 ± 56.9	534 ± 103.4	570 ± 144.3	
	OH	627 ± 128.3	758 ± 162.8	1068 ± 232.0	627 ± 91.6	609 ± 128.7	548 ± 88.2	
	F_{form} (Pr>F)	0.7215	0.4026	0.7099	0.7670	0.6292	0.6193	
	F_{dose} (Pr>F)	0.5437	0.7738	0.8233	0.6022	0.4258	0.3275	
	F_{form} x F_{dose} (Pr>F)	0.2120	0.1250	0.0544	0.0856	0.1141	0.4503	
	RSD	216.64	381.09	426.80	192.33	212.65	227.32	
Cd (µg L^{-1})	IL	0.109 ± 0.0300	0.414 ± 0.0697	0.600 ± 0.1011	0.490 ± 0.0922	0.467 ± 0.1033	0.449 ± 0.0919	**<0.001*****
	IH	0.129 ± 0.0327	0.268 ± 0.0474	0.415 ± 0.0839	0.326 ± 0.0644	0.335 ± 0.0364	0.412 ± 0.0679	
	OL	0.069 ± 0.0151	0.167 ± 0.0243	0.268 ± 0.0304	0.287 ± 0.0401	0.311 ± 0.0488	0.325 ± 0.0528	
	OH	0.156 ± 0.0194	0.360 ± 0.0508	0.511 ± 0.0752	0.380 ± 0.0215	0.423 ± 0.0389	0.342 ± 0.0426	
	F_{form} (Pr>F)	0.8474	0.1083	0.1611	0.2581	0.6056	0.0938.	
	F_{dose} (Pr>F)	0.0652	0.7790	0.7204	0.5833	0.8807	0.8555	
	F_{form} x F_{dose} (Pr>F)	0.2071	**0.0118***	**0.0177***	0.0601	0.0810	0.6237	
	RSD	0.0632	0.1341	0.1964	0.1546	0.1596	0.1328	
Mo (µg L^{-1})	IL	52.8 ± 10.12	198 ± 34.3	234 ± 43.0	190 ± 37.5	184 ± 48.6	164 ± 38.6	**<0.001*****
	IH	54.0 ± 10.78	132 ± 22.7	171 ± 36.7	121 ± 24.9	135 ± 17.6	160 ± 25.7	
	OL	39.5 ± 6.05	90.1 ± 9.48	102 ± 8.5	113 ± 15.2	123 ± 14.9	121 ± 19.0	
	OH	63.5 ± 4.92	162 ± 15.5	192 ± 29.1	145 ± 10.4	162 ± 8.6	127 ± 16.1	
	F_{form} (Pr>F)	0.8284	0.1166	0.1006	0.2931	0.5373	0.0946.	
	F_{dose} (Pr>F)	0.1715	0.9833	0.6850	0.4684	0.8575	0.9500	
	F_{form} x F_{dose} (Pr>F)	0.2128	**0.0148***	**0.0299***	0.0567	0.1335	0.8090	
	RSD	21.538	60.050	77.994	60.228	67.094	52.365	
P (mg L^{-1})	IL	31.1 ± 5.94	21.2 ± 6.80	15.9 ± 3.40	7.99 ± 1.252	6.05 ± 1.390	6.01 ± 1.431	**<0.001*****
	IH	28.2 ± 5.49	13.9 ± 3.00	9.22 ± 2.229	3.50 ± 0.979	2.65 ± 0.752	3.50 ± 0.860	
	OL	31.4 ± 10.81	9.77 ± 1.263	7.03 ± 1.307	2.84 ± 0.941	1.38 ± 0.652	2.17 ± 0.744	

	OH	33.7 ± 7.15	18.0 ± 3.12	13.4 ± 3.01	5.74 ± 1.115	4.25 ± 1.079	3.64 ± 0.954	
	F_{form} (Pr>F)	0.7126	0.3908	0.3791	0.1938	0.1506	0.1004	
	F_{dose} (Pr>F)	0.9690	0.9746	0.9572	0.4881	0.8494	0.6300	
	F_{form} x F_{dose} (Pr>F)	0.7350	0.0795	**0.0225***	**0.0038****	**0.0085****	0.0786.	
	RSD	18.674	10.284	6.296	2.650	2.5518	2.5876	
S (mg L^{-1})	IL	522 ± 118.1	1296 ± 186.9	1779 ± 235.0	1436 ± 249.8	1533 ± 323.3	1379 ± 281.7	**<0.001*****
	IH	494 ± 81.6	758 ± 113.5	1095 ± 170.4	818 ± 120.3	995 ± 92.8	1176 ± 129.2	
	OL	417 ± 81.2	679 ± 69.6	965 ± 101.1	946 ± 130.1	1036 ± 173.9	1056 ± 175.3	
	OH	660 ± 49.8	1025 ± 150.5	1460 ± 233.9	1072 ± 108.0	1229 ± 121.1	1062 ± 125.2	
	F_{form} (Pr>F)	0.7592	0.2272	0.2868	0.4947	0.5224	0.2143	
	F_{dose} (Pr>F)	0.2838	0.4133	0.6486	0.1662	0.4056	0.5697	
	F_{form} x F_{dose} (Pr>F)	0.1837	**0.0038****	**0.0109***	**0.0442***	0.0895	0.5453	
	RSD	237.96	321.80	496.93	414.43	493.35	412.67	
N (g L^{-1})	IL	3.476 ± 0.491	10.57 ± 1.858	15.46 ± 2.845	10.61 ± 2.012	10.33 ± 2.965	8.23 ± 2.714	**<0.001*****
	IH	5.599 ± 1.743	6.496 ± 0.882	9.710 ± 4.941	6.578 ± 1.123	7.119 ± 0.571	7.039 ± 0.571	
	OL	3.173 ± 0.584	5.471 ± 0.591	6.076 ± 1.300	4.130 ± 1.169	6.236 ± 1.407	5.980 ± 1.595	
	OH	4.936 ± 1.059	9.211 ± 1.599	12.29 ± 2.405	8.723 ± 1.368	8.216 ± 0.830	7.345 ± 1.812	
	F_{form} (Pr>F)	0.4378	0.3018	0.1045	0.0974	0.4086	0.5587	
	F_{dose} (Pr>F)	0.0309	0.8104	0.9068	0.9663	0.7319	0.9567	
	F_{form} x F_{dose} (Pr>F)	0.6463	**0.0024****	**0.0083****	**0.0086****	0.1614	0.4437	
	RSD	2256.1	2677.0	4819.8	3311.7	4317.0	3970.7	

The 'Pr>F' means the p-value in F-test of the factorial ANOVA test. Symbols '*', '**', '***' indicate statistical significances of the ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

