

Micronutrients in Temperate Forage Crops Grown in Sweden

Species Differences and Effects of Phenological
Development and Soil

Bodil Lindström

*Faculty of Natural Resources and Agricultural Sciences
Department of Crop Production Ecology
Uppsala*

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Abstract

Forage crops are the most important feed for ruminants and provide energy, fibre and protein as well as essential micronutrients such as cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo) and zinc (Zn). In this thesis, different aspects of the micronutrient concentrations of forage crops were investigated in temporary grasslands in Sweden. Common and novel forage grasses (cocksfoot, meadow fescue, perennial ryegrass, tall fescue, timothy), legumes (birdsfoot trefoil, red clover, white clover) and forbs (caraway, chicory, ribwort plantain, salad burnet) were grown on contrasting soils in a pot experiment (two soils) and a field experiment (timothy, meadow fescue, red clover, white clover, chicory in mixtures, three sites, two soils in common with the pot experiment). The soils were micronutrient-poor/low pH (granitic, till), micronutrient-rich/high pH (alum shale, till) and the third field site had a low soil pH but average soil micronutrient concentrations (granitic/sandstone, postglacial) compared to Swedish arable soils.

It was found that micronutrient concentrations differ between forage species, but that varietal differences are few and small. Legumes and forbs generally had higher Co, Cu, Fe and Zn concentrations compared to grasses. There were few species differences in Mn concentrations whereas plant Mo concentrations was strongly affected by soil pH. Overall, the plant micronutrient concentrations appeared to be more dependent on the soil pH than on the soil micronutrient concentration (as extracted by EDTA and 7 M nitric acid).

The micronutrient concentrations of the grass-legume mixture were affected by the differences in micronutrient concentrations between legumes and grasses, as well as by the botanical composition of the grasses and legumes in the mixture. An increased proportion of red clover was positively correlated to increased concentrations of several micronutrients in grass-legumes mixtures.

The changes in micronutrient concentrations with phenological development at five defined stages were studied in three species. The decline of micronutrient concentrations was largest in timothy, less in perennial ryegrass and least in red clover. The generally low concentrations found in timothy were largely due to the high DM proportion of micronutrient-poor stems.

It is concluded that of the forage crops, clover-rich swards harvested at an early development stage have higher micronutrient concentrations, although the soil characteristics also have a large impact.

Keywords: clover, cultivars, forbs, grass, herbs, legumes, “micro-minerals”, organic farming, ruminants, trace elements, varieties

Author's address: Bodil Lindström, SLU, Department of Crop Production Ecology
P.O. Box 7043, SE-750 07 Uppsala, Sweden
E-mail: Bodil.Lindstrom@slu.se

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Dahlin, A.S., Edwards, A.C., Lindström, B.E.M., Ramezani, A., Shand, C.A., Walker, R.L., Watson, C.A., Öborn, I. (2012). Revisiting herbage sample collection and preparation procedures to minimise the risks of trace element contamination. *European Journal of Agronomy* 43, 33-39.
- II Lindström, B.E.M., Frankow-Lindberg, B.E., Dahlin, A.S., Wivstad, M., Watson, C.A. Micronutrient concentrations in relation to phenological development of red clover (*Trifolium pratense* L.), perennial ryegrass (*Lolium perenne* L.) and timothy (*Phleum pratense* L.). *Submitted*
- III Lindström, B.E.M., Frankow-Lindberg, B.E., Dahlin, A.S., Wivstad, M., Watson, C.A. (2012). Micronutrient concentrations in common and novel forage species and varieties grown on two contrasting soils. *Grass and Forage Science*, doi: 10.1111/gfs.12006.
- IV Lindström, B.E.M., Frankow-Lindberg, B.E., Dahlin, A.S., Watson, C.A., Wivstad, M. Effect of site and red clover proportion on micronutrient concentrations in forage mixtures. *Manuscript*

Papers I and III are reproduced with the permission of the publishers.

The contribution of Bodil Lindström to the papers included in this thesis was as follows:

- I Planned the tests together with main author, carried out tests of different mills. Helped out with reviewing articles and commented on manuscript.
- II Planned the experiment, performed the pot experiment and statistical analyses and is the main author of the paper, guided by the co-authors.
- III Planned and performed the pot experiment, collected the plant sample from field, performed the statistical analyses and is the main author of the paper, guided by the co-authors.
- IV Planned the experiment, carried out the data collection from the field experiment, performed statistical analyses and is the main author of the paper, guided by the co-authors.

Abbreviations

Co	Cobalt
Cu	Copper
DM	Dry matter
Fe	Iron
Mn	Manganese
Mo	Molybdenum
N	Nitrogen
Zn	Zinc

1 Introduction

1.1 Forages – the basis of ruminant feed

The most important feed of ruminants worldwide is forages, defined as hay, silage, straw, browsing and herbage from plants that can be cut or grazed (Wilkins, 2000). Globally, forages are commonly produced in permanent grasslands whereas temporary grasslands (sown grassland in an arable rotation) are more common in, for example, the Scandinavian countries. In Sweden about 87% of the total grassland areas are temporary (Statistics Sweden, 2012). This thesis will mainly focus on temperate forage species grown in temporary grassland, with an emphasis on species and varieties grown in Sweden. Typically, forages are grown in grass-legume mixtures. This stabilizes the yield and the quality of the crop over the season and generally gives a higher DM yield than pure grass swards (Halling *et al.*, 2002; Frankow-Lindberg *et al.*, 2009).

1.1.1 The role of forages in organic farming

The standards for organic farming emphasize that “livestock diets should be in a form that allows an animal to carry out its natural feeding behaviour and meet their digestive needs” (Marley *et al.*, 2010), which is why a minimum of 60% forage should be included in feed (European Commission, 2008). Since 2011 the EC regulation also states that the forage should be 100% organically grown and preferably produced on the same farm as the animals are kept. In addition, crop rotations with short term grass/legume leys are an important part of organic farming systems because of the N fixing ability of legumes. Hence, the legume DM proportion of the leys is generally higher on organic farms than conventional farms (Pettersson *et al.*, 1998).

1.2 Micronutrients - essential, beneficial and toxic

Plant and animal production and health are multifaceted topics that depend on numerous components where one corner stone in common is the need of elements, also known as macro- and micronutrients or minerals. Soils unable to provide sufficient amounts of essential micronutrients to plants may not only lower the potential yields of the plants, but also provoke ill health in animals that depend on them as fodder.

The micronutrients essential to plants are Fe, Mn, Cu, Mo, Zn, boron (B), chlorine (Cl) and nickel (Ni) and cannot be replaced by any other nutrients (Kirkby & Romheld, 2004). They are needed to complete the vegetative or reproductive cycles of the plants. Cobalt is indirectly necessary to legumes since it is essential for the N-fixing bacteria, *Rhizobium*, living in the nodules of legume roots. Non-essential micronutrients may be beneficial and will thus enhance plant health, e.g. sodium (Na), silica (Si), selenium (Se) and aluminium (Al) (Pilon-Smits *et al.*, 2009).

Some of the non-essential nutrients for plants are, in contrast, essential for animals, i.e. Se, Na and iodine (I) (Fisher, 2004). Furthermore, the elements B, chromium (Cr), lithium (Li), Mo, Ni, rubidium (Rb), silicon (Si) and vanadium (V) are considered at least occasionally beneficial and may be essential in such small amounts that their essentiality has not yet been fully confirmed (Suttle, 2010). Although these micronutrients are essential or beneficial to plants and animals there are risks of toxicity if they are in excess. The deficiency-toxicity range is sometimes called the marginal band and may be species specific for plants (McGrath *et al.*, 2001) and animals (Suttle, 2010). This is also the case for *Rhizobium* where both micronutrient deficiencies (O'Hara *et al.*, 1988) and toxicities (Giller *et al.*, 1989; Chaudri *et al.*, 2008) may affect strains differently.

Macronutrients that are essential to both plants and animals are N, phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) but these will not be dealt with in this thesis. In both conventional and organic farming, mineral supplementations are allowed to prevent ill health of animals and production loss due to macro or micronutrient deficiencies. Hence, micronutrients are returned with the manure and may accumulate in the soil and affect soil microbes such as *Rhizobium* (Pain, 2000).

The main focus of this thesis is on Co, Cu, Fe, Mn, Mo and Zn concentrations in forage crops and how well they meet the minimum requirements of dairy cows, as specified by the National Research Council (2001).

1.3 Investigating plant micronutrient content – risks of sample contamination and other sources of error

Micronutrients and other trace elements are typically found in such small concentrations that the units used are milligram per kg of plant DM. Because of the low concentrations, experiments dealing with micronutrients in plants require awareness of potential sources of contamination and error from the point of sampling, during handling and preparation to analyses of the plant material. Unless care is taken throughout this whole chain the results will be unreliable despite the quality control and quality assurance which is common in analytical laboratories.

When large plant samples need to be collected, which is often the case with field experiments concerning forages, the samples are usually bulky and heterogeneous. This requires extraction of representative sub-samples and homogenization in such a way that additional variation is not added. Variation can be minimized by standardization of the cutting height at harvest and by a sub-sampling method of fresh and dry material which ensures that, for example, the proportions of stems and leaves are not distorted by chance.

Micronutrient concentrations in soil (*e.g.* Co, Cu, Fe and Zn) are several times higher than in plants, hence contamination by soil particles and dust needs to be avoided (Fleming *et al.*, 1986; Wyttenbach & Tobler, 2002). Plant samples may be contaminated by the splashing from heavy rains or tramping by grazing cattle, which is why it has been suggested waiting with sampling for two weeks after such events (Scott *et al.*, 1971). Different techniques of washing and brushing of the plant samples have been studied with varying results (*e.g.* Porter, 1986; Markert, 1995). However, if high concentrations of Al, Fe, Sc and Ti are found in the plant analyses this can be a sign of soil contamination (Wyttenbach & Tobler, 2002). Other precautionary measures such as only using new sample bags, keeping surfaces for sample handling clean, using separate ovens when plant and soil materials are dried and using new or acid-washed containers for storage, are important. Another source of contamination may be from cutting or grinding and milling equipment made of metals (*e.g.* stainless steel) which can become worn during use (*e.g.* Allan *et al.*, 1999). Therefore, plant samples from other experiments, not initially planned for analysis of micronutrients, may be of limited value due to uncertainties regarding sample handling, milling and storage.

