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ORIGINAL ARTICLE OPEN ACCESS

Species Composition Influences Zinc and Selenium Uptake in Multispecies Grasslands

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Received: 20 September 2024 | **Revised:** 28 March 2025 | **Accepted:** 27 April 2025

Funding: This work was supported by the European Union's Horizon 2020 Research and Innovation Programme (Marie Skłodowska-Curie Actions) under grant agreement No. 814030 and Devenish Nutrition.

Keywords: grasslands | multispecies mixtures | selenium | species composition | zinc

ABSTRACT

The aim of this study was to assess and analyse the effect of species richness on zinc (Zn) and selenium (Se) uptake and concentrations in grasslands while investigating the potential role of grass species in facilitating their uptake in mixtures. We conducted a grassland experiment at both pot and field scales. In the greenhouse pot experiment, three grasses, four legumes and five forb species were grown as monocultures and mixtures along a species richness gradient. In the field experiment, we compared a monoculture of *Lolium perenne* sward with two multispecies mixtures (comprising grasses, legumes and forbs). At harvest, Zn and Se uptake values were determined for all the monocultures and mixtures in both experiments. Zinc and Se uptake increased with increasing species richness ($p < 0.05$). Higher Zn and Se uptake in the multispecies mixtures was primarily due to high aboveground biomass production and/or high shoot Zn and Se concentrations of some of the component species in the mixtures. However, there was insufficient evidence supporting the facilitation of Zn and Se uptake by grass species. To optimise Zn and Se uptake by multispecies mixtures, it is suggested that the choice of forage species should be based on biomass production potential as well as Zn and Se concentrations of the component species in the mixture.

1 | Introduction

The establishment of multispecies plant communities is considered an important approach for promoting sustainability in agricultural grasslands (Finn et al. 2013). Sown multispecies grasslands typically consist of a combination of plant species from more than one functional group: grasses, legumes and forbs (Daly et al. 1996; Grace et al. 2018). Previous studies showed that more biomass can be produced from multispecies swards with lower nitrogen (N) fertiliser input, thereby reducing the environmental impact associated with the cultivation of perennial ryegrass (*Lolium perenne*) monoculture with high N input (Baker et al. 2023; Grace et al. 2018; Moloney et al. 2020). Multispecies grasslands can also increase herbage N yield (Abalos et al. 2021), improve N use efficiency (Carswell et al. 2019) and reduce N leaching and N_2O emission (Bracken et al. 2022; Carlton et al. 2019; Cummins et al. 2021). While these studies have provided more insights into the potential of species-rich grassland communities in the cycling of N, there is limited research on the role of multispecies swards in enhancing the uptake of micronutrients.

Micronutrients are important for forage quality in grasslands (Gupta et al. 2001; Kao et al. 2020; Vejnovic et al. 2018). Since forages from grasslands represent a significant part of livestock feedstuff, their micronutrient content influences livestock productivity and health (Gupta et al. 2008; Lindström, Frankow-Lindberg, Dahlin, Wivstad, et al. 2014). Zinc (Zn) and selenium (Se) are important micronutrients which are crucial for molecular and cellular functions in ruminants because of their unique role in enzyme activity and regulation of the antioxidative status of animals, which ultimately affect the quality of the animal-derived products (Chauhan et al. 2014; Stanton et al. 2022). Zinc is essential for boosting immunity and increasing disease resistance (Hill and Shannon 2019). Its deficiency can lead to stunted growth, poor reproduction, and weakened bones (Hill and Shannon 2019). Similarly, Se is vital for improving productive efficiency, fertility and enhancing immune response (Hefnawy and Tórtora-Pérez 2010). Selenium deficiency in cattle has been linked to placental retention, reduced fertility (Mehdi and Dufrasne 2016), poor seminal quality (Beckett and Arthur 2005), smaller litter size, reduced weight gains and lower milk production (Hefnawy and Tórtora-Pérez 2010).

