Sveriges lantbruksuniversitet
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Title: **Red clover increases micronutrient concentrations in forage mixtures**

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Highlights:

- Four grass-clover-chicory mixtures were grown on three contrasting sites.
- Chicory and clovers had higher micronutrient concentrations than grasses.
- Mixture micronutrient concentrations increased with red clover proportion.

- Site properties affected overall micronutrient levels in species and mixtures.

Keywords: grass, legume, ley, trace element, soil, herb

Abstract

Forage crops provide micronutrients as well as energy, protein and fiber to ruminants. However, the micronutrient concentrations of forage plant species differ, legumes generally having higher concentrations than grasses. In addition to that there are also strong effects of soil type.Typically, the concentrations of one or several micronutrients in forage are too low to meet the nutritional requirement of dairy cows. We hypothesized that the overall micronutrient (Co, Cu, Fe, Mn, Mo, Zn) concentrations of forage mixtures are affected by the red clover dry matter (DM) proportion and site effects. This hypothesis was tested at three contrasting sites. The results showed that increased red clover proportion increased the overall concentrations of several micronutrients in the mixtures at all sites. At the site with the widest range of red clover proportion (0-70%) in the mixture, the Co, Cu and Fe concentrations more than doubled between the lowest and highest red clover DM proportion. At the other two sites a smaller increase in red clover proportion (from 10% to 25% or from 25% to 50%) also increased the overall concentrations of Co by up to 80% but less for other micronutrients. One of the sites generally had higher micronutrient concentrations in the crop and removed larger amounts of micronutrients with the harvested biomass compared to the other two sites. This could be explained by differences in pH and micronutrient concentrations of the soils at the sites. We conclude that increased red clover proportion in the sward has the potential to increase the overall micronutrient concentrations but that the effect of the soil is also a controlling factor.

1 Introduction

Forages are important in ruminant production. In addition to energy, fiber and protein, the forages provide macro- and micronutrients required for sustainable animal production and health (*Suttle*, 2010). Thus, in livestock production systems which mainly rely on forage the plants are the main source of the essential micronutrients such as cobalt (Co), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) as well as the beneficial molybdenum (Mo). However, the micronutrient concentrations of forage vary with site (*Hopkins* et al., 1994), largely due to the influence of differences in soil properties such as texture, organic matter, pH, total and available micronutrient concentrations of the soil (e.g. *Kähäri* and *Nissinen*, 1978; *Paasikallio*, 1978). Thus farms who base feed on locally produced forage, for example organic farms, depend on the soil properties of the farm.

To ensure that feed rations meet livestock requirements, as specified by e.g. *National Research Council* (2001), mineral supplementations are allowed in both conventional and organic livestock systems. Such supplementation may lead to a relatively rapid increase in micronutrient concentrations in the soils of livestock farms (*Andersson*, 1992; *Knutson*, 2011) which in the long term may lead to excessive concentrations affecting important microbial processes on some soils (*Giller et al.*, 1998). However, the use and dependency of mineral supplementation may be reduced by altering the species mixture of the sward. Studies on different species mixtures have shown that grass-legume mixtures have higher micronutrient concentrations (*Govasmark* et al., 2005; *Kunelius* et al., 2006) and higher micronutrient removals in the harvested biomass (*Høgh-Jensen* and *Søegaard*, 2012) than pure grass swards. This is because of the generally higher micronutrient concentrations found in legumes compared to grasses (e.g. *Lindström* et al., 2012; *Pirhofer-Walzl* et al., 2011). However, the relationship between the legume proportion and the overall micronutrient concentrations of the mixed sward has rarely been evaluated. Furthermore, the strong link between plant micronutrient concentrations and soil properties needs to be taken into account in studies regarding micronutrient concentrations in forage. A field experiment with a range of timothy-red clover dominated mixtures established at three contrasting sites provided an excellent opportunity to explore this. The hypothesis tested was that the overall micronutrient concentrations of forage mixtures are affected by the red clover dry matter (DM) proportion and by site effects.

2 Materials and methods

A field experiment was established in 2010 at three sites with contrasting soils in Sweden: Rådde (57°36'N, 13°15'E), Lillerud (59°38'N, 13°23'E) and Ås (63°14'N, 14°33'E). The soil at Rådde is a till with sandy loam texture developed from mainly granitic parent material, at Lillerud the soil is a postglacial silty loam originating from mainly granitic and sandstone bedrock, and at Ås a loamy till developed from alum shales. A composite soil sample, taken along a transect of each field before the trials were sown, was analysed for pH, total C and N, pseudo-total macro- and micronutrient concentrations and of "plant available" micronutrient concentrations (Tab. 1). Soil pH was first analysed in deionized water and then in 0.01 M calcium chloride solution according to *Sumner* (1994). Total N and C concentrations in soil samples were analysed by high temperature (1250°C) induction furnace combustion using LECO CN2000 (LECO Corporation, St Joseph, MI, USA). Pseudo-total macro- and micronutrient concentrations were extracted with concentrated nitric acid and hydrogen peroxide and analysed on ICP-SFMS at ALS Scandinavia AB in Luleå, Sweden (same laboratory and method as the Swedish arable soil monitoring program). "Plant available" soil micronutrients were extracted with 0.05 M EDTA (pH 7) and analysed by ICP-MS (*Ure* and *Berrow*, 1970).

The experiment included five species: timothy (*Phleum pratense* L., cv. Grindstad), meadow fescue (*Festuca pratensis* Huds., cv. Sigmund at Rådde and Lillerud, cv. Kasper at Ås), red clover (*Trifolium pratense* L., cv. Ares at Rådde and Lillerud, cv. Torun at Ås), white clover (*Trifolium repens* L., cv. Ramona at Rådde and Lillerud, Undrom at Ås) and chicory (*Cichorium intybus* L., cv. Grassland's Puna). All species except chicory have the bulk of their root system in the upper 25 cm of the soil profile. These species were sown in four different mixtures; i) timothy and red clover (15 and 5 kg ha^{-1}, respectively); ii) timothy, red clover and meadow fescue (4.2, 10.8 and 5 kg ha^{-1}, respectively); iii) timothy, red clover and white clover (2, 15, 3 kg ha^{-1}, respectively); and iv) timothy, red clover and chicory (3, 15, 5 kg ha^{-1}, respectively). The experimental design was a randomized block design with three replicates. Plot size harvested was 12.0, 14.0 and 13.5 m^2 at Rådde, Lillerud and Ås, respectively.