Hence, it is important to review aspects of quality assurance and to develop routines with the aim of minimizing contamination and error with regard to micronutrient concentrations of the plant samples before starting experiments. This includes an evaluation of the milling equipment to be used.

1.4 Micronutrient concentrations in forage species

Several studies have investigated different aspects of micronutrient concentrations in forage species and varieties in order to find out how well they meet ruminant requirements. In addition to the commonly sown grasses and legumes, forbs (non-legume dicotyledones) have been investigated. Pirhofer-Walzl *et al.* (2012) found that forbs as a group had similar concentrations to the legumes but higher concentrations of Cu, Fe and Zn than grasses. Similar tendencies have been noted in other studies with forbs, in particular chicory has been shown to have higher Cu and Zn concentrations than perennial ryegrass (Forbes & Gelman, 1981; Eekeren *et al.*, 2006; Harrington *et al.*, 2006) and white clover has been found to have higher concentrations of Cu than perennial ryegrass (Forbes & Gelman, 1981; Jarvis & Whitehead, 1983; Brink *et al.*, 2001). But there is also evidence that there are species differences between grasses. Cocksfoot has been found to have higher Cu and Mn but similar Zn, Mo and Co concentrations to perennial ryegrass (Forbes & Gelman, 1981) and higher Cu, Mn and Zn but lower Co and Fe concentrations than meadow fescue (Rinne *et al.*, 1974). Although most species comparisons have been done with pure stand grown species, there are also experiments which investigated species mixtures. Kunelius *et al.* (2006) found differences in Cu, Fe, Mn and Zn concentrations between a selection of species mixtures but the main differences were between grass-only mixtures and grass-legume mixtures. This is similar to results by Høgh-Jensen & Søgaard (2012) who found that species mixtures with one grass and one legume had higher micronutrient accumulation than the pure grass stands.

However, conclusions regarding actual species differences and effects of species mixtures are sometimes difficult to draw between different studies since the micronutrient concentration in a specific plant is influenced by a number of factors, i.e. phenological development of the species and time of season (Fleming & Murphy, 1968), root competition (Schenk, 2006), botanical composition and harvest regime (Belesky *et al.*, 2001), weather (Roche *et al.*, 2009), fertilizing regime (Mayland & Sneva, 1983) and, last but not least, soil micronutrient concentrations and their availability which are affected by soil properties (*e.g.* Paasikallio, 1978; Kähäri & Nissinen, 1978). In Sweden, as elsewhere, the regional patterns in parent material and micronutrient concentrations in the soil are more or less reflected in the micronutrient concentrations of crops, for example cereals (Eriksson *et al.*, 2010). Hence, in regions with low micronutrient concentrations in the soil, the micronutrient concentration differences between forage species may be used to increase the overall micronutrient status of the sward.

1.4.1 Effect of phenological development

Micronutrients are transported from the roots to the shoots through mass flow in the xylem, which is driven by transpiration or is due to root pressure (Kirkby & Romheld, 2004). Redistribution within the plant, from old senescing leaves to younger ones, may occur, as well as from leaves to reproductive tissue like pods (McGraw *et al.*, 1986). Manganese and Cu have limited mobility in the phloem, while Fe and to some extent Zn are considered phloem mobile (Kirkby & Romheld, 2004). Molybdenum has been shown to be mobile in some legumes. Since young leaves have low transpiration rates this redistribution is dependent on xylem/phloem exchanges of micronutrients.

Studies investigating the changes in micronutrient concentrations of forage species with increased phenological development have been conducted either by harvesting at regular time intervals (Anke *et al.*, 1994; Brink *et al.*, 2006) or at specified phenological stages (Fleming & Murphy, 1968; Whitehead & Jones, 1969). Anke *et al.* (1994) found that red clover concentrations of Fe, Mn, Mo, Ni and Zn decreased with time during phenological development whereas Whitehead and Jones (1969) found stable concentrations of Cu, Fe, Mn, Zn but a decrease of Co and Mo concentrations. In the same study (Whitehead & Jones 1969), white clover, lucerne and sainfoin showed similar patterns to red clover, with regard to the examined micronutrient concentrations.

During phenological development, Cu, Fe, Mn, Ni and Zn concentrations decreased in meadow fescue (Anke *et al.*, 1994) and Co, Cu and Fe concentrations generally decreased in perennial ryegrass (Fleming & Murphy, 1968). In both grasses the Mo concentrations diverged most from the pattern of the other micronutrients, being constant in perennial ryegrass and with a short period of increase before decrease in meadow fescue.

In conclusion, forage species tend to have stable or decreasing micronutrient concentrations with advancing phenological development. How the phenological development and the changes in DM proportion between stems, leaves and flowers affect the overall micronutrient concentrations of the sward is unclear, but Fleming (1963) and Pederson *et al.* (2002) found that stems typically have lower concentrations than leaves which may partly explain the changes in micronutrient concentrations with phenological development.

1.4.2 Re-growth and sward age

The micronutrient concentrations at the first harvest occasion and those of the re-growths have been shown to differ with species and the micronutrient in question. These varying results between studies may depend on harvesting

being done at different development stages and with a different number of harvest occasions. This may affect the proportions of mainly leaves, stems, and to some extent buds and flowers, of the total DM yield which have an impact on the overall trend of micronutrient concentrations. In addition, soil conditions and growing conditions may fluctuate during the growth season and between years, due to, for example, precipitation and temperature, which could affect the soil conditions.

Timothy grown in pure stands and meadow fescue in a mixture with either cocksfoot or timothy had decreasing Fe, Mn and Mo concentrations whereas different trends were found for Cu and Zn concentrations between the first and following harvest occasions (Rinne *et al.*, 1974; Ylärinta & Sillanpää, 1984; Fystro & Bakken, 2005). Repeated harvesting of white clover resulted in decreasing Cu concentrations (Jarvis & Whitehead, 1983).

Even though a grassland may be temporary, it is usually kept for a number of years which is why there may be differences in micronutrient concentrations between years. In a meadow fescue-cocksfoot sward in Finland Fe, Mn and Mo concentrations decreased, whereas concentrations of Cu and Co increased and that of Zn showed no difference between the years (Rinne *et al.*, 1974). However, the decrease in Cu and Zn concentrations of the biomass harvested from a chicory-cocksfoot-lucerne sward between first and third harvest year was thought to be a result of the decrease in DM proportion of chicory in the sward (Belesky *et al.*, 2001).

1.5 Micronutrient availability in soils

Low plant micronutrient concentrations may either be due to low concentrations in the soil or low plant availability of the micronutrients in the soil solution. The micronutrients originate from minerals and organic matter in the soil and are released as ions through the processes of weathering, desorption and mineralization. These processes are affected by soil pH, redox potential (pE), organic matter availability and chelates, which the plants themselves can modify in the rhizosphere, for example excreting exudates with chelates that bind specific elements (Hinsinger, 1998; Kirkby & Romheld, 2004). The ions are transported to the roots either with the soil solution through mass flow or the ions themselves move in the soil solution through diffusion. At the root surface, the ions from the soil solution are taken up through different processes in the roots that are passive (mass flow of water and ions through root surface) or energy demanding (selective). However, macro- and micronutrient ions may negatively or positively interact with each other affecting the micronutrient incorporation into plants (Russelle & McGraw,

1986). An additional pathway for (micro)nutrient acquisition is through symbioses with mycorrhiza (Marschner, 1995), where forbs have been found to have a higher degree of colonization than legumes and grasses (Karanika *et al.*, 2007). Mycorrhiza can also protect the plant against toxic concentrations of *e.g.* Zn (Zhu *et al.*, 2001).

There are several analytical methods for the estimation of short term and long term plant availability of micronutrients in the soil, but there is not one ideal method that covers all soils and micronutrients partly due to the diversity of soils and chemistry of micronutrients (Bussink & Temminghoff, 2004). Jarvis and Whitehead (1983) found that the mean Cu concentrations of white clover shoots grown in twenty-one soils were significantly correlated with the Cu extracted from the soils with three different methods (EDTA (pH 7); DTPA+CaCl₂ (pH 7.3); 1.95% HNO₃). However, the Cu concentrations of perennial ryegrass shoots were only significantly correlated with Cu extracted by the DTPA and HNO₃ methods (Jarvis & Whitehead, 1981). This suggests that there are also plant species differences which might make interpretations and generalizations of plant-soil correlations with regard to micronutrient concentrations difficult.

While most micronutrients have an increased solubility with decreased pH, the opposite is true for Mo. The highest plant availability of most micronutrients is at, roughly, a pH between 5.5 and 6.5 (McBride, 1994). In addition to pH, the availability of Fe and Mn in the soil solution is also affected by redox potentials.

In a large study performed in Finland, the Cu, Mn and Zn concentrations of timothy were positively correlated with the soil concentration of the micronutrient when extracted by AAAC-EDTA and negatively correlated to pH and to some extent soil organic C (Paasikallio, 1978; Kähäri & Nissinen, 1978). Molybdenum concentrations of timothy were also positively correlated to the extractable concentrations in the soil, and, in contrast to the other micronutrients, positively correlated to pH. Correlations between pH and plant micronutrient concentrations were also seen in a Swedish study with cereals (Eriksson *et al.*, 2010). In this study, a strong extraction reagent (7 M HNO₃) was used, but nevertheless some correlations to Cu, Mo, Ni and Zn concentrations in the cereals were found, particularly in oats.

The effect of pH was also seen in a liming experiment with a mixed grass sward where the increased pH had a negative effect on plant uptake of Mn, Zn and Co but increased uptake of Mo (mg m⁻² cut⁻¹), even when the dilution effect due to DM yield increase was accounted for (Fystro & Bakken, 2005). Decreased plant concentrations of Mn, Ni, Zn with increased liming rates were also seen in a longterm field experiment at six sites across Sweden (Andersson

& Siman, 1991). Similarly, chicory grown in soils with various pH had decreased concentrations of Zn, B, Cu and Mn with increasing pH while the Fe concentration was unaffected (Crush & Evans, 1990). Furthermore, N fertilization may reduce soil pH and affect the micronutrient concentrations of forages (Rinne *et al.*, 1974; Mayland & Sneva, 1983).