Table B.9 Micronutrient concentrations in grass of the first cut

Treatments	Cd (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Mo (mg kg^{-1})	P (mg kg^{-1})	S (mg kg^{-1})	Se (mg kg^{-1})	Zn (mg kg^{-1})	N (% DM)
A-CK	0.031±0.0016	5.50±0.311	75.8±27.35	53.9±1.87	0.459±0.1189	2869±117.8	1861±77.1	0.029±0.0023	16.3±0.97	1.74±0.131
A-U-O	0.125±0.0134	10.5±0.57	77.7±7.80	113±18.5	0.253±0.0421	3043±304.5	2645±196.1	0.042±0.0132	36.1±3.18	3.87±0.153
A-U-I	0.108±0.0074	10.3±0.53	81.5±11.97	122±11.7	0.223±0.0381	2788±196.0	2674±71.4	0.032±0.0109	34.5±1.88	4.12±0.194
A-F-O	0.037±0.0030	6.12±0.720	47.9±6.40	40.9±2.45	0.494±0.0351	3163±154.2	1695±137.3	0.012±0.0018	18.5±0.96	1.70±0.143
A-F-I	0.041±0.0055	6.88±1.140	46.4±4.37	44.8±3.03	0.544±0.0354	3118±186.0	1800±129.7	0.014±0.0025	19.3±1.93	1.78±0.217
A-UF-O	0.085±0.0077	10.4±0.44	78.5±9.73	88.4±9.62	0.441±0.0232	4596±166.9	2553±108.8	0.019±0.0022	40.4±1.41	3.53±0.176
A-UF-I	0.096±0.0076	10.5±0.43	70.5±2.72	118±7.9	0.438±0.0268	4463±87.2	2691±91.6	0.016±0.0035	40.8±2.03	3.77±0.152
G-CK	0.062±0.0037	7.24±0.265	56.5±7.35	42.5±1.56	0.317±0.0210	3122±157.6	1342±59.1	0.008±0.0031	30.4±1.65	2.09±0.185
G-U-O	0.153±0.0080	12.1±0.36	101±4.0	51.2±2.23	0.253±0.0148	4660±203.1	2808±120.4	0.014±0.0046	44.9±1.97	4.44±0.104
G-U-I	0.164±0.0139	11.8±0.39	127±31.2	53.8±4.50	0.261±0.0295	4328±160.2	2781±41.8	0.016±0.0029	45.2±1.30	4.33±0.105
G-F-O	0.113±0.0069	10.6±0.52	66.3±3.72	45.9±2.71	0.825±0.0305	4160±160.0	2306±101.4	0.010±0.0025	45.7±3.00	3.22±0.194
G-F-I	0.087±0.0171	8.47±1.151	57.5±5.94	42.7±4.71	0.750±0.0363	3524±324.5	1841±205.6	0.007±0.0026	35.3±4.86	2.44±0.404
G-UF-O	0.144±0.0771	12.5±0.37	106±15.1	56.7±1.57	0.380±0.0338	5178±302.1	3220±172.2	0.010±0.0020	49.1±1.23	4.38±0.103
G-UF-I	0.151±0.0122	11.9±0.39	92.7±4.44	55.3±2.94	0.392±0.0473	5075±129.8	3098±34.3	0.012±0.0027	48.8±1.80	4.31±0.091
Excreta type (ET)	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***
Supplemental mineral form (Form)	0.9667	0.3228	0.9943	0.2546	0.8662	0.0525	0.4227	0.5496	0.2585	0.5761
Soil	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***
ET x Form	0.3655	0.8536	0.2213	0.3794	0.9262	0.6822	0.4551	0.8819	0.2913	0.2250
ET x Soil	0.3413	0.1739	0.4647	**<0.001** ***	**<0.001** ***	**0.0011** **	0.0635	0.0544	**<0.001** ***	0.0554
Form x Soil	0.7931	0.0776	0.8336	0.0975	0.4154	0.3092	**0.0294** *	0.5507	0.2015	**0.0316** *
ET x Form x Soil	0.1449	0.2408	0.5633	0.3939	0.2038	0.4390	0.3189	0.5456	0.1330	0.5362

Symbols '*', '**', '***' indicate statistical significances of the ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

Table B.10 Micronutrient concentrations in grass of the second cut

Treatments	Cd (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Mo (mg kg^{-1})	P (mg kg^{-1})	S (mg kg^{-1})	Se (mg kg^{-1})	Zn (mg kg^{-1})	N (% DM)
A-CK	0.023±0.0022	5.19±0.342	58.5±11.37	106.8±11.65	0.675±0.0967	3194±184.6	2109±133.2	0.067±0.0063	17.5±1.32	1.38±0.058
A-U-O	0.069±0.0126	5.84±0.504	57.4±11.05	68.6±8.81	0.184±0.0355	2208±162.7	1687±114.1	0.031±0.0167	19.0±2.28	2.00±0.200
A-U-I	0.074±0.0130	6.35±0.816	48.0±3.69	75.7±8.06	0.162±0.0106	2120±145.8	1726±191.9	0.018±0.0014	19.6±1.69	2.17±0.323
A-F-O	0.022±0.0019	5.00±0.077	37.7±0.92	77.4±12.03	0.976±0.1104	3274±73.7	2129±112.7	0.038±0.0066	15.9±0.23	1.44±0.043
A-F-I	0.022±0.0015	5.41±0.239	40.5±1.69	74.0±9.62	0.931±0.0500	3465±172.3	2206±70.2	0.038±0.0055	16.7±0.67	1.46±0.057
A-UF-O	0.044±0.0031	5.67±0.181	43.3±4.32	57.0±3.24	0.495±0.0258	3238±104.5	1499±52.4	0.019±0.0030	18.1±0.72	1.52±0.051
A-UF-I	0.056±0.0051	6.17±0.313	44.0±1.02	74.5±6.82	0.481±0.0436	3154±45.5	1626±87.3	0.019±0.0036	19.4±0.82	1.79±0.135
G-CK	0.037±0.0028	4.54±0.108	32.1±0.39	68.0±5.76	0.756±0.0472	2596±49.2	1115±68.5	0.016±0.0018	18.0±0.32	1.38±0.038
G-U-O	0.088±0.0080	6.49±0.642	66.2±11.36	44.7±3.24	0.240±0.0454	2661±191.0	1776±110.3	0.011±0.0025	22.7±1.88	2.36±0.220
G-U-I	0.084±0.0080	5.45±0.093	54.3±3.10	37.6±2.36	0.185±0.0159	2252±84.5	1530±8.7	0.007±0.0005	20.3±0.70	2.05±0.065
G-F-O	0.047±0.0045	4.64±0.173	47.7±15.93	35.4±4.08	1.132±0.0463	2595±41.9	1506±73.7	0.006±0.0019	16.6±0.61	1.33±0.019
G-F-I	0.038±0.0042	4.39±0.111	34.0±2.03	44.1±5.76	1.031±0.0704	2489±51.0	1421±110.1	0.011±0.0010	15.0±0.66	1.30±0.022
G-UF-O	0.098±0.0071	7.19±0.705	69.6±7.95	46.7±1.03	0.509±0.0583	2966±211.9	1824±153.4	0.017±0.0086	27.6±2.89	2.38±0.273
G-UF-I	0.079±0.0039	6.02±0.075	68.1±5.52	40.5±2.71	0.428±0.0190	2755±71.7	1633±81.9	0.008±0.0011	22.7±0.73	2.19±0.153
Excreta type (ET)	**<0.001** ***	**<0.001** ***	**<0.001** ***	0.8109	**<0.001** ***	**<0.001** ***	0.0789	0.2057	**<0.001** ***	**<0.001** ***
Supplemental mineral form (Form)	0.5051	0.4670	0.1820	0.4758	0.0906	0.0822	0.4627	0.3113	0.1904	0.9174
Soil	**<0.001** ***	0.8506	**0.0075** **	**<0.001** ***	0.1169	**<0.001** ***	**0.0035** **	**<0.001** ***	**0.0014** **	**0.0392** *
ET x Form	0.8848	0.7475	0.5897	0.8357	0.8885	0.2013	0.8007	0.4263	0.7598	0.8997
ET x Soil	0.0616	0.0717	0.0614	0.3451	0.1539	**<0.001** ***	**<0.001** ***	**0.0460** *	**0.0037** **	**0.0085** **
Form x Soil	0.0576	**0.0091** **	0.3887	0.2756	0.4012	0.0672	0.0502	0.8311	**0.0189** *	0.0941
ET x Form x Soil	0.4574	0.6392	0.7140	0.1609	0.9710	0.8084	0.8670	0.5862	0.5851	0.5840