The forage species were under-sown in spring barley (*Hordeum vulgare* L.) (sown at rates of 120-200 kg seed per ha) on 7 May 2010 at Rådde, 24 May 2010 at Lillerud and 2 July at Ås. Corresponding harvest dates of barley were 6 July, 10 August and 6 September 2010. The barley crop was fertilized with 70 kg N ha^{-1}, 10 kg P ha^{-1}, 33 kg K ha^{-1} at Rådde, 60 kg N ha^{-1}, 12 kg P ha^{-1} and 15 kg K ha^{-1} at Lillerud and 40 kg N ha^{-1}, 50 kg P ha^{-1} and 95 kg K ha^{-1} at Ås. Weed ingression was controlled at Rådde by topping on 26 August.

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 after each cut except the last. In addition, the crop at Rådde was fertilized with 14 kg P ha^{-1}, 75 kg K ha^{-1} and 7 kg S ha^{-1} in the spring and 27 kg K ha^{-1} after each cut except the last. The crop at Lillerud was fertilized with 12 kg P ha^{-1} and 21 kg K ha^{-1} in the spring and 10 kg P ha^{-1} and 18 kg K ha^{-1} after each cut except the last. The amounts of P, K and S fertilizer applied were based on previous soil analyses. Different products with different combinations of N:P:K:S from Yara International ASA were used as fertilizers. With the exception of

YaraMila 22:0:12, which has 0.1% Zn and were used after first cut at Rådde (227 kg ha^{-1}), none explicitly contains micronutrients. Data from *Eriksson* (2001) have been used to estimate amounts of micronutrients found as unlabelled traces in mineral fertilizers. The year before ley establishment (2009) cereals were grown on all sites, hence any carry over effect can be considered to have affected the soil and nutrients similarly at all sites.

In the spring and summer of 2010, the mean air temperatures at all sites were close to the 30 year average but all sites received more precipitation than normal (Tab. 2). The following autumn and winter were dry, in particular at Rådde, and November-December was colder than usual at all three sites. The mean air temperature and amount of precipitation was close to the 30 years mean during the spring and summer of 2011.

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, and subsequent harvests according to farming practise at the respective sites. Plots were harvested with a Haldrup (Løgstør, Denmark) plot harvester to a stubble height of approximately 5 cm.

Two composite plant samples of forage species were taken from each plot on all harvest occasions. One sample was dried at 105 °C for at least 48 hours for DM determination. The other sample was stored cool in a perforated plastic bag (hole diameter 0.4 mm; Cryovac ®, Duncan, S.C.) and sorted fresh within 48 hours into sown components and unsown species, which were dried in a forced-draught oven (55°C, minimum 48 h). Micronutrient analyses were made on each of the sown species from the first two harvests. To this end, these samples were milled (particle size <1 mm) in a cutting mill (Grindomix GM 200, Retsch GmbH, Haan, Germany) with a titanium knife and a plastic container which ensured minimal micronutrient contamination of the samples (*Dahlin* et al., 2012). The milled samples were wet digested with 7 M ultrapure nitric acid and concentrated hydrogen fluoride at increasing temperature until boiling, then filtered and analysed for Co, Cu, Fe, Mn, Mo and Zn by ICP-SFMS at ALS Scandinavia AB in Luleå, Sweden.

3 Statistics

Micronutrient concentration and off-take (species proportion of DM yield × concentration) differences between species at all sites were analysed for each harvest with a linear mixed model with species and site as fixed factors and block as a random factor, followed by Tukey's HSD test, using JMP 8.0.1 (SAS Institute Inc., 2009). The overall micronutrient concentrations of the mixtures were calculated by taking the botanical proportion of each species in each mixture into account. Within each harvest, total DM yield and average micronutrient concentrations for each mixture were analysed with a linear mixed model with mixture and site as fixed factors and block as a random factor, followed by Tukey's HSD test. The effect of red clover proportion (of sown species) on the average micronutrient concentration was analysed per site in SAS (Institute Inc., Cary, NC, USA) with the procedure MIXED where site, mixture and the interaction between site and red clover proportion were set as fixed factors and block as a random factor. Where the residuals showed a non-normal distribution the data were ln-transformed and results are presented as back-transformed least square means.

4 Results

4.1 Dry matter yield and botanical composition

Rådde and Lillerud had similar DM yields at the first two harvests in 2011 but Lillerud had a larger DM yield than Rådde at the third harvest (Tab. 3). The DM yields were smaller at Ås than at Rådde and Lillerud at the first harvest but larger than at Rådde at the second harvest. The accumulated DM yields of the different mixtures were 11.5-13.4 t DM ha^{-1} at Rådde, 14.6-16.6 t DM ha^{-1} at Lillerud and 7.4-10.8 t DM ha^{-1} at Ås.

Timothy and red clover dominated the mixtures at all three sites with similar proportions of red clover among the mixtures at Lillerud and Rådde whereas there was greater variation in red clover proportion between mixtures at Ås (Tab. 3). The mean red clover DM proportion at all harvests was 29% (min 17- max 37%) at Rådde, 34% (min 19- max 44%) at Lillerud and 44% (min 0.1- max 73%) at Ås. The DM proportion of meadow fescue was between 10-30% at Rådde and Ås, but around 5% at Lillerud in the first two harvests. The DM proportion of white clover and chicory at all sites was well below 10%, with the exception of chicory (15%) in the second harvest at Ås.