Fertilization to increase the micronutrient concentrations of forages has given varying results. Mineral fertilizers have shown no effects (Forbes & Gelman, 1981) or have only increased the concentrations of some micronutrients (Vogt & Jakkola, 1978). Furthermore, slurry applications have been shown to both increase (McGrath *et al.*, 1982) and also decrease (Pirhofer-Walzl *et al.*, 2012) the concentration of some micronutrients in herbage.

Roche *et al.* (2009) examined weather influences on herbage quality and micronutrient concentrations and found that there was a negative correlation between rainfall and Mn concentration, while Zn concentrations increased with higher rainfall. Sunlight hours were negatively correlated with Cu and Mo concentrations while evaporation rate had a positive association with the previously mentioned micronutrients. How these weather variables affect soil conditions (*e.g.* mineralization of micronutrients into soil solution) was not investigated but is certainly part of the explanation.

In conclusion, site and soil factors clearly affect plant micronutrient concentrations which is why it is important to include these in experiments concerning micronutrients in forages.

2 Aim

Research for this thesis was done within the projects: “Micronutrient management strategies in organic systems: How to utilize local and site specific resources for sustainable crop and animal production of high quality products?” and “How can we choose species, varieties and mixtures to optimize forage quality and micronutrient concentration on contrasting soils?”. The aim was to investigate the micronutrient concentrations of species and varieties commonly grown as forage crops in temporary grasslands in Sweden. Due to increased demand for locally and/or organically produced dairy and beef products, which mainly depend on locally produced forage, there was a need to increase knowledge of how well forage crops can meet the micronutrient requirements of ruminants. Apart from plant physiological and management factors, the micronutrient concentrations of the forage depends on a number of site factors, in particular the micronutrients of the soil. This triggered the question of how applicable results from other countries are to forage crops grown in Sweden when it comes to species differences of micronutrient concentrations and the effects of soil and site. Hence, a range of common and novel temperate forage species were pot grown in two contrasting soils and plant samples of several species were collected from varietal trials. The choice of species variety is important to obtain a high DM yield and good overwintering. This also necessitated comparisons of micronutrient concentrations in varieties commonly used in Sweden.

The actual effect of legumes, which are reported to have higher micronutrient concentrations than grasses, on the overall micronutrient concentrations of mixtures was also investigated in more detail with a range of DM proportions of red clover. This was done at three contrasting sites representing regions where forage production is common.

Since many of the other nutritive properties of the forage changes with phenological development, its impact on micronutrient concentrations was also

examined. A particular focus was on how the DM proportion between stems, leaves and flowers, with their respective micronutrient concentrations, affected the overall plant concentrations at the flowering stage.

Furthermore, to do accurate plant micronutrient analyses and obtain reliable results requires a high level of quality assurance when handling plant samples. This was reviewed at the start of the project and different mills were tested so as to select one which did not contaminate our samples. This also highlighted the problems with unknown or insufficient standards for handling and preparation of archived plant samples, compared to the high standards needed for reliable micronutrient analyses. Hence, this thesis only contains results obtained from newly sampled plant material.

The specific questions/hypotheses posed were:

- I How can we avoid contamination and ensure high data quality in forage research, with regard to micronutrients and other non-nutrient trace elements? (Paper I)
- II Micronutrient concentrations change with the phenological development of the plants. The overall plant micronutrient concentrations depend on the concentrations and DM proportions of the stems, leaves and flowers/panicles/spike. (Paper II)
- III Forage micronutrient concentrations are affected by the interaction between plant species and soil properties. There are differences between varieties of the same species. (Paper III)
- IV The red clover DM proportion of a species mixture affects the overall micronutrient concentrations of the biomass harvested. (Paper IV)

3 Materials and methods

3.1 Contamination tests of mills (Paper I)

Contamination by micronutrients and some non-nutrient trace elements from mills during plant sample preparation was tested with a number of mills (Table 3). These were compared with plant samples prepared using a plastic knife or a pair of plastic scissors as controls. Each mill and control was tested with five replicated sub-samples and evaluated by ANOVA.

3.2 Plant species (Paper II, III, IV)

Plant samples were evaluated from one outdoor pot experiment, one greenhouse pot experiment and three field trials as well as plant samples taken from the official Swedish variety field trials. Species and varieties used are presented in Table 1.

3.3 The sites and soils (Paper II, III, IV)

Figure 1 shows the three sites in Sweden where field experiments were conducted or from where soils were taken for the pot experiments. Plant samples were taken from variety field trials at Rådde research station. The pot experiment which tested a range of species used soils from Rådde and Ås (referred to as granitic and alum shale soils, respectively in Paper III), but was conducted outdoors in a netted yard at Ultuna, Uppsala. The soil from Ås was also used in the experiment on phenological stages (Paper II), which was performed in a greenhouse at Ultuna.

Table 1. Forage species and varieties used in the pot and field experiments

Species	Variety			
	Greenhouse (Paper II)	Outdoor pot experiment (Paper III)	Variety field trial (Paper III)	Field experiment (Paper IV)
Timothy <i>Phleum pratense</i> L.	Grindstad	Dolina, Grindstad	Alexander, Grindstad, Lischka, Ragnar	Grindstad
Perennial ryegrass <i>Lolium perenne</i> L.	Helmer	Helmer	Birger, Calibra, Helmer	
Meadow fescue <i>Festuca pratensis</i> Huds.		Sigmund	Sigmund	Sigmund /Kasper ¹
Tall fescue <i>Festuca arundinacea</i> Schreb.		Swaj	Kora, Swaj	
Festulolium hybrid <i>Festuca arundinacea</i> x <i>Lolium multiflorum</i>			Hykor	
Cocksfoot <i>Dactylis glomerata</i> L.		Luxor	Dactus, Luxor	
Ribwort plantain <i>Plantago lanceolata</i> L.		wild type		
Salad burnet <i>Sanguisorba minor</i> Scop.		wild type		
Caraway <i>Carum carvi</i> L.		Volhouden		
Chicory <i>Cichorium intybus</i> L.		Grasslands Puna		Grasslands Puna
Birdsfoot trefoil <i>Lotus corniculatus</i> L.		Oberhaunstaedter		
Red clover <i>Trifolium pratense</i> L.	Ares	Ares, Merviot, Nancy	Fanny	Ares /Torun ¹
White clover <i>Trifolium repens</i> L.		Lena, Ramona, Riesling		Ramona /Undrom ¹

¹Varieties adapted to the different sites were used.

The field trials (Paper IV) were established at Rådde, Lillerud and Ås. At Ås, the soil for the two pot experiments (Paper II, III) was collected from the same area of the field where the field experiment later was established. The Rådde soil used in the pot experiment (Paper III) was collected from a neighbouring

field to where the field experiment was established. Only the topsoil (0-25 cm depth) was collected for the pot experiments. Each soil was sieved through an aluminium mesh (8 mm × 18 mm) and then thoroughly mixed. A representative subsample for soil analyses was taken from the mixed soil before it was poured into pots. Soil samples from the field experiments (top soil *i.e.* 25 cm deep) were taken from transects across the fields before sowing. Soil analyses are described below and presented in Table 2. The soil at Rådde is a till with sandy loam texture developed from granitic parent material, at Lillerud the soil is a postglacial silty loam originating from mainly granitic and sandstone bedrock, and at Ås the soil is a loamy till developed from alum shales. Although organic farming was one of the targets for these investigations, mineral macronutrient fertilizers were used in all experiments to avoid plant macronutrient deficiencies and to more clearly see the soil effect on the plant micronutrient concentration.



Figure 1. Sites where field or pot experiments were conducted and from where soils for the two pot experiments were taken. Site 1, Rådde (57°36'N, 13°15'E), site 2, Lillerud (59°38'N, 13°23'E), site 3, Ås (63°14'N, 14°33'E) and site 4, Uppsala (59°49'N, 17°39'E), Sweden.

3.4 Greenhouse pot experiment (Paper II)

On 16 October 2010, three species (Table 1) were sown as pure stands with four replicated pots for harvests at each of five phenological stages. The pots were arranged in a randomized block design. Each pot was filled with 5 L soil

over 1 L Leca® (light expanded clay aggregate). To induce vernalization of perennial ryegrass (Aamlid *et al.*, 2000) all pots were placed in a greenhouse with a temperature of +20/15°C day/night temperatures and additional light of approximately 100 micromol m⁻² at the soil level of the pots (and increasing to more than 300 micromol m⁻² at the top of the canopy) for 20 hours day⁻¹ for eight weeks followed by a cool period of 12 weeks with no additional light and low temperature (approximately +6°C). All plants were cut back to a height of 5 cm before the lights were turned on and the temperature was increased again (18 h day⁻¹, +18/14°C) on 11 March 2011. The plants were watered with deionized water as necessary and fertilizers were mixed from laboratory grade compounds (K₂PO₄, KCl, NH₄NO₃). During the early growth all plants were fertilized with the equivalent of 18 kg P ha⁻¹ and 60 kg K ha⁻¹ and the grasses received 60 kg N ha⁻¹. After the cold period the plants were again fertilized with the same amount of nutrients. The plants were watered with deionized water. Harvests (cutting height approximately 5 cm) were carried out when the designated phenological stage was reached in all replicated pots.

The first harvest was done at the stem elongation stage and the second set of pots was harvested when the ears were visible on the grasses and the buds were visible on red clover. The third stage was defined as when half of the ear had emerged on the grasses and individual buds were visible on the red clover. The fourth and fifth stages were defined as a visible stem underneath the ear of the grasses and open flowers on the main stem of red clover, and visible stamen on the grasses and open flowers on side stems on red clover, respectively.

At the final harvest (at the flowering stage), red clover plants were separated into i) flowers and clearly visible buds, ii) leaves with petioles and stipules and iii) stems. The grasses were similarly separated into three categories; i) spike or panicle (hereafter called flowers), ii) leaves, and iii) true stems + leaf sheaths. The effect of species, phenological stage and their interaction were analysed with regard to micronutrient concentrations and DM harvested. The proportions of stems, leaves and flowers in the total DM harvested were compared between species for each plant component.