Symbols '*', '**', '***' indicate statistical significances of the ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

Table B.11 Micronutrient concentrations in grass of the third cut

Treatments	Cd (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Mo (mg kg^{-1})	P (mg kg^{-1})	S (mg kg^{-1})	Se (mg kg^{-1})	Zn (mg kg^{-1})	N (% DM)
A-CK	0.024±0.0023	5.43±0.158	90.7±32.00	166±11.4	0.878±0.0877	3486±126.9	2660±80.8	0.064±0.0044	17.6±0.50	1.39±0.017
A-U-O	0.046±0.0038	6.02±0.235	54.2±3.11	127±11.1	0.334±0.0207	3374±155.9	1607±95.0	0.037±0.0018	18.7±0.33	1.39±0.060
A-U-I	0.056±0.0031	6.12±0.446	56.3±9.40	132±15.7	0.407±0.0704	3350±290.5	1476±84.9	0.035±0.0028	19.2±0.81	1.41±0.030
A-F-O	0.021±0.0028	5.23±0.169	76.8±21.41	125±13.9	1.556±0.1876	3730±118.5	2644±168.8	0.061±0.0179	16.7±0.41	1.44±0.028
A-F-I	0.021±0.0007	5.47±0.145	69.7±5.64	134±14.1	1.475±0.0751	3829±93.3	2735±98.3	0.047±0.0026	17.7±0.41	1.50±0.067
A-UF-O	0.036±0.0021	6.79±0.129	56.1±3.55	116±9.8	1.241±0.0358	4396±78.5	1816±44.4	0.046±0.0075	19.9±0.29	1.50±0.024
A-UF-I	0.049±0.0057	6.95±0.348	61.0±5.57	126±3.3	1.148±0.0989	4154±128.3	1666±42.0	0.053±0.0112	21.4±1.43	1.46±0.027
G-CK	0.047±0.0011	5.04±0.206	145±67.4	149±4.6	1.137±0.0682	2987±139.0	1366±73.9	0.019±0.0046	19.7±0.71	1.22±0.020
G-U-O	0.067±0.0097	5.73±0.350	67.7±13.98	105±12.8	0.519±0.1073	3306±137.1	1623±95.6	0.026±0.0108	21.1±1.62	1.40±0.033
G-U-I	0.060±0.0052	5.37±0.180	69.5±13.72	93.3±8.74	0.369±0.0747	3030±98.0	1534±37.2	0.013±0.0014	20.0±1.23	1.30±0.017
G-F-O	0.047±0.0031	5.07±0.137	86.8±23.20	95.7±6.89	1.974±0.0656	3266±81.2	1827±61.3	0.014±0.0013	17.9±0.32	1.28±0.042
G-F-I	0.044±0.0036	4.85±0.085	146±53.3	105±3.9	1.779±0.1615	3169±61.5	1837±146.5	0.017±0.0058	16.3±0.76	1.23±0.036
G-UF-O	0.058±0.0051	5.76±0.175	71.6±1.25	72.9±7.10	0.983±0.1245	3271±81.0	1549±35.2	0.023±0.0108	21.0±0.64	1.39±0.045
G-UF-I	0.055±0.0057	5.21±0.309	56.8±3.36	72.6±7.96	0.801±0.1281	3173±112.5	1470±61.6	0.011±0.0011	19.9±1.03	1.35±0.036
Excreta type (ET)	**<0.001** ***	**<0.001** ***	**0.0273** *	**0.0282** *	**<0.001** ***	**<0.001** ***	**<0.001** ***	0.4428	**<0.001** ***	**0.0252** *
Supplemental mineral form (Form)	0.6034	0.4444	0.4933	0.5306	0.1072	0.1663	0.2486	0.2723	0.7574	0.2361
Soil	**<0.001** ***	**<0.001** ***	0.0693	**<0.001** ***	0.4896	**<0.001** ***	**<0.001** ***	**<0.001** ***	0.4094	**<0.001** ***
ET x Form	0.5930	0.8286	0.4952	0.6936	0.7612	0.6020	0.3123	0.8949	0.9202	0.4782
ET x Soil	0.1544	**0.0123** *	0.3544	0.3504	**<0.001** ***	**<0.001** ***	**<0.001** ***	0.1307	0.3190	**0.0043** **
Form x Soil	**0.0368** *	0.0567	0.4915	0.4620	0.2699	0.5029	0.9156	0.6016	**0.0417** *	0.0553
ET x Form x Soil	0.4981	0.9199	0.2635	0.8494	0.8998	0.5152	0.8014	0.3361	0.9106	0.3405

Symbols '*', '**', '***' indicate statistical significances of the ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