Zinc is also an essential micronutrient for plants. It plays a key role in regulating plant growth and development (Hill and Shannon 2019). It is the only nutritionally relevant metal found in all the six major enzyme classes and has an important catalytic and structural role in enzyme activities, underscoring its fundamental role in plant metabolism (Gupta et al. 2016; Stanton et al. 2022). It is predominantly taken up by plants as a divalent cation (Zn^{2+}) through root cells via Zn transporters (Gupta et al. 2016). Most soils contain sufficient total amounts of Zn to sustain multiple crops, but only a small proportion is potentially available to plants due to the strong binding of Zn^{2+} to organic matter and metal(hydr)oxides, particularly in high pH soils (Van Eynde et al. 2022). A sufficient supply of Zn to plants has been shown to enhance the yield of grassland species (Grewal 2001; Radujković et al. 2021) as well as fodder crops (Kumar et al. 2017; Tondey et al. 2021). Severe Zn deficiency in plants could cause leaf chlorosis, a reduction in plant growth, and increased susceptibility to certain infections (Sharma et al. 2013).

Selenium is not considered an essential nutrient for plants (Broadley et al. 2012), but it can offer certain benefits to plants (Vejnovic et al. 2018). For instance, Se can induce pathogenic resistance in plants, protect them from heavy metal toxicity and other abiotic stress such as extreme weather conditions that could lead to desiccation or cold (Gupta and Gupta 2017; White 2016). Selenium is taken up by plants as selenate (SeO_4^{2-}, the dominant species in soil solution) or selenite (SeO_3^{2-}) (Weng et al. 2011). Similar to Zn, it adsorbs to soil organic matter (SOM) and metal(hydr)oxides (Fernández-Martínez and Charlet 2009).

Multispecies swards could enhance uptake of Zn and Se. The positive relationship between species richness and productivity is often attributed to selection and complementarity effects (Loreau and Hector 2001). These effects could also potentially explain the higher Zn and Se uptake and concentrations observed in diverse grasslands compared to monocultures. Selection effects occur when a species in the mixture exhibits dominant trait(s) that influence the overall performance of the mixture; it is mainly due to the increased probability of including highly productive species in more species-rich mixtures (Huston 1997; Loreau and Hector 2001). Complementarity effects refer to enhanced performance of a mixture due to niche partitioning or facilitation (Loreau and Hector 2001; Tilman et al. 1997). Niche partitioning refers to complementary resource use by their constituent species as a result of trait variation (Tilman et al. 1997). Facilitation occurs when a particular plant species and/or functional group within a plant community provides benefit(s) to another plant species in the community, for instance by increasing nutrient availability (Callaway 1995; Loreau and Hector 2001; Xue et al. 2016).

A selection effect on micronutrient uptake was implicated in multispecies grasslands by inclusion of species or functional groups with high shoot micronutrient concentrations. For instance, Lindström, Frankow-Lindberg, Dahlin, Watson et al. (2014) attributed the increased micronutrient uptake in grassland mixtures to high micronutrient concentration and biomass of legumes, particularly red clover. The study by Høgh-Jensen et al. (2006) also showed that forbs, especially chicory, had higher concentrations of Zn, thereby increasing the overall micronutrient concentration of the multispecies swards. A selection effect on Se uptake and concentration also seems likely, as plant species differ strongly in Se uptake and accumulation in their shoots (Broadley et al. 2012). Specifically, members of the Brassicaceae family accumulate relatively large amounts of Se.

Some studies have attributed the increase in Zn uptake in agricultural intercropping systems to facilitation, in which one species mobilises limited soil Zn and makes it available to the neighbouring species (Li et al. 2003, 2014; Zuo and Zhang 2008). Although Zn is primarily taken up by plants as Zn^{2+}, it can also be taken up as organic ligand–Zn complex, a process dependent on the type of ligand secreted by plant roots (Gupta et al. 2016). Zinc–ligand complexes are usually negatively charged, which reduces adsorption to the soil's solid phase and accelerates transport towards the root (Northover et al. 2022). Two main mechanisms underlie ligand-mediated Zn uptake. The first one entails the secretion of phytosiderophores such as deoxymugineic acid, which form complexes with Zn, facilitating its solubilisation and uptake (Gupta et al. 2016). The study of Inal et al. (2007) showed that intercropping maize (a graminaceous crop) with peanut (a non-graminaceous crop) increased the Zn concentration of peanut. The release of phytosiderophores is limited to graminaceous monocotyledonous species (Gupta et al. 2016; Stanton et al. 2022). The presence of graminaceous species belonging to *Poaceae* family, like grasses, could facilitate Zn uptake for a non-graminaceous species (Wei et al. 2022). The second mechanism involves the release of protons and organic anions which can convert Zn into available form that can be readily taken up by roots (Gupta et al. 2016; Stanton et al. 2022). The release of protons can lower the rhizosphere pH and result in desorption of soil-bound Zn (Rehman et al. 2018; Van Eynde et al. 2022). Legumes are known to release protons via their roots, thereby acidifying the rhizosphere (Yan et al. 1996). This acidification as well as Zn complexation by organic anions could increase Zn bioavailability and subsequently promote Zn uptake by neighbouring species.