3.5 Outdoor pot experiment (Paper III)

Pure stands of twelve forage species (Table 1) were sown in 7 L plastic pots which were placed outdoors in a netted yard in Uppsala in a randomized block design. The plants were sown in June 2009, stored in a dark freezer (-1°C) during the winter and harvested the following year on 8 June and 20 July. The plants were watered with deionized water as necessary and fertilizers were mixed from laboratory grade compounds (K₂PO₄, KCl, NH₄NO₃). In 2009, all

pots received P and K at rates equivalent to fertilization with 30 kg P ha⁻¹ and 100 kg K ha⁻¹. The grasses and forbs also received the equivalent of 100 kg N ha⁻¹. In 2010, all species were fertilized with the equivalent of 27 kg P ha⁻¹ and 90 kg K ha⁻¹ before the first cut. After the first cut an additional 33 kg P ha⁻¹ and 110 kg K ha⁻¹ were provided. The grasses and forbs also received 90 and 110 kg N ha⁻¹. Micronutrient concentrations and total DM biomass were analysed as a linear mixed model with origin of soil and plant species and their interaction as fixed factors, and block and varieties nested within species as random factors. Varietal differences were analysed with variety and soil as fixed factors.

3.6 Variety field trial (Paper III)

All species taken from the Swedish variety trials (Table 1) at Rådde research farm were sown in spring 2008 with the exception of cocksfoot and red clover, which were sown in 2007. Each variety was sown in 15 m² plots in a randomized block design (one trial per species), with three replicates. The samples were collected from the first cut in 2009 (cocksfoot 26 May, red clover 11 June, the other species 9 June). In early spring cocksfoot was fertilized with 100, 26, 100 and 11 kg ha⁻¹ of N, P, K and S, respectively, while the other grasses received 100, 20, 75 and 11 kg ha⁻¹ of N, P, K and S, respectively. Red clover was fertilized with 26 kg P ha⁻¹ and 100 kg K ha⁻¹ on the same occasions as the other species. A Haldrup plot harvester (Haldrup, Løgstør, Denmark) was used to cut the sward to a stubble height of approx. 5 cm. The grasses were cut at heading and red clover at the initial budding stage. Micronutrient concentrations and DM yield were analysed as a linear mixed model with species as a fixed factor, and block and variety nested within species as random factors.

3.7 Field experiment (Paper IV)

The five species (Table 1) were grown in four different mixture types; i) timothy and red clover (15 and 5 kg ha⁻¹, respectively); ii) meadow fescue, timothy and red clover (4.2, 10.8 and 5 kg ha⁻¹, respectively); iii) white clover, timothy and red clover (2, 15, 3 kg ha⁻¹, respectively); and iv) chicory, timothy and red clover (3, 15, 5 kg ha⁻¹, respectively). The experimental design was a randomized block design with three replicates and the plot size harvested was 12.0, 14.0 and 13.5 m² at Rådde, Lillerud and Ås, respectively. The forage species were under-sown in spring barley (*Hordeum vulgare* L.). In the spring of 2011 the crops at all sites received 60 kg N ha⁻¹ and another 50 kg N ha⁻¹

was applied to each re-growth. Fertilizer P, K and S were added in accordance to the site-specific needs of the soils. In 2011, the plots were harvested three times at Rådde (8 June, 20 July and 14 September) and Lillerud (7 June, 19 July and 4 October) and twice at Ås (16 June and 30 August). The first harvest was carried out at the ear emergence stage of timothy. Harvesting was done with a Haldrup plot harvester which gave a stubble height of approximately 5 cm. Red clover DM proportion was correlated with the concentrations of each micronutrient in the mixtures by linear regression.

3.8 Chemical analyses

3.8.1 Plant samples

All plant samples were dried at 50°C for at least 48 hours before being weighed and milled to a particle size below 1 mm with a cutting mill (Grindomix GM 200, Retsch GmbH, Haan, Germany). The exception was the plant samples in Paper I which were milled on different devices as shown in Table 3 and described in 3.1. The plant samples were wet digested with 7 M ultrapure nitric acid (HNO₃) and hydrogen fluoride (HF) in a microwave oven followed by filtering and analysis by ICP-SFMS, at ALS Scandinavia AB in Luleå, Sweden. The exception was the plant samples in Paper I and the field-grown species in paper III which were digested with HNO₃ before analysis by ICP-MS (Dahlin *et al.*, 2012). Dry matter content was determined after drying for at least 48 hours at 105°C.

3.8.2 Soil samples

Total N and C concentration in soil samples were analysed by high temperature induction furnace combustion using LECO CN2000 (LECO Co-operation, St Joseph, MI, USA). Soil pH was measured both in deionized water and 0.01 M calcium chloride (CaCl₂) (Sumner, 1994). Soils from the field experiment and the ones used in the outdoor pot experiment were analysed for ‘pseudo-total’ macronutrients and micronutrients by HNO₃ and hydrogen peroxide (H₂O₂) extraction followed by filtration and analysis by ICP-SFMS (Swedish standard SS 02 81 50). The soil used in the greenhouse pot experiment and the field experimental soils were extracted with EDTA (pH 7) and analysed for micronutrients by ICP-MS (Ure & Berrow, 1970). The soil properties and micronutrient concentrations according to the extraction methods are presented in Table 3. Additional soil data is presented in Paper II-IV.

Table 2. Properties of soils used in the pot experiments and field experiments; texture, pH, total C and N, micronutrient concentrations (mg kg^{-1}) extracted by nitric acid/hydrogen peroxide ($\text{HNO}_3+\text{H}_2\text{O}_2$) and EDTA. Data with both extractants are shown for Ås soil

	Rådde pot (Paper III)	Rådde variety trial (Paper III)	Ås field, pot experiments (Paper II, III, IV)	Rådde field (Paper IV)	Lillerud field (Paper IV)	
Clay (%)	8	Na	24	8	27	
Silt (%)	31	Na	40	41	56	
Sand (%)	61	Na	36	51	17	
pH (H_2O)	5.5	5.6	7.5	5.8	5.6	
pH (CaCl_2)	5.2	5.2	7.2	5.3	5.3	
C (%)	3.5	3.1	3.4	3.1	1.7	
N(%)	0.27	0.24	0.31	0.22	0.14	
	$\text{HNO}_3+\text{H}_2\text{O}_2$		EDTA			
Co	2.6	2.8	12.7	0.40	0.04	0.21
Cu	5.6	9.2	17.0	3.1	0.5	2.2
Fe	11 300	9250	22 100	178	69	153
Mn	394	732	1950	125	6	31
Mo	0.61	0.85	1.06	0.04	b.d.	b.d.
Zn	25	29	104	2.69	0.69	2.01

Na: not analysed but similar soil type as Rådde pot soil; b.d.: below detection limit.

4 Results

4.1 Contamination tests of mills

Of the five milling devices investigated, the cutting mill with a titanium knife and a plastic bowl was the only one that did not contaminate the plant samples with the analyzed micronutrients and trace elements (Table 3). The largest contamination from the mills was from Cr; in particular samples treated with both the hammer mill and the ball mill had a massive increase in Cr concentration. The addition by milling of the other micronutrients was 50% or less.

4.2 Effect of phenological development

4.2.1 Changes in plant micronutrient concentrations

There was a significant increase in DM harvested between the first and fifth phenological stage for all three species and red clover had the highest DM harvested followed by timothy and then perennial ryegrass.

In the grasses, micronutrient concentrations in the whole plant decreased significantly between the first and fifth phenological stage (*i.e.* from stem elongation to visible stamen on flowers) except for Mo in timothy and Mo and Co concentrations in perennial ryegrass. Where the decreases were significant, the concentration reductions in timothy were more than 50% for the majority of micronutrients whereas the reductions in perennial ryegrass were not as pronounced. In red clover Co, Fe, Mn and Ni concentrations were more constant. In contrast the Mo concentration decreased by more than 50% between the first and fifth phenological stage in red clover. The Cu and Zn concentrations decreased with phenological development in all three species.

Table 3. Milling devices tested in three sub-studies with regard to micronutrient contamination (mg kg^{-1} DM). The same type of plant material was not used in all three tests. The third study was not reported in Paper 1

Device and material	Brand	Co	Cr	Cu	Fe	Mn	Ni	Zn
Sub-study 1								
Plexi-glass knife	(dept workshop)	0.018	0.01 ^{a1}	1.96	19.5 ^a	61.0	0.13 ^a	7.9 ^a
Hammer mill: stainless steel	Glen Creston Stanmore	0.017	0.17 ^b	2.20	22.6 ^{ab}	65.4	0.20 ^b	14.4 ^b
Hammer mill + ball mill: both made of stainless steel	Glen Creston Stanmore + Retsch Mixer Mill MM200	0.017	0.59 ^c	2.16	26.7 ^b	62.9	0.23 ^b	14.3 ^b
Ti cutting mill: Ti knives, plastic bowl	Retsch Grindomix GM 200	0.017	0.01 ^a	2.13	18.6 ^a	58.1	0.15 ^a	8.8 ^a
<i>P level</i>		<i>NS</i>	***	<i>NS</i>	**	<i>NS</i>	***	***
Sub-study 2								
Plastic scissors: polystyrene resin	Kärnan AB	0.020	0.06 ¹	4.06	33.6	33.1	0.66	15.5
Cutting mill: stainless steel	Retsch 2000 mill	0.023	0.41	4.48	36.8	36.9	0.81	16.3
<i>P level</i>		<i>NS</i>	***	**	*	*	**	*
Sub-study 3								
Plastic scissors: polystyrene resin	Kärnan AB	0.032	0.024	4.03	42.2	64.9	1.54	24.4
Cutting mill: stainless steel	Thomas Wiley laboratory mill, model 4	0.057	0.070	4.55	66.3	68.6	1.88	25.5
<i>P level</i>		**	<i>NS</i>	*	***	<i>NS</i>	**	<i>NS</i>

¹Several samples below the detection limit. NS: non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

After the vernalization period, timothy developed stems more quickly than perennial ryegrass but the latter grew more rapidly and so the stage 4 and 5 harvests of the two grasses took place on the same days. Perennial ryegrass had higher Co, Fe, Mo, Ni concentrations than timothy at each of the phenological stages ($P < 0.05$).