Table B.12 Micronutrient total uptake in grass of the first cut

Treatments	Cd (μg pot^{-1})	Cu (mg pot^{-1})	Fe (mg pot^{-1})	Mn (mg pot^{-1})	Mo (μg pot^{-1})	P (mg pot^{-1})	S (mg pot^{-1})	Se (μg pot^{-1})	Zn (mg pot^{-1})	N (g pot^{-1})
A-CK	0.064±0.0068	0.011±0.0068	0.156±0.0524	0.115±0.0157	0.915±0.1810	6.08±0.795	3.90±0.391	0.061±0.0085	0.034±0.0048	3.59±0.255
A-U-O	0.138±0.0377	0.012±0.0337	0.089±0.0249	0.117±0.0249	0.278±0.0694	3.44±0.902	3.01±0.806	0.042±0.0096	0.041±0.0112	4.49±1.294
A-U-I	0.130±0.0411	0.012±0.0411	0.101±0.0400	0.146±0.0400	0.281±0.1196	3.35±1.032	3.12±0.745	0.044±0.0260	0.040±0.0104	3.72±1.038
A-F-O	0.098±0.0106	0.016±0.0106	0.124±0.0087	0.112±0.0087	1.345±0.1987	8.44±0.864	4.46±0.343	0.034±0.0081	0.049±0.0050	4.35±0.128
A-F-I	0.106±0.0164	0.017±0.0164	0.121±0.0151	0.126±0.0151	1.498±0.2860	8.27±1.153	4.75±0.649	0.040±0.0113	0.050±0.0054	4.57±0.477
A-UF-O	0.135±0.0226	0.017±0.0226	0.126±0.0262	0.144±0.0262	0.735±0.1755	7.65±1.798	4.21±0.946	0.035±0.0138	0.067±0.0160	5.79±1.230
A-UF-I	0.119±0.0102	0.013±0.0102	0.088±0.0072	0.146±0.0072	0.545±0.0479	5.56±0.371	3.36±0.274	0.020±0.0051	0.051±0.0045	4.70±0.396
G-CK	0.231±0.0207	0.027±0.0207	0.210±0.0297	0.160±0.0297	1.175±0.1024	11.7±1.36	5.03±0.594	0.029±0.0092	0.114±0.0045	7.77±0.860
G-U-O	0.296±0.0587	0.023±0.0587	0.195±0.0359	0.099±0.0359	0.476±0.0751	8.78±1.336	5.29±0.837	0.023±0.0047	0.086±0.0151	8.53±1.558
G-U-I	0.296±0.0376	0.021±0.0376	0.237±0.0760	0.098±0.0760	0.464±0.0471	7.82±0.795	5.00±0.361	0.028±0.0044	0.081±0.0075	7.81±0.676
G-F-O	0.267±0.0259	0.025±0.0259	0.158±0.0165	0.109±0.0165	1.961±0.1506	9.89±0.785	5.47±0.411	0.024±0.0066	0.108±0.0097	7.65±0.666
G-F-I	0.228±0.0333	0.023±0.0333	0.157±0.0250	0.117±0.0250	2.212±0.6280	9.62±1.430	4.93±0.539	0.016±0.0059	0.095±0.0125	6.42±0.763
G-UF-O	0.208±0.0542	0.018±0.0542	0.145±0.0268	0.083±0.0268	0.581±0.1785	7.27±1.572	4.52±0.952	0.014±0.0033	0.071±0.0173	6.31±1.547
G-UF-I	0.221±0.0351	0.017±0.0351	0.134±0.0166	0.080±0.0166	0.566±0.0903	7.32±0.843	4.46±0.471	0.018±0.0048	0.071±0.0091	6.21±0.702
Excreta type (ET)	0.0938	0.1657	0.3046	0.9743	**<0.001** ***	**0.0027** **	0.1940	0.1931	0.2859	0.8114
Supplemental mineral form (Form)	0.8935	0.5347	0.8971	0.5807	0.7604	0.4818	0.6433	0.8326	0.4645	0.2879
Soil	**<0.001** ***	**<0.001** ***	**0.0017** **	**0.0109** *	0.0640	**0.0018** **	**0.0065** **	**0.0090** **	**<0.001** ***	**<0.001** ***
ET x Form	0.9419	0.8773	0.4780	0.8986	0.6449	0.8696	0.8856	0.7941	0.9203	0.9843
ET x Soil	0.3083	0.1928	0.1003	0.1842	0.0896	**0.0357** *	0.2341	0.8612	**0.0400** *	0.1039
Form x Soil	0.9061	0.8547	0.5816	0.5866	0.7769	0.7640	0.8380	0.8116	0.9679	0.9049
ET x Form x Soil	0.7352	0.6850	0.9342	0.8974	0.9433	0.6324	0.6612	0.4596	0.6204	0.6766

Symbols '*', '**', '***' indicate statistical significances of the ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

Table B.13 Micronutrient total uptake in grass of the second cut

Treatments	Cd (µg pot^{-1})	Cu (mg pot^{-1})	Fe (mg pot^{-1})	Mn (mg pot^{-1})	Mo (µg pot^{-1})	P (mg pot^{-1})	S (mg pot^{-1})	Se (µg pot^{-1})	Zn (mg pot^{-1})	N (g pot^{-1})
A-CK	0.030±0.0035	0.007±0.0002	0.082±0.0254	0.139±0.0130	0.920±0.2166	4.18±0.183	2.76±0.183	0.088±0.0121	0.023±0.0010	1.81±0.110
A-U-O	0.281±0.0576	0.024±0.0032	0.236±0.0536	0.281±0.0473	0.743±0.1569	8.89±0.815	6.87±0.815	0.130±0.0708	0.077±0.0122	8.22±1.224
A-U-I	0.294±0.0259	0.026±0.0030	0.197±0.0123	0.308±0.0194	0.666±0.0404	8.82±0.545	7.02±0.545	0.075±0.0061	0.080±0.0035	8.78±0.929
A-F-O	0.041±0.0069	0.009±0.0009	0.070±0.0084	0.137±0.0143	1.77±0.160	6.03±0.219	3.89±0.219	0.068±0.0110	0.029±0.0026	2.65±0.219
A-F-I	0.046±0.0040	0.011±0.0006	0.084±0.0031	0.155±0.0240	1.93±0.158	7.18±0.262	4.57±0.262	0.080±0.0131	0.035±0.0024	3.02±0.202
A-UF-O	0.179±0.0063	0.023±0.0006	0.175±0.0169	0.230±0.0078	2.00±0.073	13.2±0.17	6.07±0.166	0.077±0.0092	0.073±0.0030	6.16±0.286
A-UF-I	0.227±0.0230	0.025±0.0017	0.179±0.0103	0.301±0.0220	1.94±0.120	12.9±0.38	6.61±0.375	0.076±0.0109	0.079±0.0049	7.33±0.733
G-CK	0.115±0.0190	0.014±0.0016	0.098±0.0101	0.204±0.0188	2.33±0.349	7.90±0.831	3.40±0.415	0.047±0.0060	0.055±0.0059	4.21±0.460
G-U-O	0.656±0.0365	0.048±0.0027	0.488±0.0660	0.334±0.0173	1.78±0.306	19.8±0.66	13.2±0.34	0.083±0.0152	0.168±0.0069	17.5±0.86
G-U-I	0.577±0.0917	0.037±0.0029	0.370±0.0444	0.255±0.0288	1.27±0.187	15.1±0.80	10.3±0.61	0.046±0.0046	0.138±0.0135	13.8±0.74
G-F-O	0.166±0.0240	0.016±0.0008	0.167±0.0552	0.122±0.0101	3.95±0.128	9.11±0.527	5.25±0.140	0.023±0.0087	0.059±0.0052	4.68±0.330
G-F-I	0.139±0.0465	0.015±0.0030	0.116±0.0235	0.140±0.0104	3.44±0.521	8.50±1.685	4.67±0.566	0.039±0.0089	0.052±0.0123	4.54±1.078
G-UF-O	0.696±0.0387	0.051±0.0038	0.501±0.0699	0.334±0.0150	3.70±0.550	21.1±1.09	12.9±0.49	0.128±0.0648	0.196±0.0163	16.8±1.07
G-UF-I	0.537±0.0652	0.041±0.0030	0.456±0.0319	0.278±0.0369	2.92±0.301	18.6±1.30	11.0±0.93	0.055±0.0069	0.154±0.0133	14.8±1.34
Excreta type (ET)	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	0.2341	**<0.001** ***	**<0.001** ***
Supplemental mineral form (Form)	0.1995	0.0653	0.1038	0.9961	0.0678	**0.0430** *	**0.0326** *	0.1776	0.0549	0.2162
Soil	**<0.001** ***	**<0.001** ***	**<0.001** ***	0.5489	**<0.001** ***	**<0.001** ***	**<0.001** ***	0.2032	**<0.001** ***	**<0.001** ***
ET x Form	0.7783	0.3252	0.4976	0.4206	0.8157	0.1557	0.1628	0.3060	0.4144	0.3730
ET x Soil	**<0.001** ***	**<0.001** ***	**<0.001** ***	0.2659	**0.0412** *	**<0.001** ***	**<0.001** ***	0.3160	**<0.001** ***	**<0.001** ***
Form x Soil	**0.0377** *	**0.0019** **	0.1830	**0.0091** **	0.0595	**0.0141** *	**<0.001** ***	0.6344	**0.0084** **	**0.0112** *
ET x Form x Soil	0.3732	0.2899	0.9673	0.1552	0.9242	0.5220	0.4582	0.5164	0.4286	0.2907