Less is known about the potential facilitation of Se uptake in mixtures. Two species of the Brassicaceae family were shown to increase Se uptake by their neighbour, potentially through the release of Se chelators (El Mehdawi et al. 2012). It was also reported that the root exudates of wheat (a graminaceous species) modified the composition of dissolved organic matter (DOM) in the soil, which in turn increased Se bioavailability (Wang et al. 2020). Species that produce low molecular weight organic acids as root exudates, such as maize (Ström et al. 2001) can also promote soil Se bioavailability, which is in line with the observation that small DOM is the best predictor of Se bioavailability to *L. perenne* (Weng et al. 2011). Acidification, for instance by legumes, would, however, promote the adsorption of Se to the soil's solid phase and probably reduce uptake (Fernández-Martínez and Charlet 2009).

While these mechanisms suggest that overyielding in terms of Zn or Se uptake may occur, only a few studies have investigated this in multispecies grasslands. Given that Zn and Se uptake are relevant to productivity and forage quality, it is important to explore the effects of species richness on Zn and Se uptake and concentration in grasslands. We hypothesised that there will be increased Zn and Se uptake in species-rich grasslands, due to a selection effect and facilitation by grass species. To test this hypothesis, we conducted a grassland experiment at both pot and field scales. The pot trial enabled us to test if potential positive species richness effects are indicative of facilitation of Zn or Se uptake by grass species. The field experiment provided an opportunity to validate the results from the pot experiment, using grassland sward types that farmers can readily adopt. We compared Zn and Se uptake by two multispecies swards with a monoculture grass sward and analysed the results based on the contribution of the component species to Zn and Se uptake.

2 | Materials and Methods

2.1 | Greenhouse Experiment

2.1.1 | Experimental Design

A pot experiment was done in the greenhouse of Wageningen University and Research from July to August 2021 (day/night temperature of 27/19°C and relative humidity of 60%). We used low Zn and Se soils with $CaCl_2$-extractable Zn and $CaCl_2$-extractable Se concentrations of 6.0 μg Zn/kg and 9.9 μg Se/kg, HNO_3-extractable Zn and HNO_3-extractable Se concentrations of 7.4 mg Zn/kg and 37.9 μg Se/kg. The soil texture was sandy loam with total soil carbon content ranging from 4.2% to 5.8% and total nitrogen content between 0.4% and 0.5%. Soil pH, measured in a 0.01 M $CaCl_2$ solution at a 1:10 (w/v) ratio, was 6.3. SOM, determined using the loss-on-ignition method at 550°C, was 11.6%. The soils were collected from the topsoil layer (without the organic soil layer in the 0–5 cm depth) at the site where the field experiment was conducted, i.e., grassland research farm at Dowth, County Meath, Ireland (53°42′11.8″N, 6°26′28.9″W). The collected soils were sieved on a 6 mm mesh to remove large stones, roots and other larger particles. To increase the volume of soil available for the experiment, the collected soils were mixed with quartz sand in a ratio of 3:2 soil:quartz sand (w:w). Given that the greenhouse experiment was for 5 weeks, we applied 14 kg N/ha, 6 kg P/ha and 12 kg K/ha in the form of NH_4NO_3, KH_2PO_4 and KCl, respectively. The nutrients were thoroughly mixed with the soil and left to equilibrate for 2 days before sowing the seeds using the recommended seeding rate (Table 1). Each pot (~2.7 L, with surface area of 165.2 cm^2) was filled with 1.5 kg (dw equivalent) of the mixed soil and set to 60% of the soil water holding capacity (WHC). This moisture content was maintained throughout the experiment by weighing and watering regularly.