4.2.2 Micronutrient concentrations in stems, leaves and flowers at the flowering stage

The DM proportion of flowers and leaves of red clover (12 and 59%, respectively) and perennial ryegrass (13 and 47 %, respectively) were higher than those of timothy (3 and 33 %, respectively), at the fifth stage. Consequently, timothy had a higher stem DM proportion (64%), than perennial ryegrass (40 %) and red clover (29 %). Micronutrient concentrations differed

significantly between these plant components for each species. The flowers of red clover and timothy generally had higher micronutrient concentrations than their stems and leaves. This was also seen in perennial ryegrass for Ni and Zn concentrations. In all species the stems contained the lowest micronutrient concentrations, and these were particularly low in timothy.

4.3 Species differences and effects of soils

4.3.1 Within species – the variety effect

Varietal differences with regard to micronutrient concentrations were investigated in both the outdoor pot experiment and the variety field trial at Rådde. In the outdoor pot experiment, the only difference in concentration between varieties was for Zn and Fe (in interaction with soil type) in red clover. Timothy varieties grown in the variety field trial differed in Zn concentrations, unlike the timothy varieties grown in the outdoor pot experiment. Perennial ryegrass varieties differed with regard to Mo concentrations, and the two varieties of cocksfoot differed with respect to Cu concentration in the variety field trials. Generally, there were few within species differences between varieties and, if existing, these were smaller than the species differences. Hence, all micronutrient concentrations are presented as species means.

4.3.2 Outdoor pot experiment

The species effect was significant with respect to all micronutrient concentrations, as was the soil effect except for Cu. Furthermore, the interaction between soil type and species was significant, except for Fe and Mo. For all micronutrients, except Mo and Mn, grasses had lower concentrations than forbs and legumes (a selection of species are presented in Figure 2, see “pot”). In particular, timothy had micronutrient concentrations which were significantly lower than chicory ($P < 0.05$). Tall fescue had similar Cu and Zn concentrations to timothy. Manganese concentrations varied greatly among all species, but were highest in cocksfoot grown on the Rådde soil ($P < 0.05$). White clover and red clover had similar micronutrient concentrations except for Fe, which was significantly higher in white clover than in red clover ($P < 0.05$). Molybdenum concentrations were significantly ($P < 0.05$) higher in the two clovers and cocksfoot than in all forbs except caraway grown in Ås soil. Birdsfoot trefoil had similar Zn concentrations to the clovers, but lower concentrations of the other micronutrients, which were more similar to those of the forbs and grasses.

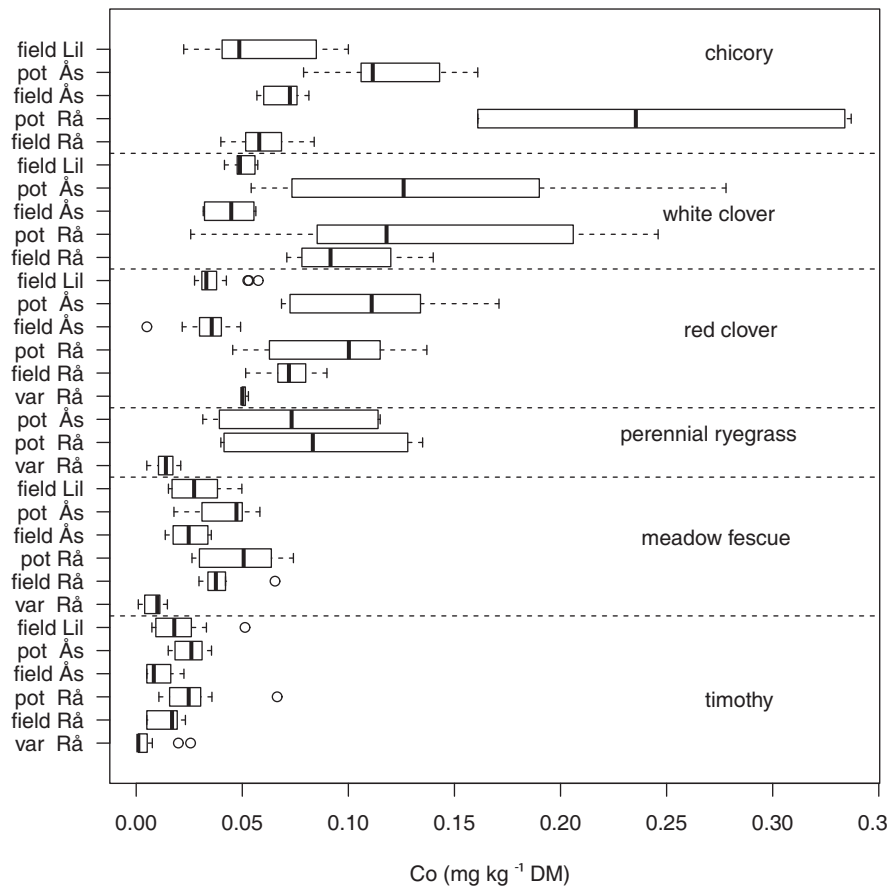


Figure 2a. Boxplot of cobalt concentrations in six species grown in different types of sites and experiment: Rå (Rådde site/soil), Ås (Ås site/soil), Lil (Lillerud site), field (Paper IV), "pot" outdoor pot experiment (Paper III), "var" variety field trial (Paper III). Includes two harvests in both the outdoor pot experiment and field experiment. Number of data points per boxplot varies with species and experiment.

With the exception of Mo, chicory generally had the highest concentrations of all micronutrients. Ribwort plantain had low Mn and Mo concentrations, similar to those of timothy, whereas Co and Zn concentrations were similar to those of the clovers.

The most prominent effect of soil type was that Mn concentrations were several-fold higher in plants grown on the Rådde soil than in plants grown on the Ås soil, whereas the reverse was true for Mo concentrations. Micronutrient concentrations were affected more by soil type in chicory than in any other species, and chicory had significantly higher concentrations of all

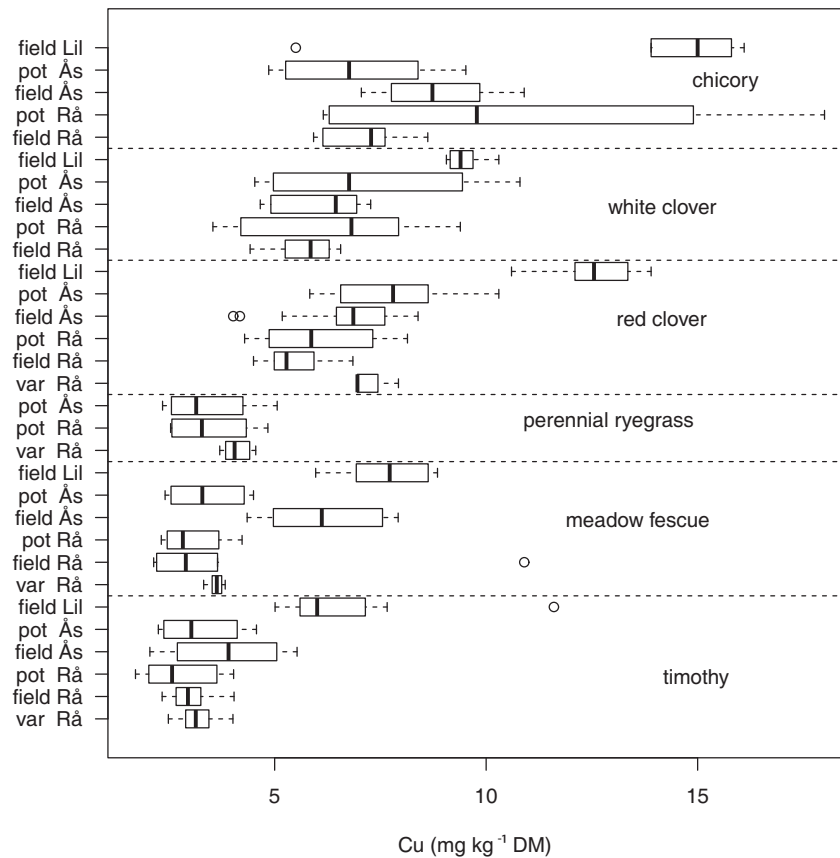


Figure 2b. Boxplot of copper concentrations in six species grown in different types of sites and experiment: Rå (Rådde site/soil), Ås (Ås site/soil), Lil (Lillerud site), field (Paper IV), “pot” outdoor pot experiment (Paper III), “var” variety field trial (Paper III). Includes two harvests in both the outdoor pot experiment and field experiment. Number of data points per boxplot varies with species and experiment.

micronutrients except Fe and Mo on the Rådde soil compared with the Ås soil ($P < 0.05$). The Zn concentration in chicory grown on the Rådde soil was higher than in any other species on either soil ($P < 0.05$).