4.2 Micronutrient concentrations and off-takes of species

Generally, chicory had the highest micronutrient concentrations of all species whereas timothy had the lowest (Tab. 4). The exception was at Rådde and Lillerud where white clover had higher Mo concentrations than chicory and timothy which had similar concentrations. Red clover and white clover had higher micronutrient concentrations than timothy with the exception of Mn and Zn. The two clovers had similar micronutrient concentrations, although there was a tendency for the concentrations to be higher in white clover. There were few clear differences between species with regard to Mn concentrations although timothy had higher concentrations than red clover at Lillerud and Rådde. Meadow fescue generally had micronutrient concentrations between those of timothy and red clover.

Despite the higher micronutrient concentrations chicory had smaller micronutrient off-take (often < 10% of total mixture) compared to timothy (<80% of total mixture) (Tab. 5), when DM yield proportion were taken into account. Further, red clover and timothy generally had similar micronutrient off-take. The exception was Rådde where timothy had larger off-take than red clover for all micronutrients but Co. In contrast, red clover had larger Co, Cu and Fe off-take than timothy in the second harvest at Ås.

4.3 Effect of site and red clover proportion on mixture micronutrient concentrations and off-takes

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 (Fig. 1). Average micronutrient off-take of mixtures also indicates that higher amounts were removed with both harvests from Lillerud compared with Rådde and Ås (Tab. 5). Molybdenum 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 (Fig. 1). 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. With one exception, the mixtures at Ås always showed a positive correlation between the red clover DM proportion and micronutrient concentrations. However, this relationship did not hold for Zn where concentration was negatively correlated with red clover DM proportion at the first harvest and unrelated to red clover DM at the second harvest. Iron and Zn concentrations at Rådde and Mo concentrations at Lillerud were positively correlated with red clover DM proportion at the second harvest occasion.

5 Discussion

5.1 Dry matter yield and botanical compositions

The accumulated DM yields recorded at all three sites were within the range previously reported for grass/clover leys in Sweden (e.g *Frankow-Lindberg* et al., 2009, *Halling* et al., 2002). The results can be considered representative for the sites, as the temperature was normal and the precipitation only slightly higher than normal compared to the long-term average (Tab. 2). The four seed mixtures produced stands of different botanical compositions at the three sites, with a wide range of red clover DM proportions at Ås and less variation at Rådde and Lillerud. The overall increase of red clover and meadow fescue DM proportion, at the expense of timothy, with each harvest is similar to the findings of *Jørgensen* and *Junttila* (1994) and *Mela* (2003). But, the overall grass proportion was similar irrespective of the mixture contained timothy only, or timothy and meadow fescue. White clover DM proportions were low at all sites and all harvests, in contrast to *Halling* et al., (2002) who found increases of white clover with each subsequent harvest. Also, the DM proportion of chicory was much lower than those reported from other sites in northern Europe (*Høgh-Jensen* et al., 2006; *Weller* and *Bowling*, 2002). This was due to the unexpectedly poor establishment of this species at all sites. Hence, the presence of chicory and white clover had little impact on the botanical composition and thus were less important with respect to micronutrient concentration of the whole mixture. This means that the proportions of red clover and timothy were the main components affecting the total micronutrient concentration of the crop.

5.2 Micronutrient concentrations

The micronutrient concentrations of the species were similar to the levels found in other studies (e.g. *Forbes* and *Gelman*, 1981; *Pirhofer-Walzl* et al., 2011). Exceptions were the generally low Co concentrations in the species at all sites and unusually high Mo concentrations at Ås. Micronutrient concentrations in the different species was generally in the order chicory>clover>grass. Amongst the grass species timothy had the lowest micronutrient concentrations. This is similar to the species rankings published by *Lindström* et al. (2012) and to conclusions regarding differences between forbs, legumes and grasses in previous studies (e.g. *Pirhofer-Walzl* et al., 2011). Furthermore, our study confirms that chicory tends to have relatively low Mo concentrations compared to other species, which could be due to the fact that it can use ammonium as an N source (*Santamaria* et al., 1998), and that there are few differences between species now studied with regard to Mn concentrations.

Red clover and timothy dominated the species mixtures and hence affected the overall micronutrient concentration and off-take of the mixtures most strongly. This was most obvious at Ås where the large variation in red clover DM proportion resulted in positive correlations between the red clover proportion and the overall concentrations of the mixtures of all micronutrients except Zn (Fig. 1). A similar pattern was observed at Rådde and Lillerud, in particular for Co where even a small increase of red clover DM proportion increased the overall Co concentration of the mixture. An increase in 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. Within the same range of red clover DM proportions, Cu and Fe concentrations increased by more than 15% and 40% at the first and second harvests, respectively, at Ås for both micronutrients, at Lillerud for Cu and at Rådde for Fe. Moreover, at Ås, the concentrations of Co, Cu and Fe more than doubled when comparing the lowest red clover DM proportion with the highest proportion. These findings support our hypothesis that the overall micronutrient concentrations of forage mixtures are affected by the red clover DM proportion and site effects. Our findings also increase the available information on the impact of clovers on the micronutrient concentration of grass-legume mixtures compared to pure grass swards, as suggested by *Govasmark* et al. (2005), *Høgh-Jensen* and *Søegaard* (2012) and *Kunelius* et al. (2006).

5.3 Site effects

The three sites were deliberately chosen to have contrasting soil micronutrient concentrations, as analysed by nitric acid. 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, according to the Swedish arable soils monitoring program (*Eriksson* et al., 2010). Lillerud has average (25-75 percentile) Co, Cu, Mn and Zn concentrations in the soil. Rådde has Co, Cu and Zn concentrations within the lowest 25% but more average concentrations of the other micronutrients studied. However, plant micronutrient concentrations are also affected by a range of other site factors including soil organic matter (*Adriano*, 2001), proportion of clay (*McBride*, 1994) and the weather during the experimental period (*Roche* et al., 2009).

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 soil at Ås had higher micronutrient concentrations (pseudo-total concentrations extracted by nitric acid and EDTA used as a proxy for the plant available fraction) than Lillerud but the micronutrients were obviously less plant available. 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. However, we cannot exclude temperature effects (*Whitehead*, 2000). 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.