Symbols '*', '**', '***' indicate statistical significances of the ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

Table B.14 Micronutrient total uptake in grass of the third cut

Treatments	Cd (μg pot^{-1})	Cu (mg pot^{-1})	Fe (mg pot^{-1})	Mn (mg pot^{-1})	Mo (μg pot^{-1})	P (mg pot^{-1})	S (mg pot^{-1})	Se (μg pot^{-1})	Zn (mg pot^{-1})	N (g pot^{-1})
A-CK	0.016±0.0031	0.003±0.0005	0.063±0.0282	0.103±0.0110	0.543±0.0502	2.20±0.287	1.69±0.234	0.040±0.0033	0.011±0.0015	0.89±0.124
A-U-O	0.092±0.0176	0.012±0.0016	0.105±0.0150	0.237±0.0213	0.650±0.1015	6.46±0.798	3.03±0.279	0.069±0.0072	0.036±0.0051	2.70±0.398
A-U-I	0.120±0.0169	0.013±0.0011	0.116±0.0145	0.273±0.0139	0.826±0.0534	7.00±0.440	3.13±0.308	0.074±0.0101	0.041±0.0046	3.05±0.401
A-F-O	0.027±0.0048	0.007±0.0004	0.101±0.0334	0.162±0.0255	1.98±0.2951	4.74±0.285	3.37±0.303	0.080±0.0277	0.021±0.0018	1.83±0.113
A-F-I	0.029±0.0010	0.008±0.0003	0.098±0.0087	0.189±0.0194	2.08±0.1074	5.40±0.177	3.86±0.164	0.067±0.0040	0.025±0.0009	2.12±0.114
A-UF-O	0.069±0.0071	0.013±0.0007	0.105±0.0091	0.214±0.0100	2.31±0.1108	8.21±0.598	3.38±0.189	0.088±0.0174	0.037±0.0024	2.81±0.172
A-UF-I	0.108±0.0122	0.015±0.0012	0.133±0.0129	0.276±0.0184	2.50±0.1916	9.09±0.576	3.65±0.215	0.114±0.0196	0.047±0.0050	3.20±0.193
G-CK	0.079±0.0058	0.008±0.0006	0.230±0.0941	0.248±0.0098	1.93±0.2376	4.99±0.243	2.29±0.157	0.032±0.0093	0.033±0.0019	2.05±0.146
G-U-O	0.265±0.0475	0.022±0.0027	0.264±0.0562	0.392±0.0241	1.99±0.4367	12.9±1.47	6.31±0.704	0.098±0.0406	0.083±0.0116	5.50±0.695
G-U-I	0.211±0.0085	0.019±0.0016	0.238±0.0293	0.330±0.0106	1.26±0.1469	10.9±0.92	5.54±0.493	0.048±0.0039	0.072±0.0061	4.74±0.478
G-F-O	0.067±0.0163	0.007±0.0014	0.122±0.0423	0.128±0.0223	2.71±0.5332	4.50±0.913	2.48±0.462	0.020±0.0040	0.025±0.0055	1.77±0.357
G-F-I	0.076±0.0116	0.008±0.0011	0.231±0.0758	0.181±0.0186	3.15±0.6120	5.48±0.590	3.10±0.129	0.031±0.0121	0.029±0.0047	2.13±0.225
G-UF-O	0.253±0.0209	0.026±0.0024	0.320±0.0370	0.317±0.0219	4.25±0.3063	14.5±1.28	6.86±0.639	0.088±0.0314	0.094±0.0115	6.18±0.625
G-UF-I	0.244±0.0237	0.023±0.0019	0.252±0.0202	0.319±0.0304	3.44±0.3238	14.2±1.39	6.57±0.585	0.050±0.0050	0.089±0.0077	6.07±0.625
Excreta type (ET)	**<0.001** ***	**<0.001** ***	**0.0484** *	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**0.0340** *	**<0.001** ***	**<0.001** ***
Supplemental mineral form (Form)	0.8141	0.8550	0.6676	0.0838	0.5676	0.7708	0.7636	0.3499	0.8073	0.2162
Soil	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**0.0187** *	**<0.001** ***	**<0.001** ***
ET x Form	0.5883	0.6217	0.3248	0.1203	0.3529	0.4292	0.3053	0.6804	0.7088	0.3730
ET x Soil	**<0.001** ***	**<0.001** ***	0.2013	**<0.001** ***	0.3781	**<0.001** ***	**<0.001** ***	0.1726	**<0.001** ***	**<0.001** ***
Form x Soil	0.0709	0.1010	0.8704	0.0561	0.1625	0.2624	0.3654	0.1460	0.1508	**0.0112** *
ET x Form x Soil	0.2637	0.4216	0.1231	0.0775	0.2561	0.5011	0.6766	0.1858	0.5863	0.2907