4.3.3 Variety field trials

All micronutrient concentrations were significantly affected by species. Cocksfoot had the highest Mn concentration ($P < 0.05$) and red clover the highest Co and Cu concentrations ($P < 0.05$; a selection of species are presented in Figure 2, see “var”). Timothy generally tended to have the lowest

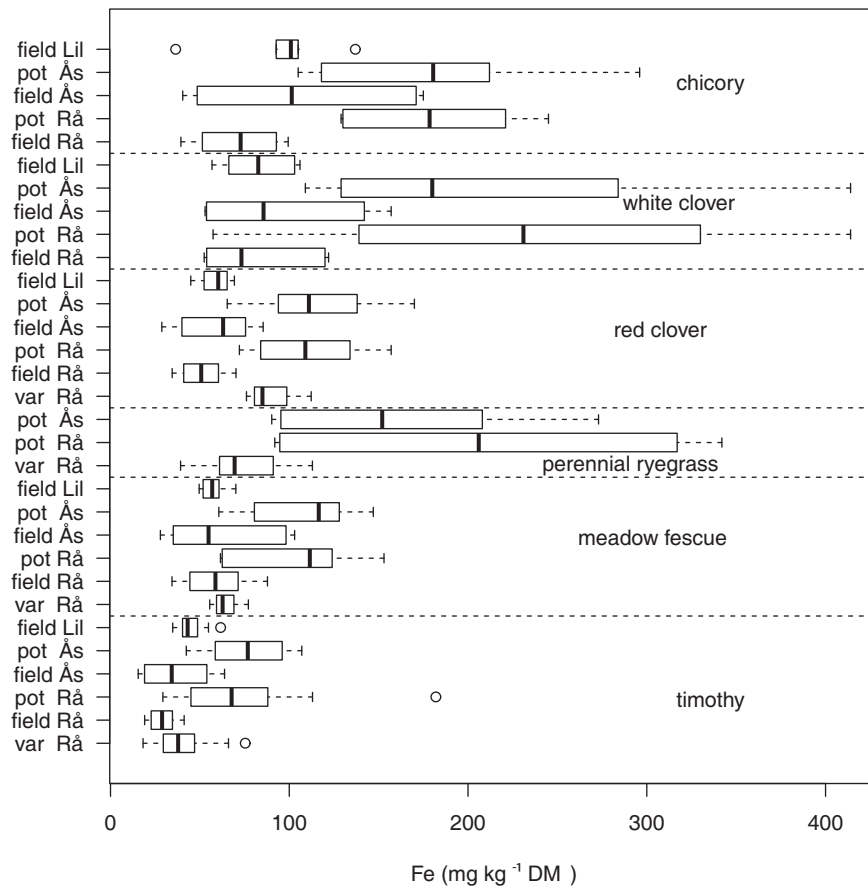


Figure 2c. Boxplot of iron concentrations in six species grown in different types of sites and experiment: Rå (Rådde site/soil), Ås (Ås site/soil), Lil (Lillerud site), field (Paper IV), "pot" outdoor pot experiment (Paper III), "var" variety field trial (Paper III). Includes two harvests in both the outdoor pot experiment and field experiment. Number of data points per boxplot varies with species and experiment.

micronutrient concentrations of all species, but the levels were not significantly lower than in the three *Festuca* species except for Mo ($P < 0.05$), and not significantly lower than in perennial ryegrass except for Cu and Mn ($P < 0.05$).

4.4 Effect of red clover proportion in species mixtures

Timothy and red clover dominated the mixtures at all three sites and the mean red clover DM proportion of all harvest occasions was 29% (min 17 - max

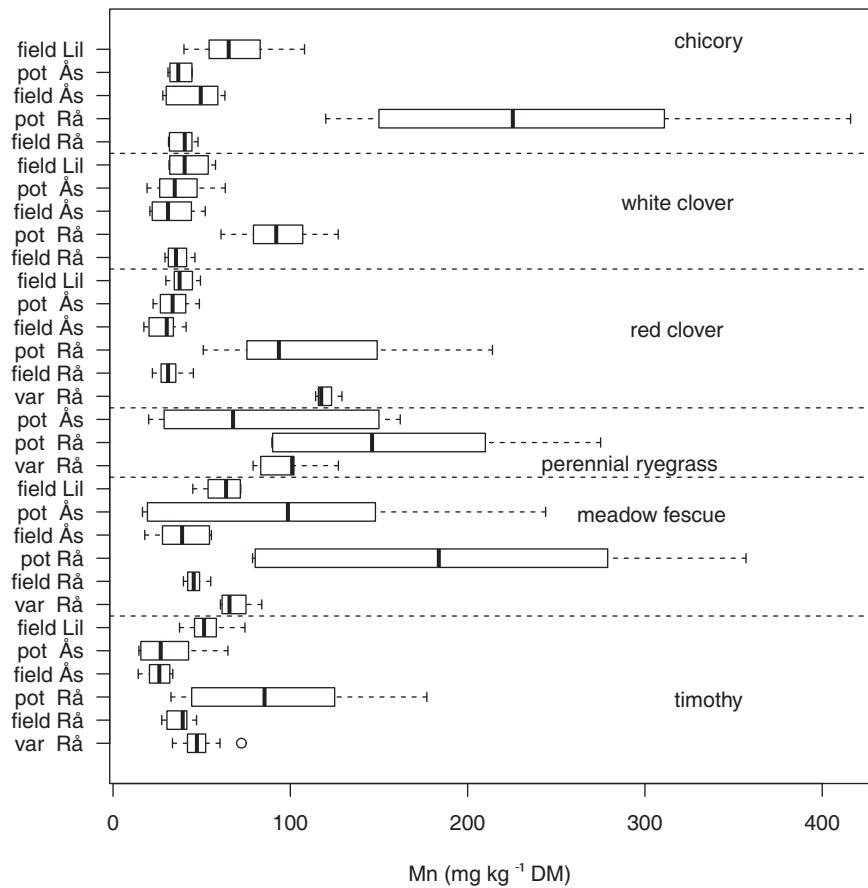


Figure 2d. Boxplot of manganese concentrations in six species grown in different types of sites and experiment: Råde (Råde site/soil), Ås (Ås site/soil), Lil (Lillerud site), field (Paper IV), “pot” outdoor pot experiment (Paper III), “var” variety field trial (Paper III). Includes two harvests in both the outdoor pot experiment and field experiment. Number of data points per boxplot varies with species and experiment.

37%) at Råde, 34% (min 19 - max 44%) at Lillerud and 44% (min 0.1 - max 73%) at Ås.

Similar to the outdoor pot experiment, chicory had the highest micronutrient concentrations of all species whereas timothy, with the exception of Mo (Figure 2, see “field”), had the lowest. Red clover and white clover had higher micronutrient concentrations than timothy, except for Mn and Zn. The two clovers had similar micronutrient concentrations, although there was a tendency for the concentrations to be higher in white clover. Meadow fescue generally had micronutrient concentrations in between those of timothy and red

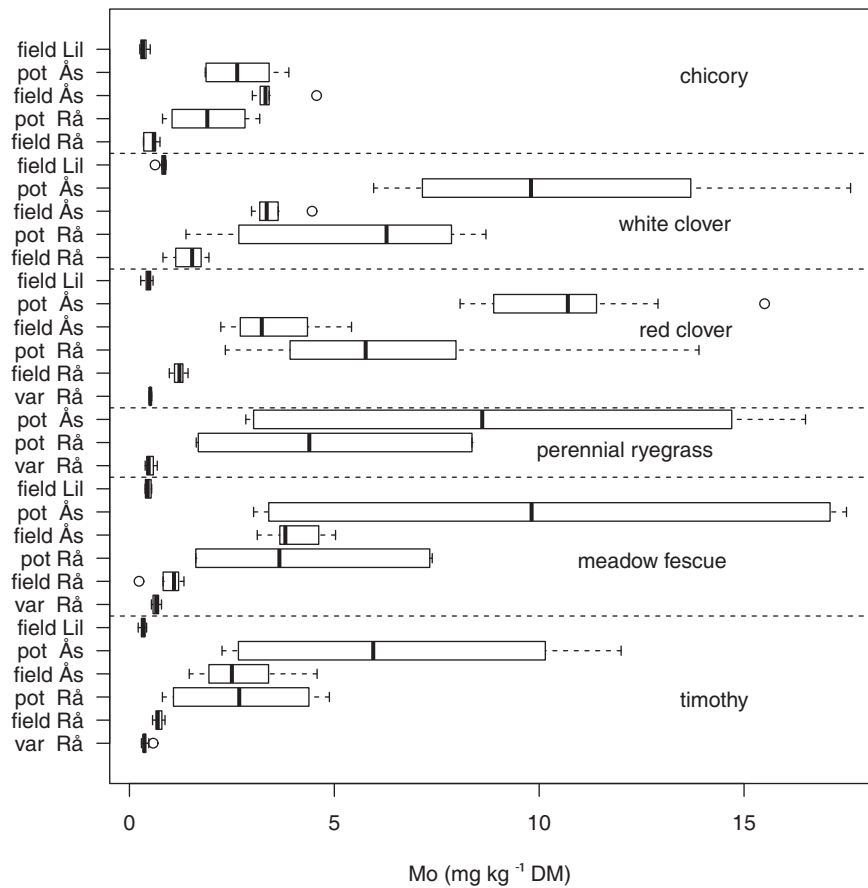


Figure 2e. Boxplot of molybdenum concentrations in six species grown in different types of sites and experiment: Rå (Rådde site/soil), Ås (Ås site/soil), Lil (Lillerud site), field (Paper IV), “pot” outdoor pot experiment (Paper III), “var” variety field trial (Paper III). Includes two harvests in both the outdoor pot experiment and field experiment. Number of data points per boxplot varies with species and experiment.

clover. There were few clear differences between species with regard to Mn concentrations.

The overall micronutrient concentrations of the mixtures were always significantly affected by site but there were few differences between mixture types. The mixtures at Lillerud generally had higher micronutrient concentrations than those at Rådde and Ås, in particular at the second harvest. The Mo concentration showed the largest variation between sites and was always significantly higher in the mixtures grown at Ås compared to those at Rådde and Lillerud.