The Rådde soil had a similar pH to the Lillerud soil but a higher total C concentration, lower clay proportion and lower soil micronutrient concentrations. The DM yields at the two sites were similar but the micronutrient concentrations of the plants were lower at Rådde. The availability of micronutrients may be negatively or positively correlated with the organic C of a soil depending on the affinity of the respective micronutrient for the organic matter (*Adriano*, 2001) and whether there is a net immobilization into or mineralization from the soil organic matter pool. Further, a high clay proportion typically gives a high micronutrient availability (*McBride*, 1994). In addition to the higher micronutrient concentrations in the soil at Lillerud compared to that of Rådde, this could be the reason for the higher micronutrient concentrations in the biomass harvested at Lillerud than at Rådde.

Our results exemplify the difficulty in interpreting soil micronutrient analysis since the uptake by plants is a continuous biochemical process in contrast to soil analysis which is purely chemical processes and presents a snapshot of the soil micronutrient status (*Bussink* and *Temminghoff*, 2004). As seen in studies by *Jarvis* and *Whitehead* (1981; 1983) the variation in soil Cu concentrations between the twenty-one 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. A similar comparison between species mixtures in this study (at a common red clover DM proportion of 25%) shows that the largest variations in EDTA-extracted soil occurred for Co and Mn concentrations which varied by a factor 10 – 20 between the three sites, while plant concentrations varied at most 2.5 times. The largest variation between mixtures due to red clover DM proportion was 8.5 times for Co concentration and 1.2 times for Mn concentrations, at Ås at the second harvest. This was due to the large differences in Co concentrations but small differences in Mn concentrations between red clover and timothy. On the other hand, Mo concentrations varied little between soils (the EDTA-extractable concentrations were below detection limit, but nitric acid extractable concentrations varied 4.2

times) while there was a 12-fold difference in plant Mo concentrations due to sites. In conclusion, the variation between species grown on the three study sites with regard to Co and Mn as well as Cu, Fe and Zn were smaller than the variation between the micronutrient concentrations extracted from the soil, whereas the opposite was true for Mo. A small variation in plant micronutrient concentrations was expected since plants can actively regulate their uptake of most micronutrients (*Marschner*, 1995).

Mineral N, P and K fertilizers may contain traces of micronutrients (*Eriksson*, 2001) which may affect the nutrient balance of the fields (e.g. *Bengtsson* et al., 2003). The current field experiments were N fertilized in a similar way at all sites. In contrast, the timing and amounts of P and K fertilizer differed between sites, partly due to soil status, which demanded products with different P:K ratios. One of the fertilizers contained a known, low concentration of Zn but traces of micronutrients may also have been present in all the used fertilisers. This might have affected the micronutrient uptake by the forage crop and resulted in site differences. However, the amounts of micronutrients estimated to have been added by the mineral fertilizers were small in comparison to the amounts of removed in the harvested crop.

5.4 Implications of the results

The high DM yield proportion of timothy resulted in similar or higher micronutrient off-take despite its overall low concentration, compared to red clover. However, it is the concentration of micronutrients that determines the feed quality. Compared to the demands of lactating dairy cows (*National Research Council*, 2001), the requirements for Fe and Mn concentrations were met irrespective of red clover DM proportion and site whereas Co, Cu and Zn concentrations were generally too low. Despite the positive correlations between increased red clover DM proportions and increased Co concentrations of the mixtures at all sites, the concentrations were never more than half of the requirements of dairy cows (0.11

mg Co kg^{-1} DM). However, plant material grown at Lillerud was close to the requirements of 11 mg Cu kg^{-1} DM and 43-55 mg Zn kg^{-1} DM (low to high lactating cows). This was because Cu and Zn concentrations were higher in herbage at Lillerud than at the other sites. At Lillerud, the required Cu concentration of dairy cows was met where the red clover DM proportion at the second harvest exceeded 50%. The red clover DM proportion was also important for Fe and Mn concentrations at Ås at the second harvest. This was because decreased red clover DM proportion decreased the Fe and Mn concentrations close to the minimum requirement of 18 mg Fe kg^{-1} DM and 14 mg Mn kg^{-1} DM. In practise, other options are available to the farmer to provide animals with the required micronutrients where soils are deficient in some element, such as fertilization of the crop. Still, as the required concentrations in plants are frequently lower than those recommended for livestock feed supplements are generally given in conventional farming. However, in systems such as organic farming alternatives to dependency of external inputs are favoured. Furthermore, at farms with high soil concentrations of e.g. Cu and Zn, consideration of long-term soil health may call for other means of meeting animal micronutrient demands than fertilizing the soil or supplementing the feed and thereby generating Cu and Zn rich manure.

In order to favour the clover proportion in the sward, large applications of N fertilizer should be avoided or grasses will easily out-compete legumes. Even so, red clover proportion generally declines with sward age (*Mela*, 2003) which could result in a decline of micronutrient concentrations in the harvested plant material. However, white clover DM proportion tends to increase with time and is as rich in micronutrients as red clover. Consequently, a grass mixture with red and white clover gives a higher yield stability of clovers (*Frankow-Lindberg* et al., 2009), and such a mixture may also result in more stable micronutrient concentrations in the forage over time.

6 Conclusions

The generally high micronutrient concentrations of red clover compared to timothy resulted in a positive correlation between red clover DM proportion and the overall micronutrient concentration of the mixture. This was seen for several micronutrients at three contrasting sites. The micronutrient concentration levels in the harvested biomass also differed between the sites. Thus, our results suggest that increased red clover DM proportion in the sward have a potential to increase the overall micronutrient concentrations but that the effect of soil is also very important.

Acknowledgements

We thank SW Seed, Svalöv Sweden, for providing the seeds and everyone at the Rådde, Örebro and Ås research stations who helped out with the field experiments. This study was funded by Swedish Farmers' Foundation for Agricultural Research (SLF) project H0841014, the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS) project 2007-1636 and co-funded by the Swedish University of Agricultural Sciences (SLU).

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Table 1: Soil characteristics of the experimental soils (top soil depth 25 cm): particle size distribution, pH in water (H_2O) and calcium chloride ($CaCl_2$) solution, total C and N, micronutrient concentrations in EDTA extracts and macro- and micronutrient concentrations in nitric acid and hydrogen peroxide (HNO_3+H_2O_2) extracts.