The treatments in the pot experiment comprised 12 monocultures, six two-species mixtures, two three-species mixtures,

TABLE 1 | Plant species and the seeding rate for greenhouse and field experiments.

Plant species			Seeding rate (kg/ha)
Functional group	**Common name**	**Latin name**	
Grasses	Perennial ryegrass	*Lolium perenne* L.	35.0
	Timothy	*Phleum pratense* L.	20.0
	Cocksfoot	*Dactylis glomerata* L.	20.0
Legumes	White clover	*Trifolium repens* L.	12.0
	Red clover	*Trifolium pratense* L.	15.0
	Birdsfoot trefoil	*Lotus corniculatus* L.	12.5
	Sainfoin	*Onobrychis viciifolia* Scop.	87.5
Forbs	Chicory	*Cichorium intybus* L.	15.0
	Plantain	*Plantago lanceolata* L.	10.0
	Yarrow	*Achillea millefolium* L.	2.5
	Sheep's parsley	*Petroselinum sativum* Hoffm.	10.0
	Salad burnet	*Sanguisorba minor* L.	22.5

two six-species mixtures and one 12-species mixture (Table 2). The species combinations in each treatment were chosen based on the potential interaction between the species in different functional groups. The experiment had a randomised complete block design with four replicates. The multispecies mixtures of grasses, legumes and forbs species were sown at a sowing proportion of 40:30:30 respectively and adjusted to account for the number of species in each mixture (Table S1).

A preliminary experiment was set up where the 12 species were grown as monocultures in the same soil under similar conditions, with or without Zn and Se fertilisation in the form of Zn-EDTA (equivalent to 5 kg/ha) and Na_2SeO_4 (10 g/ha) salts. This was to confirm that the soils were Zn and Se limited and to determine whether Zn and Se were growth-limiting micronutrients or not.

2.1.2 | Aboveground Biomass Sampling and Analysis

All plants were harvested 5 weeks after sowing. The multispecies were sorted into individual species and their aboveground biomass was dried at 70°C for 72 h to determine dry weight. Dried samples were ground, digested, and subsequently analysed to determine total Zn and Se concentration following the method described by Novozamsky et al. (1996) with slight modification. Briefly, 300 mg of ground plant samples was weighed into a tube and 5 mL of 14 M HNO_3 and 0.3 mL of 12 M HCl was added. Subsequently, 1.5 mL of H_2O_2 was added to remove the nitrous vapour. The suspension was then placed in the microwave rotor for digestion. After the run was completed, the digestion container was carefully opened and the final suspension volume was made up to 20 mL using Milli-Q water. The digestate was subsequently analysed on ICP-MS to determine the total Zn and Se concentration of the samples. Some species were excluded from the data analyses either due to handling error during Zn and Se determination in the laboratory or insufficient sample or no biomass for digestion. Hence, we excluded 10 out of 216 samples analysed for Zn and Se concentration.

2.2 | Field Experiment

2.2.1 | Site Description

The grassland field experiment was established at Dowth, County Meath, Ireland, 53°42′11.8″N, 6°26′28.9″W in 2020. The site has been a permanent pasture for over 40 years, comprised of native grassland species which include: *Lolium perenne* L., *Phleum pratense* L., *Dactylis glomerata* L., *Trifolium repens* L., *Cichorium intybus* L., *Plantago lanceolata* L., *Holcus lanatus* L., *Poa annua* L., *Alopecurus pratensis* L., *Festuca arundinacea* Schreb., *Festuca rubra* L., *Rumex acetosa* L., *Veronica persica* Poir., *Luzula campestris* L. The soil texture of the field was sandy loam. The total soil carbon content ranged from 4.2% to 5.8%, and the total soil nitrogen content ranged from 0.4% to 0.5%. Soil pH measured in 0.01 M $CaCl_2$ at 1:10 (*w/v*) was 6.3, and soil organic matter (SOM) determined using the loss on ignition method (550°C) was 11.6%.