Symbols '*', '**', '***' indicate statistical significances of the ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

Table B.15 Total amount of micronutrients taken up by grass across the three cuts

Treatments	Cd (μg pot^{-1})	Cu (mg pot^{-1})	Fe (mg pot^{-1})	Mn (mg pot^{-1})	Mo (μg pot^{-1})	P (mg pot^{-1})	S (mg pot^{-1})	Se (μg pot^{-1})	Zn (mg pot^{-1})	N (g pot^{-1})
A-CK	0.110±0.0067	0.022±0.0010	0.301±0.0448	0.357±0.0200	2.38±0.328	12.3±0.83	8.35±0.417	0.189±0.0121	0.068±0.0055	6.29±0.182
A-U-O	0.510±0.0493	0.048±0.0020	0.430±0.0547	0.635±0.0576	1.67±0.192	18.8±1.11	12.9±0.54	0.242±0.0752	0.155±0.0094	15.4±0.62
A-U-I	0.510±0.0097	0.051±0.0039	0.421±0.0444	0.731±0.0444	1.81±0.185	19.7±1.58	13.3±0.68	0.191±0.0265	0.160±0.0073	15.6±0.92
A-F-O	0.166±0.0149	0.032±0.0013	0.296±0.0527	0.412±0.0527	5.09±0.584	19.2±0.59	11.7±0.54	0.182±0.0426	0.100±0.0040	8.82±0.202
A-F-I	0.181±0.0203	0.036±0.0019	0.303±0.0685	0.470±0.0685	5.51±0.341	20.8±1.61	13.2±0.96	0.187±0.0263	0.109±0.0068	9.71±0.642
A-UF-O	0.383±0.0152	0.053±0.0040	0.405±0.0443	0.588±0.0443	5.05±0.150	29.0±2.25	13.7±0.93	0.199±0.0163	0.178±0.0177	14.8±1.17
A-UF-I	0.453±0.0415	0.053±0.0034	0.401±0.0449	0.723±0.0449	4.98±0.277	27.5±1.46	13.6±0.66	0.209±0.0180	0.177±0.0123	15.2±1.03
G-CK	0.425±0.0426	0.049±0.0044	0.537±0.0502	0.612±0.0502	5.44±0.677	24.6±2.22	10.7±1.14	0.108±0.0103	0.202±0.0210	14.0±1.43
G-U-O	1.217±0.0860	0.094±0.0038	0.947±0.0449	0.825±0.0449	4.25±0.754	41.5±1.38	24.8±0.63	0.205±0.0383	0.337±0.0144	31.5±0.34
G-U-I	1.085±0.1225	0.078±0.0034	0.845±0.0394	0.683±0.0394	2.99±0.319	33.9±1.10	20.9±0.58	0.122±0.0057	0.291±0.0155	26.3±0.61
G-F-O	0.500±0.0618	0.048±0.0039	0.447±0.0277	0.360±0.0277	8.62±0.576	23.5±1.98	13.2±0.84	0.067±0.0105	0.192±0.0192	14.1±1.22
G-F-I	0.443±0.0791	0.046±0.0058	0.505±0.0365	0.438±0.0365	8.80±1.289	23.6±2.52	12.7±0.77	0.087±0.0170	0.175±0.0234	13.1±1.75
G-UF-O	1.158±0.0337	0.094±0.0039	0.966±0.0472	0.733±0.0472	8.53±0.964	42.8±1.74	24.3±0.65	0.230±0.0586	0.360±0.0182	29.3±0.49
G-UF-I	1.002±0.1050	0.081±0.0050	0.843±0.0697	0.677±0.0697	6.92±0.669	40.2±1.72	22.1±1.24	0.123±0.0081	0.313±0.0195	27.1±1.58
Excreta type (ET)	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**<0.001** ***	**0.0357** *	**<0.001** ***	**<0.001** ***
Supplemental mineral form (Form)	0.4682	0.1561	0.6226	0.3162	0.3889	0.2583	0.1889	0.1028	0.1653	0.0509
Soil	**<0.001** ***	**<0.001** ***	**<0.001** ***	0.3723	**<0.001** ***	**<0.001** ***	**<0.001** ***	**0.0042** **	**<0.001** ***	**<0.001** ***
ET x Form	0.9182	0.2787	0.5240	0.4114	0.3950	0.2327	0.13719	0.2865	0.6092	0.2098
ET x Soil	**<0.001** ***	**<0.001** ***	**0.0018** **	0.2241	0.2312	**<0.001** ***	**<0.001** ***	0.2847	**<0.001** ***	**<0.001** ***
Form x Soil	0.0659	**0.0040** **	0.5105	**0.0253** *	0.1347	0.0604	**0.0028** **	0.2814	**0.0255** *	**0.0062** **
ET x Form x Soil	0.7083	0.4584	0.6300	0.1591	0.6906	0.2020	0.4314	0.4256	0.8055	0.4381

Symbols '*', '**', '***' indicate statistical significances of the ANOVA test at p-value<0.05, <0.01, <0.001, respectively.