Soil properties	Site		
	Rådde	Lillerud	Ås
Clay (%)	8	27	24
Silt (%)	41	56	40
Sand (%)	51	17	36
pH (H_2O)	5.78	5.63	7.45
pH ($CaCl_2$)	5.25	5.25	7.18
C (%)	3.1	1.7	3.4
N (%)	0.22	0.14	0.31
EDTA extractable elements (mg kg^{-1} DM)			
Co	0.04	0.21	0.40
Cu	0.5	2.2	3.1
Fe	69	153	178
Mn	6	31	125
Mo	0.00	0.00	0.04
Zn	0.69	2.01	2.69
HNO_3+H_2O_2 extractable elements (mg kg^{-1} DM)			
P	727	791	1 050
K	395	1200	1280
S	320	186	465
Ca	1860	2660	9870
Mg	916	2340	4090
Co	2.8	5.1	12.7
Cu	5.4	11.0	17.0
Fe	10100	11900	22100
Mn	254	473	1950
Mo	0.51	0.25	1.06
Zn	22	69	104

Table 2: Monthly total precipitation and mean air temperature during the experimental period 2010-2011 and the 30 years mean (1961-1990) at the field experiment sites Rådde, Lillerud and Ås.

	Precipitation (mm)						**Temperature (°C)**					
	2010-2011			**30 year mean**			**2010-2011**			**30 year mean**		
Month	Rådde[a]	Lillerud[b]	Ås[c]	Rådde[d]	Lillerud[b]	Ås[e]	Rådde[a]	Lillerud[b]	Ås[e]	Rådde[d]	Lillerud[b]	Ås[e]
April	missing	25	26	54	38.2	32.4	5.3	5.2	2.6	3.5	3.8	1.3
May	86	missing	100	60	42.3	39.3	9.2	9.7	6.6	9.2	10	7.6
June	58	50	125	75	56	58.3	13.2	14.1	10.3	13.5	14.8	12.5
July	160	125	87	94	63.2	86.1	17.4	17.7	15.5	14.7	16.1	13.9
Aug	133	111	78	91	72.2	59.9	15	15.5	13.2	13.5	15	12.7
Sept	66	71	60	102	73.1	64.5	10.4	10.5	8.8	10	11	8.2
Oct	60	57	13	98	68.2	44.9	5.2	5.2	4.1	6.1	6.6	3.8
Nov	63	64	18	104	72.5	40.4	-0.2	-2	-6.2	1.2	1.3	-2.4
Dec	21	32	46	87	51.2	44	-8.5	-10.8	-13.4	-2.1	-2.6	-6.3
Jan	44	56	34	78	45.3	35.6	-2.85	-3.8	-4.7	-3.9	-4.4	-8.9
Feb	38	43	25	51	32.5	28.5	-4	-5.7	-7.6	-3.9	-4.5	-7.6
March	34	23	13	59	38.5	30	0.5	0	-1.8	-0.6	-1	-3.5
April	20	18	18	54	38.2	32.4	8.6	8.8	5.3	3.5	3.8	1.3
May	55	57	76	60	42.3	39.3	10	10.3	8.2	9.2	10	7.6
June	97	52	55	75	56	58.3	14.7	15.7	13.6	13.5	14.8	12.5
July	96	79	64	94	63.2	86.1	16.4	17.4	15.8	14.7	16.1	13.9
Aug	192	113	95	91	72.2	59.9	14.8	15.4	14	13.5	15	12.7
Sept	126	126	78	102	73.1	64.5	12.2	12.6	10.5	10	11	8.2
Oct	93	65	10	98	68.2	44.9	7.2	7.1	5.7	6.1	6.6	3.8

[a] data from Rådde reseach station, 1 km from field
[b] data from Karlstad airport, ca 15 km from field
[c] data from Rösta, ca 2 km from field
[d] data from Borås, ca 30 km from field
[e] data from Frösön airport, ca 6 km from field

Table 3: Dry matter yield (t DM ha^{-1}) and species proportions (% of DM) of mixtures with two or three species grown at three sites (Rådde, Lillerud and Ås) and harvested at two or three occasions (1st, 2nd and 3rd). Dry matter yield presented as least square means (n=3). Values within the same column followed by the same letter are not significantly different at $P < 0.05$.

Site	Mix	Yield (t DM ha^{-2})			Timothy (%)			Red clover (%)			3rd sown species (%)			Unsown (%)		
		1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Rådde	timothy + red clover	6.6^{a}	3.5^{cde}	2.9^{b}	82	81	58	17	17	42	-	-	-	1	2	0
Rådde	+ meadow fescue	7.1^{a}	3.4^{de}	3.2^{b}	59	67	56	24	18	24	17	10	20	0	5	0
Rådde	+ white clover	6.7^{a}	3.4^{de}	3.0^{b}	82	72	65	13	12	27	4	4	8	1	12	0
Rådde	+ chicory	6. 9^{a}	3.4^{de}	2.9^{b}	75	80	60	19	16	38	5	3	2	1	1	0
Lillerud	timothy + red clover	6.3^{a}	4.8^{bc}	4.4^{a}	58	49	47	37	50	53	-	-	-	5	1	0
Lillerud	+ meadow fescue	5.8^{a}	4.6^{bcd}	4.1^{a}	48	48	27	43	47	50	5	4	23	4	1	0
Lillerud	+ white clover	6.1^{a}	4.5^{bcd}	4.5^{a}	61	60	54	20	32	37	7	7	9	12	1	0
Lillerud	+ chicory	6.4^{a}	4.5^{bcd}	4.4^{a}	57	57	32	37	38	61	4	1	7	2	4	0
Ås	timothy + red clover	3.4^{b}	6.9^{a}	-	45	30	-	48	67	-	-	-	-	7	3	-
Ås	+ meadow fescue	3.4^{b}	5.7^{ab}	-	22	10	-	56	60	-	20	28	-	2	2	-
Ås	+ white clover	2.5^{b}	5.6^{ab}	-	75	80	-	7	1	-	2	2	-	16	17	-
Ås	+ chicory	3.4^{b}	6.2^{ab}	-	35	23	-	57	54	-	2	15	-	6	8	-
P-value																
Site		<0.001	<0.001	0.007												
Mixture		0.394	0.026	0.956												
Site × Mixture		0.342	0.673	0.357												