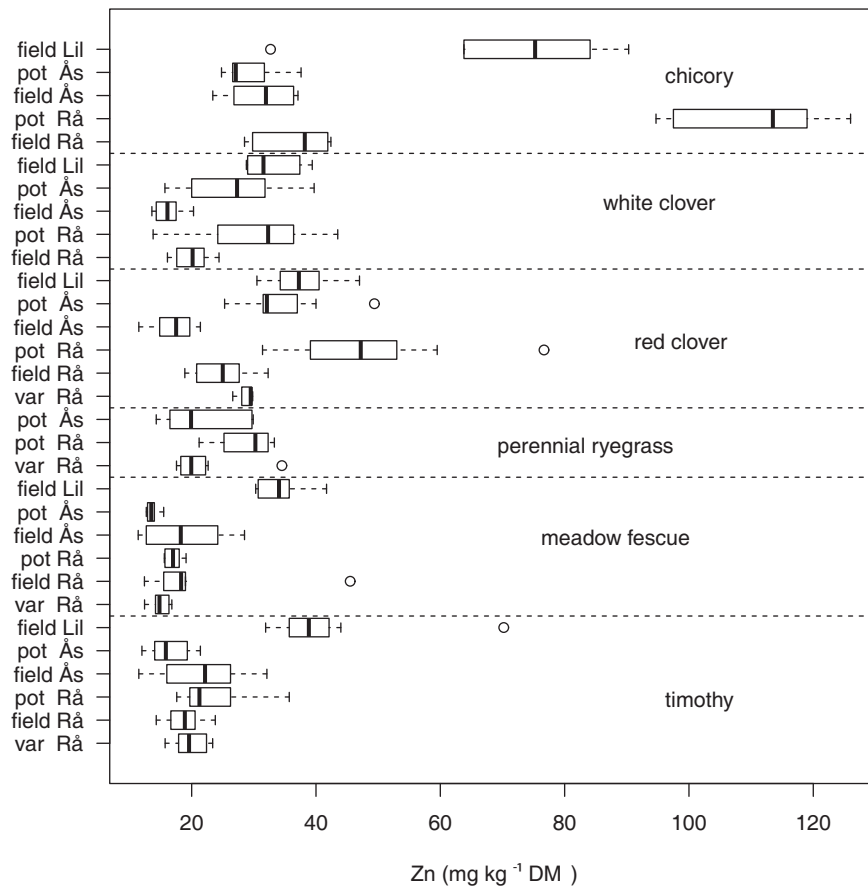


Figure 2f. Boxplot of zinc concentrations in six species grown in different types of sites and experiment: Rå (Rådde site/soil), Ås (Ås site/soil), Lil (Lillerud site), field (Paper IV), "pot" outdoor pot experiment (Paper III), "var" variety field trial (Paper III). Includes two harvests in both the outdoor pot experiment and field experiment. Number of data points per boxplot varies with species and experiment.

The Co concentration of the mixtures was always positively correlated with the red clover proportion in the harvested biomass. This was also the case for Cu concentrations in mixtures grown at Ås and Lillerud. The mixtures at Ås almost always showed a positive correlation between the red clover DM proportion and micronutrient concentrations. The exception was Zn concentrations which were negatively correlated with the red clover DM proportion at the first harvest and not significant at the second harvest. Iron and Zn concentrations at Rådde and Mo concentrations at Lillerud were furthermore positively correlated with the red clover DM proportion at the

second harvest. Generally, micronutrient concentrations at the second harvest increased more with an increase in the proportion of red clover DM than at the first harvest.

5 Discussion

5.1 Methodologies for investigating plant micronutrients

There are many potential risks of contamination and other sources of error when micronutrients in plant materials are investigated, as seen in the literature review in Paper I and as outlined in the Introduction of this thesis. One of the sources which is assessable is that of contamination from the milling equipment. The tests of milling devices confirm that these are potential sources of contamination of several micronutrients (of varying degree) to plant material. This implies that there is a risk in using archived plant samples from experiments not initially planned for analysis of micronutrients as they might not have been handled with enough caution to prevent contamination. To enable future micronutrient analyses of plant material from ongoing experiments, the sampling protocol needs to cover all the aspects of quality assurance. Furthermore, the protocol used, with thorough descriptions of potential sources of error, needs to be easily accessible for anyone intending to use the archived samples. However, all these additional precautions may be time consuming and the requirements for, for example, milling equipment may be difficult to meet unless these have already been tested. On the other hand, the interest in micronutrient concentrations of different types of crops is increasing (Watson *et al.*, 2012) which is why additional precautions might be needed to enable future micronutrient investigations of plant samples from, for example, long-term field experiments. The problems with insufficient information on plant sample handling are partly the reason why this thesis includes only new experiments with a short time perspective.

5.1.1 Precautions taken in this project

In addition to the sometimes general information from the literature review, some hands-on routines were developed at the beginning of the studies.

Throughout the project, harvesting, plant sample handling and preparations were planned to minimize handling and exposure to contamination. Furthermore, if soil contamination of a plant sample was suspected, this was carefully followed up throughout the handling and analysis. Before the fields were harvested all plots were visually examined for soil disturbances from, for example, moles and voles which may have created soil heaps above the cutting height of the plot harvester. This was found once in one plot and here the sub-sampling from the pile of harvested biomass were done carefully to avoid soiled plants, then the sample was washed in deionized water before dried. No visual traces of soil was found in the plastic bag after drying, and analyses of Al and Fe concentrations of this sample did not indicate soil contamination. For the same reason, the plants in the outdoor pot experiment were already showered before harvest because of suspected soil dust contamination from the construction of a new building close to the netted yard where the pots were kept. Analyses of Al and Fe in the plant samples were within the normal range, hence there seemed not to be any problem with soil dust contamination in the pot experiment.

The sub-sampling technique chosen for the field experiments has been shown to give botanically representative samples from mixed swards. This technique includes a Haldrup plot harvester which potentially could add contamination of some micronutrients to the plant samples. Since the amount of harvested biomass from each plot was of similar size, it may be assumed that the samples were contaminated to a similar degree.

5.1.2 Pot grown plants compared to field grown plants

The interaction effect of soil and species on micronutrient concentrations of forages was investigated in both Paper III and IV and included both pot-grown and field-grown species on two and three soils, respectively. Since two of these soils were represented in both pot and field experiments with five species each, comparisons can be made regarding the effect of methodology on plant micronutrient concentrations (Figure 2). The pot-grown species showed greater variation between the replicates compared to the field-grown species. In addition, the pot-grown species generally had higher micronutrient concentrations than the field-grown species. This could be due to higher rates of mineralization of organic matter in the pots due to soil sieving and homogenization at the start of the experiment. Furthermore, the soils of the pots were probably exposed to higher temperatures and a more constant humidity since they were watered regularly which also is beneficial for mineralization. In particular, this could explain the high Mo concentrations in the pot-grown species (Wichard *et al.*, 2009).

The plant Mn concentrations in the outdoor pot experiment were consistently higher in the Rådde soil than the Ås soil which was thought to be partly due to differences in pH but also redox potential since the Rådde soil appeared more compact in the pots. Compacted soil may inhibit proper aeration and increase the Mn availability of the soil (McBride, 1994). The field-grown species at Rådde did not show the same consistently higher Mn concentrations compared to those at Ås (Figure 2) which suggests that soil compaction and changes in the redox potential in the pots could have given a high plant Mn concentration in the Rådde soil. However, despite differences in experimental types, plant ranking from high to low micronutrient concentrations between these five species was similar both in the pots and the field.

The greenhouse pot experiment had three species grown on the Ås soil. The plant micronutrient concentration in this experiment was closer to that of the field-grown species and hence lower than in the outdoor pot experiment. The reason for this is unclear but could be due to the fact that the soil had been standing for a year before the start of the greenhouse experiment.

5.2 Effect of phenological development

The changes that occurred during advancing phenological development with regard to micronutrient concentrations and DM accumulation differed between the species. Between the first and last phenological stage investigated in Paper II, the concentrations decreased more than 50% for some micronutrients in timothy but less in perennial ryegrass, whereas red clover had stable Co, Fe and Mn concentrations. These patterns resemble those found in other studies, although the reports on red clover are to some extent conflicting (Fleming & Murphy, 1968; Whitehead & Jones, 1969; Anke *et al.*, 1994; Brink *et al.*, 2006). The species differences (as well as conflicting results) could be due to species specific changes in the proportions between leaves and stems, since flowers and leaves generally had higher micronutrient concentrations than stems. Similar to other studies (Bartholomew & Chestnutt, 1978; Nissinen *et al.*, 2010), timothy had a stem proportion around 65% whereas it was 40% in perennial ryegrass. Hence, the results suggest that a harvest at the early phenological stages, with a high proportion of leaves, would give higher micronutrient concentrations in the sward. This will also result in a biomass rich in energy and protein suitable as a feed for high-yielding animals.

5.3 Species differences

The DM harvest of the forage species grown in the field experiment was generally similar to that normally found in these regions (*e.g.* Halling *et al.*, 2002; Frankow-Lindberg *et al.*, 2009). The micronutrient concentrations of these species can therefore be considered to be indicative of on-farm values, and not a result of non-representative growth which could lead to, for example, a dilution or accumulation effects (Jarrell & Beverly, 1981). The potential reasons for the somewhat higher micronutrient concentrations of the outdoor pot experiment have already been discussed but the species differences found are confirmed by the field experiment.

The few and small differences between varieties in terms of micronutrient concentrations that were found in the studies in Paper III agree with findings by Forbes and Gelman (1981) for perennial ryegrass, white clover and cocksfoot. Since these differences were smaller than those between species with regard to micronutrient concentrations, the varietal differences will be disregarded henceforth.

In both the field experiment and the outdoor pot experiment, chicory, red clover and white clover generally had higher concentrations of Co, Cu, Fe and Zn than timothy and meadow fescue. On the other hand, Mn concentrations were similar in all species. Perennial ryegrass was not included in the field experiment but had a micronutrient concentration pattern similar to the other grasses in the pot experiment. These species, and the other species used in the studies reported in Paper III, have not been compared together in one single study before, but two or more species have been compared with roughly the same trends and concentrations (Forbes & Gelman, 1981; Harrington *et al.*, 2006; Pirhofer-Walzl *et al.*, 2011). Plant Mo concentrations varied most strongly between soils, particular in the pot experiment, and were highest in plants grown in the Ås soil. However, chicory Mo concentrations were generally low and least affected by soil. Apart from Mo, micronutrient concentrations in chicory seem to be more readily affected by soil (or site) than concentrations of the other species (Figure 2). In particular Zn concentrations in chicory were high in some soils, which confirm earlier findings (Crush & Evans, 1990). Timothy, on the other hand, appeared less affected by soil and site.