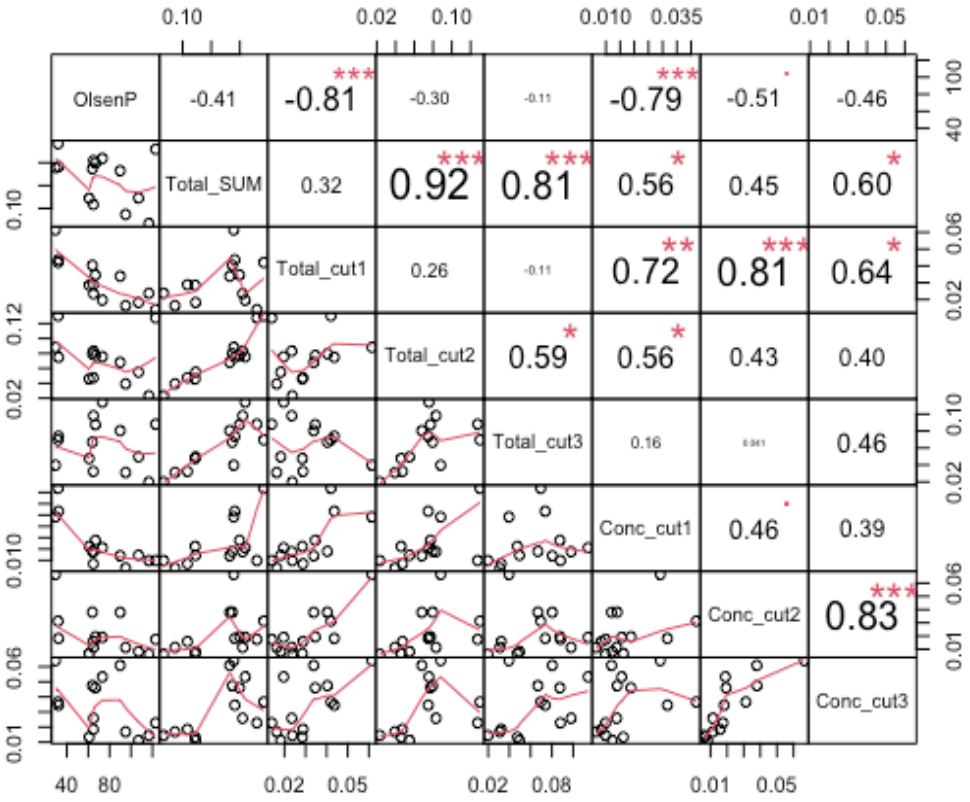

Figure B.3 The correlation map of Olsen-P and Se uptake or Se concentration in grass of different cuts. Total: total uptake of Se in grass; Conc: Se concentration in grass; SUM: The summary accumulation across the three cuts; The star-symbols: '*', '**', '*' represent significant result of Pearson's correlation test with a p-value <0.001, <0.01, <0.05, respectively.**

Table B.16 Soil pH and TOC of fields under different forage management practices at North Wyke Farm Platform (Southwest, England)

	Soil pH (mean ± SE)	Soil TOC (g kg^{-1} ± SE)
Permanent pasture managed	5.8 ± 0.04	56.5 ± 1.70[a]
Grass-legume mixture	6.0 ± 0.04	41.0 ± 1.66[b]
Reseeded grass monoculture swards	5.9 ± 0.08	37.5 ± 1.35[b]
p-value (>F)	0.07963	<0.001

The soil samples were collected in three seasons (April-May, July-August and October) in 2018; in each season, seven bulked samples, composed of 20 sub-samples collected at random at each field, were collected from seven fields of the same forage treatment.

Table B.17 Mineral requirement of growing lambs of different weight

Body weight	Dry matter intake	Zn		Cu		Mn		Se from forage		Se from concentrate	
kg	kg d^{-1}	mg $kg\text{-}DM^{-1}$*	mg d^{-1}	mg kg-DM-1*	mg d^{-1}	mg $kg\text{-}DM^{-1}$*	mg $kg\text{-}DM^{-1}$*	mg $kg\text{-}DM^{-1}$*	mg d^{-1}	mg $kg\text{-}DM^{-1}$*	mg d^{-1}
20	0.63	20.63	13	4.92	3.1	19.05	12	0.29	0.18	0.14	0.09
20	0.74	22.97	17	5.41	4.0	20.27	15	0.36	0.27	0.18	0.13
20	0.82	25.61	21	5.98	4.9	21.95	18	0.43	0.35	0.22	0.18
20	1.09	26.61	29	6.06	6.6	22.02	24	0.48	0.52	0.24	0.26
30	1.1	21.82	24	5.00	5.5	19.09	21	0.33	0.36	0.16	0.18
30	1.05	26.67	28	6.10	6.4	22.86	24	0.42	0.44	0.21	0.22
30	1.22	26.23	32	5.98	7.3	22.13	27	0.43	0.53	0.21	0.26
30	1.55	25.81	40	5.87	9.1	21.29	33	0.45	0.69	0.23	0.35
40	1.44	31.25	45	4.93	7.1	18.06	26	0.31	0.45	0.16	0.23
40	1.54	33.12	51	5.19	8.0	18.83	29	0.34	0.53	0.18	0.27
40	1.62	38.89	63	5.99	9.7	22.22	36	0.43	0.70	0.22	0.35
40	1.96	38.27	75	5.87	12	21.43	42	0.44	0.87	0.22	0.43
50	1.51	32.45	49	5.17	7.8	19.21	29	0.30	0.46	0.15	0.23
50	1.73	31.79	55	4.97	8.6	18.50	32	0.31	0.54	0.16	0.27
50	1.75	38.29	67	5.94	10	21.71	38	0.41	0.71	0.20	0.35
50	2.03	38.92	79	6.01	12	22.17	45	0.43	0.88	0.22	0.44
50	2.37	38.40	91	5.86	14	21.52	51	0.44	1.04	0.22	0.52
60	1.83	28.96	53	4.59	8.4	17.49	32	0.26	0.47	0.13	0.23
60	1.81	32.60	59	5.14	9.3	19.34	35	0.30	0.55	0.15	0.28
60	2.18	32.57	71	5.09	11	18.81	41	0.33	0.72	0.17	0.36
60	2.17	38.25	83	5.90	13	21.66	47	0.41	0.88	0.20	0.44
60	2.44	38.93	95	5.98	15	22.13	54	0.43	1.05	0.22	0.53
70	1.86	24.19	45	3.92	7.3	15.05	28	0.17	0.31	0.08	0.15
70	1.96	26.02	51	4.18	8.2	15.82	31	0.20	0.39	0.10	0.20
70	2.18	28.90	63	4.59	10	16.97	37	0.26	0.56	0.13	0.28
70	2.33	32.19	75	5.02	12	18.88	44	0.31	0.73	0.15	0.36
70	2.59	33.59	87	5.21	14	19.31	50	0.34	0.89	0.17	0.45
80	1.94	24.74	48	4.12	8.0	15.98	31	0.16	0.32	0.08	0.16
80	2.04	26.47	54	4.36	8.9	16.67	34	0.20	0.40	0.10	0.20
80	2.39	27.62	66	4.44	11	16.74	40	0.24	0.57	0.12	0.28
80	2.4	32.50	78	5.17	12	19.17	46	0.30	0.73	0.15	0.37
80	2.87	31.36	90	4.95	14	18.47	53	0.31	0.90	0.16	0.45
	Min	20.63	13	3.92	3.1	15.05	12	0.16	0.18	0.24	0.10
	Max	38.93	95	6.10	15	22.86	54	0.48	1.05	0.08	0.50

*These data is calculated from the data of daily mineral requirement of sheep (mg d^{-1}) and the data of DM intake in the same table reported by (NRC, 2007).