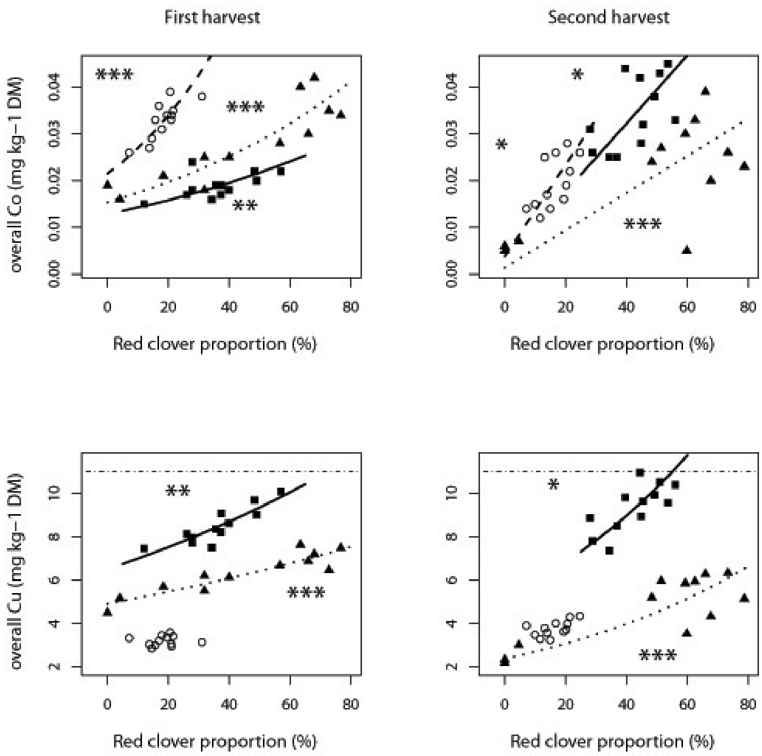
Table 4: Micronutrient concentrations (mg kg^{-1} DM) in timothy, red clover, meadow fescue, white clover and chicory at the experimental sites Rådde, Lillerud and Ås, first and second harvest occasion in 2011. Least square means of timothy and red clover (n= 12), other species (n=3). Values within the same column followed by the same letter are not significantly different at $P < 0.05$.