2.2.2 | Experimental Design

The field experiment was set up with three sward types as the fixed factor with four replicates. The size of each plot was 3 m x 10 m and all treatments were established in July 2020 in a randomised complete block design. The three sward types investigated were: perennial ryegrass (PRG) monoculture, six-species (6S) and 12-species (12S) swards (Table 2). The PRG sward was sown as a monoculture of *L. perenne* while 6S and 12S were

TABLE 2 | Composition of mixtures in the greenhouse and field experiments.

# of species	Treatment code	Species composition	Experiment[a]	Functional groups[b]
2	2Sa	*L. perenne* + *T. repens*	Gh	G + L
2	2Sb	*P. pratense* + *T. pratense*	Gh	G + L
2	2Sc	*L. perenne* + *P. lanceolata*	Gh	G + F
2	2Sd	*P. pratense* + *C. intybus*	Gh	G + F
2	2Se	*T. pratense* + *P. lanceolata*	Gh	L + F
2	2Sf	*T. repens* + *C. intybus*	Gh	L + F
3	3Sa	*L. perenne* + *T. repens* + *P. lanceolata*	Gh	G + L + F
3	3Sb	*P. pratense* + *T. pratense* + *C. intybus*	Gh	G + L + F
6	6Sa	*L. perenne* + *P. pratense* + *T. repens* + *T. pratense* + *P. lanceolata* + *C. intybus*	Gh + F	2G + 2L + 2F
6	6Sb	*D. glomerata* + *L. corniculatus* + *O. viciifolia* + *A. millefolium* + *P. sativum* + *S. minor*	Gh	2G + 2L + 2F
12	12S	*L. perenne* + *P. pratense* + *T. repens* + *T. pratense* + *P. lanceolata* + *C. intybus* + *D. glomerata* + *L. corniculatus* + *O. viciifolia* + *A. millefolium* + *P. sativum* + *S. minor*	Gh + F	3G + 4L + 5F

[a] Experiment: field (F) and/or greenhouse (Gh).
[b] Functional group: Grass (G) or legume (L) or forb (F).

sown as multispecies mixtures of grasses, legumes and forb species at a sowing proportion of 40:30:30 respectively (Table S2). We compared the multispecies grasslands with the monoculture of *L. perenne* because the monoculture of *L. perenne* is conventionally used in many agricultural grasslands in the temperate region because of its high yield potential, palatability and digestibility (Hannaway et al. 1999; Baker et al. 2023). For any multispecies mixture to be considered relevant in real-world grassland systems, the advantages of the multispecies alternative would need to be comparable to or outperform the conventionally used species (Finn et al. 2013), such as *L. perenne*. In the 12S sward, five species did not persist, namely: *Lotus corniculatus*, *Onobrychis viciifolia*, *Achillea millefolium*, *Petroselinum sativum* and *Sanguisorba minor* as these species were not observed in the swards in the establishment year. Hence, the 12-species sward was almost comparable to the six-species sward as the only difference was the presence of *Dactylis glomerata* in the 12-species sward. The seeding rates used were similar to those of the greenhouse experiment (Table 1).

The N fertiliser treatment used was chemical N fertiliser applied as granulated protected urea. A total N fertiliser rate of 70 kg N/ha/year was applied across all treatments except for PRG sward, which received 170 kg N/ha/year to compensate for the absence of N_2 fixation in the sward. All swards received 35 kg N/ha at the beginning (March) and middle (May) of the growing season, except PRG sward, which received 42.5 kg N/ha in March and May but 21.25 kg N/ha in April, June, August and September.