In Paper IV, the effect of species differences on the overall micronutrient concentrations in a species mixture was studied more in detail. It was shown that the red clover DM proportion was positively correlated to several micronutrients at the three sites. In particular, Co concentrations were strongly affected and showed that an increased red clover DM proportion from 10% to 25% at Rådde or from 25% to 50% at Lillerud and Ås increased the average Co

concentration of the mixture by more than 30% at the first harvest and more than 80% at the second harvest. These results are in accordance with the findings of Kunelius *et al.*, (2006) and Høgh-Jensen & Søgaard (2012) of higher micronutrient concentrations and accumulations in grass-legume mixtures compared to pure grass swards. However, since the red clover proportion generally declines with sward age (Mela, 2003) this could result in a decrease of micronutrient concentrations in the harvested plant material over the years. This problem could be solved by adding white clover in the species mixture as white clover DM proportion tends to increase with time. Hence, a grass-legume mixture with red and white clover which can be expected to give a higher yield stability of clovers (Frankow-Lindberg *et al.*, 2009), will also give a benefit in terms of more stable micronutrient concentrations.

5.4 Effect of soils

The effect of soil is only one aspect of the site effect on plant micronutrient, but in these studies the focus has been on soil properties. The soils at Rådde, Lillerud and Ås, used in the field experiment, and the two soils used in the pot experiment (from Rådde and Ås) were deliberately chosen to be contrasting in their concentrations of micronutrients. These soils were chosen from a larger set of soil samples collected at the start of the project. The soils were analysed with the same method (7 M HNO₃ extraction) used for the Swedish arable soils monitoring programme (Eriksson *et al.*, 2010) to enable comparisons on the national scale. This method gives the pseudo-total micronutrient concentrations of the soil and has shown correlation with, for example, Cu and Zn concentrations of cereals (Eriksson *et al.*, 2010).

The soil at Ås belongs to the 10% of Swedish soils with the highest Co, Mn and Zn concentrations and has above average Cu and Mo concentrations (Eriksson *et al.*, 2010). Lillerud has average (25-75 percentile) Co, Cu, Mn and Zn concentrations in the soil. The soils at Rådde have Co, Cu and Zn concentrations within the lowest 25% but more average concentrations of the other micronutrients studied.

In addition, the field experiment soils were analysed with a method using EDTA (adjusted to pH 7) as an extracting agent which is supposed to give a better estimate of the plant available micronutrient concentrations than, for example, the stronger extractant (HNO₃) used by Eriksson *et al.* (2010). Although the precise protocols could differ, the use of EDTA for soil micronutrient analyses is common in the literature. However, EDTA extraction may provide limited information about the availability of micronutrients (Bussink & Temminghoff, 2004). Jarvis and Whitehead (1981; 1983) extracted

twenty-one soils with EDTA and showed that the variation in soil Cu concentrations between the soils they studied was wider than between the Cu concentrations of the plants grown on them, in this case pure stands of perennial ryegrass and white clover. This was also the case in the field experiment presented in Paper IV where the variation between plants grown on the three study sites with regard to Co, Mn, Cu, Fe and Zn concentrations was smaller than the variation between the micronutrient concentrations extracted from the soils, whereas the opposite was true for Mo. Since plants can actively regulate their uptake of most micronutrients, a smaller variation in plant micronutrient concentrations than in the soil could be expected (Marschner, 1995).

The generally higher micronutrient concentrations in the forage species grown at Lillerud indicated that soil micronutrients were relatively available at this site compared to the other sites. The higher soil micronutrient concentrations at Ås, as extracted by both HNO₃ and EDTA, were, on the contrary, not as plant available as they were at Lillerud. This might be explained by the high pH (above 7) of the Ås soil since this limits the availability of most micronutrients except Mo (McBride, 1994). The high plant Mo concentration at Ås is a further sign of this. Another explanation of the relatively low micronutrient concentrations of the mixtures at the second harvest at Ås could, at least partly, be due to a dilution effect since the DM yield of this harvest was larger than at the other sites. In addition, the harvest was done rather late in the season. However, the soils also differed in soil organic matter and proportion of clay (Table 2), which are important soil properties that affect micronutrient availability (McBride, 1994).

5.5 Practical implications

In a Swedish study comparing organically and conventionally managed fields, the red clover DM proportion in organic swards was 61% (mean of two harvests) compared to 37% in the conventional fields (Pettersson *et al.*, 1998). Thus, the organic farmers are to some extent already exploiting the species differences with regard to micronutrient concentrations with their red clover rich swards. This difference between swards on organic and conventional farms is partly due to a higher reliance on N₂-fixation by legumes on organic farms. On the other hand, red clover proportion may be decreased due to grass competition if the temporary grasslands are heavily fertilized with N. From the ruminant feeding point of view, the voluntary intake of legumes is high, the nutritional qualities are good overall and legumes can be fed purely to

ruminants, although increased N concentrations in the feed may lead to increased N losses with faeces and urine (Bertilsson & Murphy, 2003).

Diversifying the sward with forbs is another possible option to increase the overall micronutrient concentration, as indicated by the high concentrations found in chicory and other forbs (Paper III; Forbes & Gelman, 1981; Harrington *et al.*, 2006). This has been found with regard to the more abundant macronutrients, where the overall concentrations of these were increased with increased chicory proportion in the sward (Belesky *et al.*, 2001; Weller & Bowling, 2002). Apart from the macro- and micronutrients, studies on other nutritive values show that forbs, such as chicory, meet the ruminant requirements of these (Barry, 1998; Sanderson *et al.*, 2003). In particular many organic farmers consider forbs, such as caraway, in the sward to be beneficial because of their macro- and micronutrients but also because they think forbs improve animal health in other ways (Smidt & Brimer, 2005; Zollitsch *et al.*, 2008). However, too high a proportion of chicory in feed may give the milk a bitter taint (Barry, 1998). Another potential drawback is that the pot-grown chicory not only had high micronutrient concentrations, but also a high concentration of the potentially toxic heavy metal Cd (Paper III) which was above the limit set by the EC directive for feed (2002). Furthermore, the DM proportion of chicory in the mixtures reported in Paper IV was low and it tended to develop fibrous stems. Chicory is not a common species in seed mixtures sold in Sweden, and there is a lack of agronomic experience of the species. As the milk yield per cow in Sweden is among the highest in Europe the need for a high quality forage with respect of energy and protein content is paramount. Hence, the agronomy of potential new species for inclusion in seeding mixtures needs to be investigated to ensure that the necessary high-quality feed is achieved.

Overall, the species and mixtures investigated and presented in Papers II, III and IV met the required concentrations of Fe and Mn whereas Co, Cu and Zn in most cases were below the requirements (0.11 mg Co kg⁻¹, 11 mg Cu kg⁻¹, 12-18 mg Fe kg⁻¹, 14 mg Mn kg⁻¹, 43-55 mg Zn kg⁻¹) of low (25 kg milk day⁻¹) and high yielding (54 kg milk day⁻¹) dairy cows (National Research Council, 2001). This was further aggravated by the decrease of Cu and Zn concentrations in all three species studied in Paper II with advancing phenological development. Low Cu concentrations could become an even greater problem if the forage at the same time has high Mo and S concentrations, because they reduce the bioavailability of Cu in the rumen (Suttle, 2010). From this point of view, the low Mo concentrations of chicory but relatively high Co, Cu and Zn concentrations are an advantage.

Nevertheless these results imply that mineral supplementation may be reduced with a high red clover proportion and an early harvest, but that some supplementation may still be needed in order to meet the requirements of Co, Cu and Zn of dairy cows. However, mineral supplementation may in the long run increase the total soil micronutrient concentrations, through the spreading of e.g. Zn enriched manures. This could become a problem particularly in areas with high soil micronutrient concentrations but also in areas with initially moderate soil micronutrient concentrations since Zn has been found to negatively affect the populations of *Rhizobium* after application via organic material (sewage sludge) (Chaudri *et al.*, 2008). On the other hand, in areas with low total soil micronutrient concentrations there is a potential risk of depleting soil of micronutrients by growing crops with high DM yields and micronutrient concentrations (Paper III), unless animal manure is returned to the same land.

Farm-gate and field nutrient balances can help in understanding the broader implications of the results presented here. In particular, these approaches could assist in planning the use of manures and fertilizers to recirculate and replace the micronutrients removed by the harvested forage and avoid micronutrient accumulation. Farm-gate budgets are especially important in production system with high yields of milk or meat and which rely mainly on local forage production (Watson *et al.*, 2012).

6 Conclusions

Legumes and forbs generally had higher Co, Cu, Fe and Zn but similar Mn and Mo concentrations compared to grasses. Chicory had the highest and timothy the lowest micronutrient concentrations. The overall micronutrient concentrations of a grass-legume sward were increased by increasing the red clover DM proportions. This implies that seed mixtures with red clover and white clover could be used when higher and more stable sward micronutrient concentrations are desired. If micronutrient-rich forbs, such as chicory, are to be successfully included in sward mixtures in Sweden, further investigation of, for example, establishment techniques, management, fertilization, harvesting regime, and feeding value may be needed.

The varietal differences in micronutrient concentrations were few and small, and consequently this quality characteristic does not interfere with the choice of a variety that is persistent and gives high yields of good quality, which are the most important criteria when choosing varieties for a specific site.

In contrast to Fe and Mn, the concentrations of Co, Cu and Zn of the forage species grown on the three experimental soils and sites were generally low in comparison with dairy cow requirements. This was further aggravated by the decrease of Cu and Zn concentrations with phenological development, particularly in timothy. Leaves had higher micronutrient concentrations than stems. This implies that in order to obtain high micronutrient concentrations a harvest should be done at an early phenological stage when the proportion of leaves is high. The results suggest that mineral supplements of Co, Cu and Zn are needed to meet the dairy cow requirements, although there might be areas in Sweden where it is not needed, in particular when clover-rich forages is used.

The strongest effect of soil on plant micronutrient concentrations was that of the soil pH. The soil micronutrient concentration as extracted by

concentrated nitric acid and EDTA gave little information regarding the plant availability of these micronutrients.

The accuracy of plant micronutrient analyses is highly dependent on the sampling strategy, sample handling and any sample preparation. This implies that results from studies lacking this awareness must be considered with caution.

The pot-grown and field-grown species showed a similar ranking from high to low micronutrient concentrations, irrespective of experimental type. However, the pot experiment overestimated the micronutrient concentrations in plants but also gave larger variation between the replicates. This implies that future studies on micronutrient concentrations in forage crops and other plants need to consider the choice of experiment type depending on the hypothesis posed.

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