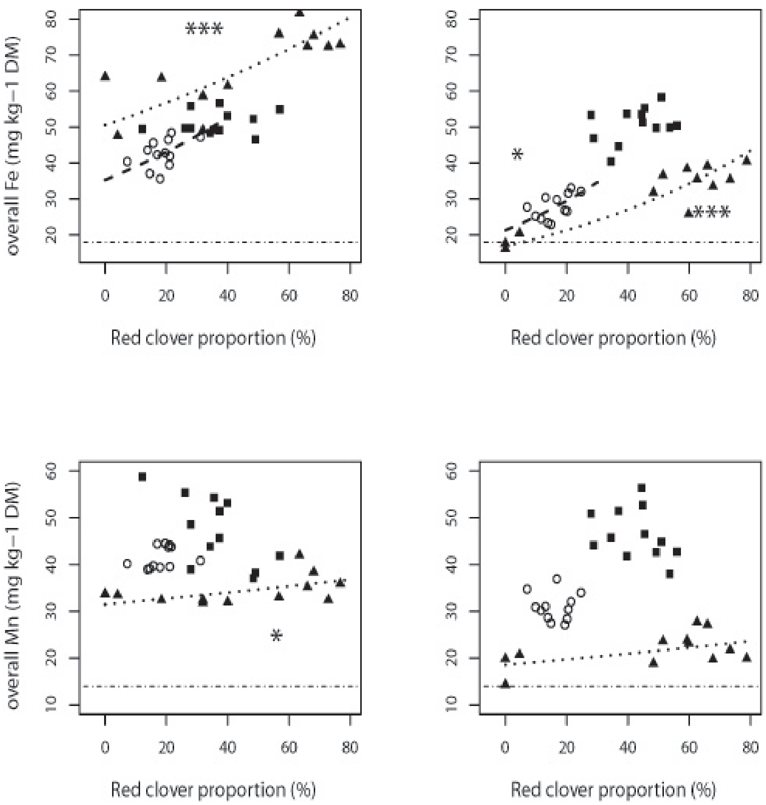
Site Species	Co		Cu		Fe		Mn		Mo		Zn	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Rådde												
timothy	0.020 ^{g}	0.008 ^{g}	2.66 ^{i}	3.29 ^{fg}	34.8 ^{h}	23.5 ^{fg}	42.6 ^{abdefg}	31.2 ^{de}	0.64 ^{g}	0.78 ^{cd}	20.5 ^{ghi}	17.0 ^{fg}
meadow fescue	0.038 ^{def}	0.044 ^{cde}	2.24 ^{i}	5.14 ^{de}	62.6 ^{efg}	51.2 ^{bcde}	45.8 ^{abcdefg}	45.9 ^{bc}	0.99 ^{de}	0.73 ^{bcde}	18.5 ^{hi}	20.6 ^{cdefg}
red clover	0.077 ^{b}	0.067 ^{ab}	5.29 ^{h}	5.53 ^{e}	61.3 ^{f}	41.2 ^{cde}	36.1 ^{chi}	26.5 ^{defg}	1.24 ^{d}	1.16 ^{b}	28.0 ^{def}	21.2 ^{cef}
white clover	0.121 ^{a}	0.076 ^{a}	5.41 ^{gh}	5.99 ^{cde}	108 ^{bc}	55.7 ^{bcd}	42.2 ^{abcdefghi}	30.7 ^{def}	1.78 ^{c}	1.09 ^{bc}	22.4 ^{ghi}	17.7 ^{efg}
chicory	0.070 ^{bc}	0.050 ^{cd}	7.73 ^{de}	6.52 ^{cde}	92.9 ^{cd}	49.1 ^{bcde}	45.3 ^{abdefg}	33.4 ^{cde}	0.66 ^{fg}	0.41 ^{efg}	41.4 ^{bc}	31.4 ^{bd}
Lillerud												
timothy	0.010 ^{h}	0.028 ^{ef}	5.71 ^{fh}	7.07 ^{cde}	40.7 ^{h}	47.9 ^{bcd}	48.4 ^{abcei}	54.9 ^{b}	0.38 ^{i}	0.30 ^{g}	39.3 ^{bc}	39.5 ^{b}
meadow fescue	0.018 ^{g}	0.040 ^{cdef}	6.81 ^{eg}	8.46 ^{bcd}	58.7 ^{fg}	55.9 ^{bcd}	54.7 ^{abc}	67.6 ^{ab}	0.43 ^{hi}	0.47 ^{defg}	37.1 ^{bcd}	31.6 ^{bcd}
red clover	0.031 ^{f}	0.041 ^{d}	12.89 ^{b}	12.09 ^{ab}	64.6 ^{f}	52.3 ^{bc}	42.6 ^{dfgh}	34.6 ^{cd}	0.49 ^{h}	0.41 ^{f}	40.7 ^{b}	34.5 ^{b}
white clover	0.051 ^{cd}	0.049 ^{bcd}	9.68 ^{c}	9.32 ^{abc}	101 ^{bc}	64.4 ^{b}	50.8 ^{abcdeghi}	33.9 ^{cde}	0.86 ^{ef}	0.76 ^{bcde}	33.9 ^{cde}	31.5 ^{bcd}
chicory	0.046 ^{de}	0.091 ^{a}	14.63 ^{a}	15.91 ^{a}	98.0 ^{c}	119 ^{a}	55.2 ^{ab}	94.2 ^{a}	0.41 ^{hi}	0.29 ^{fg}	76.0 ^{a}	78.4 ^{a}
Ås												
timothy	0.016 ^{g}	0.005 ^{g}	5.03 ^{h}	2.63 ^{g}	52.2 ^{g}	19.9 ^{g}	31.6 ^{f}	20.2 ^{g}	1.88 ^{c}	3.40 ^{a}	26.2 ^{efg}	16.0 ^{fg}
meadow fescue	0.031 ^{ef}	0.018 ^{fg}	7.48 ^{e}	4.85 ^{ef}	91.0 ^{cd}	32.6 ^{ef}	51.2 ^{abcdgh}	25.6 ^{defg}	3.53 ^{a}	4.44 ^{a}	24.7 ^{fgh}	12.8 ^{g}
red clover	0.039 ^{de}	0.029 ^{def}	7.56 ^{e}	5.82 ^{de}	75.5 ^{de}	38.6 ^{de}	34.8 ^{efi}	21.2 ^{fg}	2.73 ^{b}	4.26 ^{a}	19.4 ^{i}	14.5 ^{g}
white clover	0.054 ^{cd}	0.035 ^{def}	7.02 ^{ef}	5.17 ^{de}	137 ^{ab}	53.6 ^{bce}	44.2 ^{bcgh}	22.4 ^{efg}	3.29 ^{ab}	3.64 ^{a}	17.4 ^{i}	15.0 ^{fg}
chicory	0.075 ^{bc}	0.064 ^{abc}	9.10 ^{cd}	8.42 ^{bcd}	160 ^{a}	50.5 ^{bcd}	59.2 ^{ad}	33.2 ^{cd}	3.28 ^{ab}	3.60 ^{a}	36.2 ^{bcd}	26.3 ^{bcde}
P-values												
Site	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.171	<0.001	<0.001	<0.001	<0.001	<0.001
Species	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Site × Species	<0.001	<0.001	<0.001	0.006	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.003

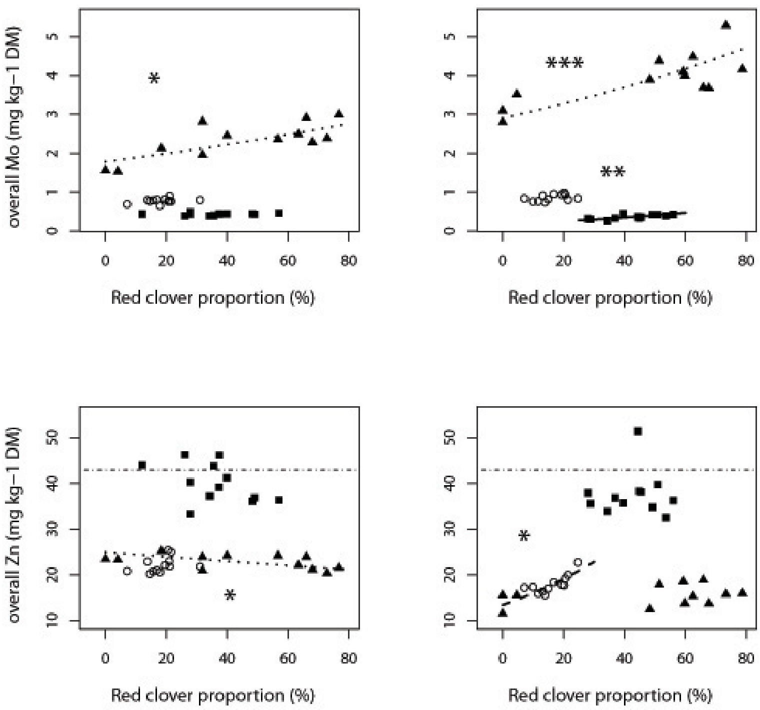
Table 5: Average micronutrient off-take (g ha^{-1}) of all mixtures as well as in each species: timothy, red clover, meadow fescue, white clover and chicory, at the experimental sites Rådde, Lillerud and Ås, first and second harvest occasion in 2011. Least square means of mixtures (n=12) and of species: timothy and red clover (n= 12), other species (n=3). Values within the same column followed by the same letter are not significantly different at $P < 0.05$, for comparisons of site effects of mixtures (X, Y, Z) and site effects of species and species differences (a, b, c *etc.*).