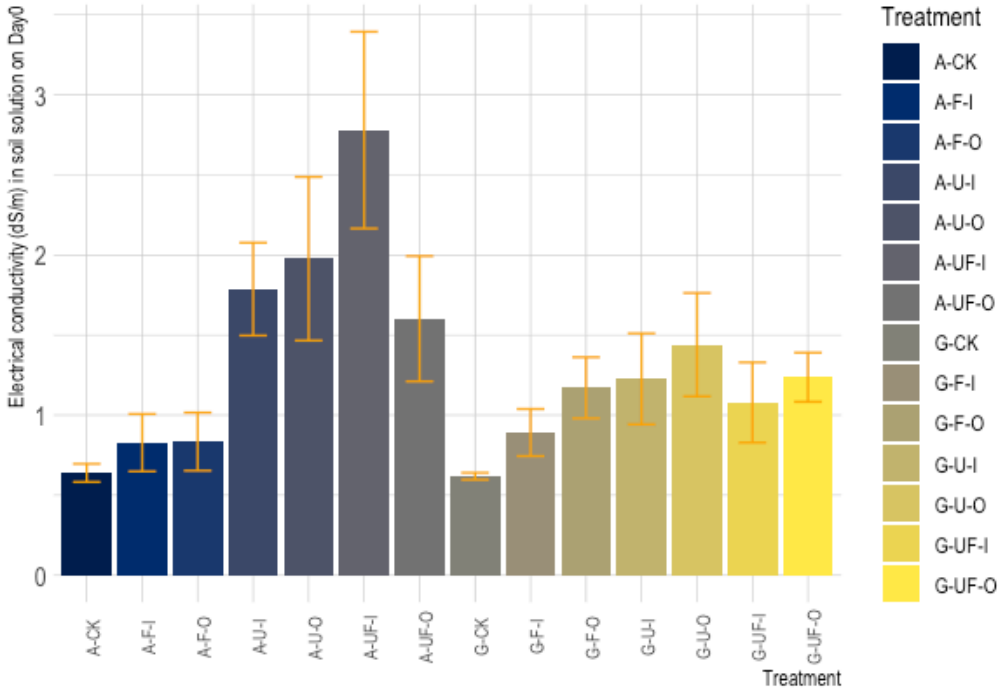

Figure B.4 Electrical conductivity (dS m^{-1}) of soil solution collected on Day 0.

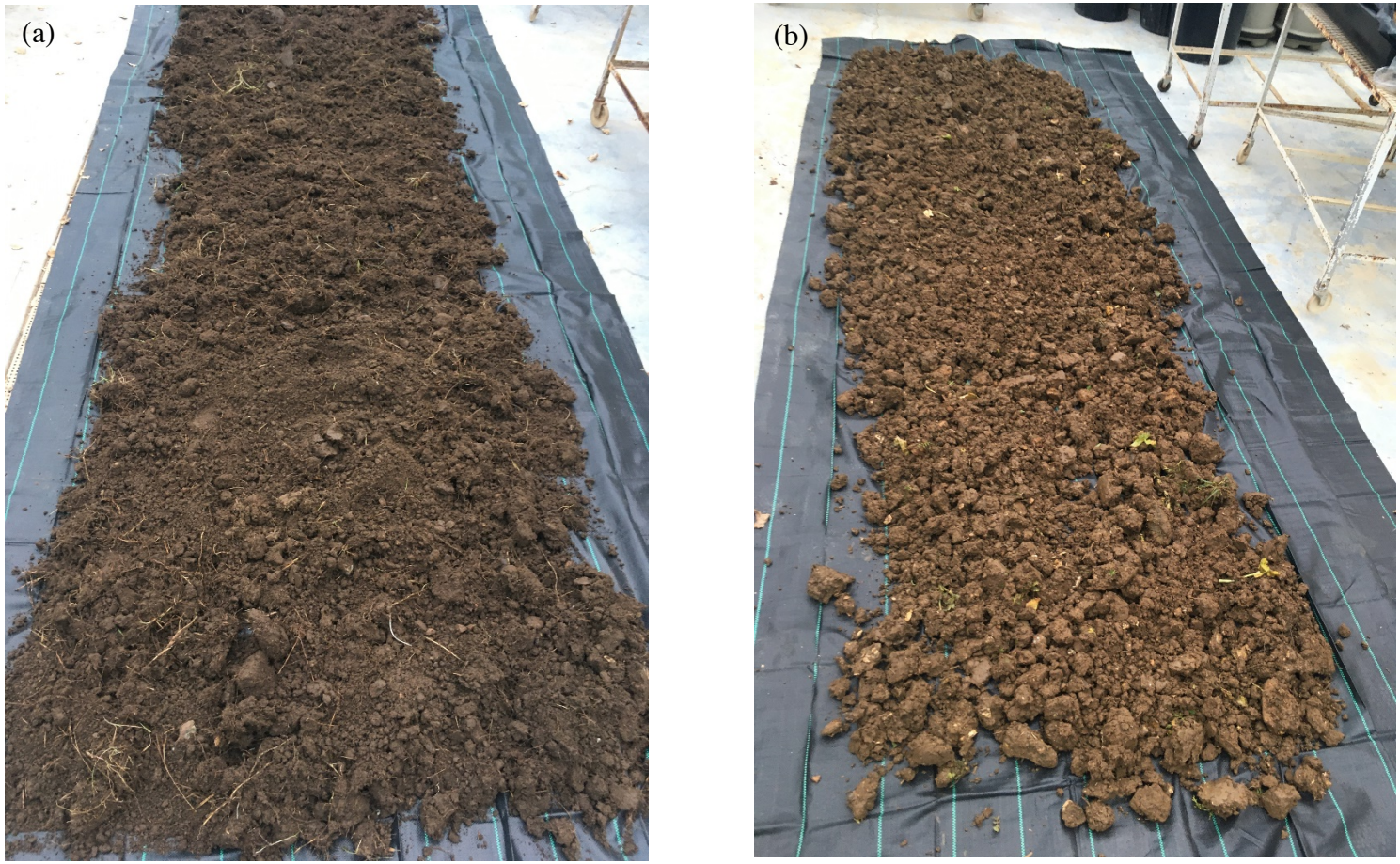

Figure B.5 Photos of soil collected from (a) the grassland and (b) the arable land, respectively.

Appendix C

Publications

Publication C.1

Article category: Review paper

Title: Factors influencing elemental micronutrient supply from pasture systems for grazing ruminants

Journal: Advances in Agronomy

Publication date: August 2020

DOI: 10.1016/bs.agron.2020.06.004

Author contribution:

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Tegan Darch	Investigation, Writing - Original Draft (Chapter 7), Visualization	Supervisor
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Heather L. Buss	Writing - Review & Editing	Supervisor
Helen Warren	Writing - Review & Editing, Funding acquisition	Supervisor and funder
M. R.F. Lee	Writing - Review & Editing, Project administration	Supervisor

Publication C.2

Article category: Conference paper

Conference: British Society of Animal Science

Title: Impact of supplementation dose and form on selenium partitioning and composition in urine and faeces of sheep

Journal: Animal - science proceedings

Publication date: April 2021

DOI: 10.1016/j.anscip.2021.03.155

Author contribution:

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Helen Warren	Writing - Review & Editing, Resources Funding acquisition	Supervisor and funder
M. R.F. Lee	Writing - Review & Editing, Project administration	Supervisor