Site	**Species**	**Co**		**Cu**		**Fe**		**Mn**		**Mo**		**Zn**	
		1st	**2nd**	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**
Rådde	**mixture**	**0.22^{X}**	**0.06^{Y}**	**22^{Y}**	**12^{Z}**	**288^{X}**	**89^{Z}**	**229^{XY}**	**45^{Y}**	**5.2^{X}**	**2.7^{Y}**	**140**	**25^{Z}**
	timothy	0.1^{a}	0.02^{bc}	13^{ab}	8^{bc}	175^{a}	59^{ab}	214^{a}	79^{ab}	3^{a}	2^{b}	103^{a}	43^{bc}
	meadow fescue	0.04^{abcd}	0.01^{bcd}	3^{cdef}	2^{defg}	73^{abcd}	17^{cdef}	53^{bcde}	15^{defg}	1^{abcdef}	0.2^{cde}	226^{bc}	7^{efgh}
	red clover	0.09^{a}	0.03^{ab}	6^{bcd}	3^{def}	73^{bc}	20^{cd}	43^{cd}	13^{ef}	2^{bcd}	0.6^{cd}	33^{b}	10^{ef}
	white clover	0.03^{bcde}	0.01^{bcd}	1^{efg}	0.9^{fg}	22^{cdef}	8^{def}	9^{fgh}	4^{fghi}	0.4^{efg}	0.2^{def}	5^{cde}	3^{gh}
	chicory	0.02^{cdefg}	0.004^{d}	2^{defg}	0.5^{g}	22^{cdef}	4^{f}	11^{efgh}	3^{hi}	0.2^{g}	0.03^{fg}	10^{bcd}	3^{gh}
Lillerud	**mixture**	**0.11^{Y}**	**0.15^{X}**	**50^{X}**	**42^{X}**	**298^{X}**	**228^{X}**	**268^{X}**	**210^{X}**	**2.5^{Y}**	**1.6^{Z}**	**229**	**170^{X}**
	timothy	0.03^{bcd}	0.07^{a}	19^{a}	17^{ab}	137^{ab}	116^{a}	163^{ab}	133^{a}	1^{cde}	0.7^{c}	132^{a}	96^{a}
	meadow fescue	0.005^{fg}	0.006^{cd}	2^{defg}	1^{efg}	16^{def}	9^{def}	15^{defgh}	11^{defgh}	0.1^{g}	0.08^{ef}	10^{bcd}	5^{fgh}
	red clover	0.06^{ab}	0.08^{a}	25^{a}	23^{a}	125^{ab}	98^{a}	83^{bc}	65^{abc}	1^{def}	0.8^{c}	79^{a}	65^{ab}
	white clover	0.02^{bcdef}	0.02^{bcd}	4^{bcde}	3^{cde}	41^{bcdef}	19^{bcde}	21^{defg}	10^{defghi}	0.4^{fg}	0.2^{cde}	14^{bcd}	10^{defg}
	chicory	0.01^{defg}	0.003^{d}	3^{cdef}	0.6^{g}	21^{cdef}	4^{ef}	12^{defgh}	3^{ghi}	0.09^{g}	0.01^{g}	17^{bc}	3^{gh}
Ås	**mixture**	**0.083^{Y}**	**0.12^{X}**	**19^{Y}**	**27^{Y}**	**196^{Y}**	**179^{Y}**	**120^{Y}**	**178^{X}**	**6.5^{X}**	**22^{X}**	**70**	**120^{Y}**
	timothy	0.02^{de}	0.008^{cd}	6^{cd}	4^{cde}	61^{bc}	32^{bc}	37^{cde}	32^{cd}	2^{abc}	5^{a}	31^{bc}	25^{cd}
	meadow fescue	0.02^{bcdef}	0.03^{abc}	5^{bcde}	7^{abcd}	57^{abcde}	49^{abc}	32^{cdef}	39^{bcde}	2^{abcd}	7^{ab}	16^{bc}	20^{bcdef}
	red clover	0.04^{abcd}	0.07^{a}	9^{bc}	17^{ab}	83^{ab}	107^{a}	39^{cde}	61^{abc}	3^{ab}	12^{a}	22^{b}	41^{bc}
	white clover	0.003^{g}	0.004^{d}	0.4^{g}	0.5^{g}	8^{f}	5^{ef}	3^{h}	2^{i}	0.2^{g}	0.4^{cde}	1^{e}	2^{h}
	chicory	0.006^{efg}	0.06^{ab}	0.8^{fg}	8^{abcd}	13^{ef}	47^{abc}	5^{gh}	31^{bcde}	0.3^{fg}	3^{ab}	3de	24^{bcde}
P-value site effects of species and species differences													
Site		<0.001	0,065	<0.001	<0.001	0,012	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Species		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Site × Species		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
P-value site effects of mixtures													
Site		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.103	<0.001

Figure 1: Overall micronutrient concentration in relation to red clover proportion (% of DM of the sown species) of species mixture at first (left row) and second (right row) harvest occasions, in 2010, at Rådde (○-----), Lillerud (■———) and Ås (▲ ⋯⋯). Regression lines indicate significant (*: *P*< 0.05; **: *P*< 0.01-0.001; ***: *P*< 0.001) and near-significant (p-values in figure) relationships. Horizontal dashed-dotted line indicate minimum dairy cow requirement for low lactating cows; for Co this falls above the graph range (0.11 mg kg^{-1} DM). With the exception of Co at the second harvest, all data were ln-transformed during statistical analyses but the graph presents actual values, hence the lines are presented back-transformed (n=12).




overall Fe (mg kg−1 DM)
overall Mn (mg kg−1 DM)
Red clover proportion (%)
Red clover proportion (%)
Red clover proportion (%)
Red clover proportion (%)


overall Mo (mg kg−1 DM)
overall Zn (mg kg−1 DM)
Red clover proportion (%)
Red clover proportion (%)
Red clover proportion (%)
Red clover proportion (%)