2.2.3 | Aboveground Biomass Sampling and Analysis

The aboveground biomass was harvested six times during the growing seasons of 2021 and 2022, corresponding to an ~35 days harvesting interval by cutting the swards to 6 cm sward height. Only the July harvest was used for this study as precipitation, soil temperature and moisture were optimal for all species at that moment. A $50 \times 50\,\text{cm}^2$ quadrat was placed at two random locations in the middle of each plot to determine the sampling location for the harvest of the aboveground biomass. The dry weight of the harvested aboveground plant biomass was determined by oven-drying approximately 250 g at 55°C until constant weight was reached. The dried samples were ground and subsequently analysed to determine Zn and Se concentrations in the sward. From another subsample, the individual species were separately analysed for Zn and Se concentration. The procedure for Zn and Se determination in the samples from the field experiment was described in Ligowe et al. (2020) which was similar to that of the procedure used for the analysis of the samples from the greenhouse experiment. To ensure that there is minimal discrepancy between the results of the two procedures used, a subset of the plant samples was analysed using the two procedures and there was no significant difference in the results obtained.

2.2.4 | Calculations and Statistical Analysis

Zinc and Se uptake were calculated by multiplying the aboveground biomass of each species or sward by their corresponding Zn and Se concentration respectively and expressed in g/ha. The combination of micronutrient concentration and harvested biomass to represent micronutrient uptake is being used in many studies (Brink et al. 2001; Pederson et al. 2002). In the greenhouse experiment, the net effect (*NE*) of mixtures was calculated as the difference between the observed and expected Zn or Se uptake:

$$NE = Y_o - Y_e$$

where Y_o is the total observed Zn or Se uptake of species in the mixture; and Y_e is the total expected Zn or Se uptake. The total observed Zn and Se uptake values were the cumulative Zn and Se uptake of all species in the mixture while the expected Zn and Se uptake values were calculated by multiplying the Zn and Se uptake of each species grown as monocultures by their respective sowing proportion. When the net effect was positive, it was partitioned into complementarity (*CE*) and selection effects (SE) following the additive partitioning method described by Loreau and Hector (2001).

$$NE = CE + SE$$

$$CE = N.\,\overline{\Delta RY}.\,\overline{M} = (RYT - 1)\,\overline{M}$$

where *RYT* is the cumulative relative Zn/Se uptake; and $\overline{M}$ is the average Zn/Se uptake in the monocultures. The relative Zn/Se uptake was calculated as the ratio of the observed Zn/Se uptake in the mixture to the Zn/Se uptake in corresponding monoculture. SE was calculated as the difference between NE and CE. We interpreted a positive complementarity effect as indicative for niche differentiation and/or facilitation of Zn or Se uptake.

All statistical analyses were performed in R version 4.2.2. In the greenhouse experiment, the effect of species richness (i.e., 1, 2, 3, 6, 12) on aboveground biomass, Zn and Se uptake was tested using a linear mixed effect model, lme from package ‘nlme’ (Pinheiro et al. 2023), fitted with type III SS ANOVA. Similarly, the effect of sward type on aboveground biomass, Zn and Se concentration and uptake was also tested with a linear mixed effect model, in both the greenhouse and field experiments. Sward type was used as a fixed factor and block as a random factor. Tukey HSD test was used to test differences between levels of the fixed factor using package ‘emmeans’ (Lenth et al. 2023). All model residuals were assessed for normality and homogeneity graphically and/or using the ‘performance’ package (Lüdecke et al. 2021) in R. When the model residuals are not normally distributed or heterogeneous, we either transformed the data using log- or square root-transformation or included a variance structure in the model using the function varIdent from package ‘nlme’. Student *T*-test was used to examine whether the net effect was significantly greater than zero.

3 | Results

3.1 | Greenhouse Experiment

Shoot Zn and Se concentration as well as uptake varied among the 12 species after 5 weeks of growth as monoculture in the greenhouse experiment ($p < 0.01$). *Onobrychis viciifolia* had the highest shoot Zn concentration, which was about 3.5 times higher than *P. sativum*, which had the lowest Zn concentration (Figure 1a). A different pattern was observed with Se concentration, in which *S. minor* had the highest shoot Se concentration and *L. perenne* had the lowest Se concentration (Figure 1c). *Dactylis glomerata* had the highest Zn and Se uptake and *S. minor* had the lowest (Figure 1b,d). The 12 plant species also varied in aboveground biomass ($p < 0.01$). The largest biomass was produced by *D. glomerata*, whereas *S. minor* produced the least biomass (Figure 1e).

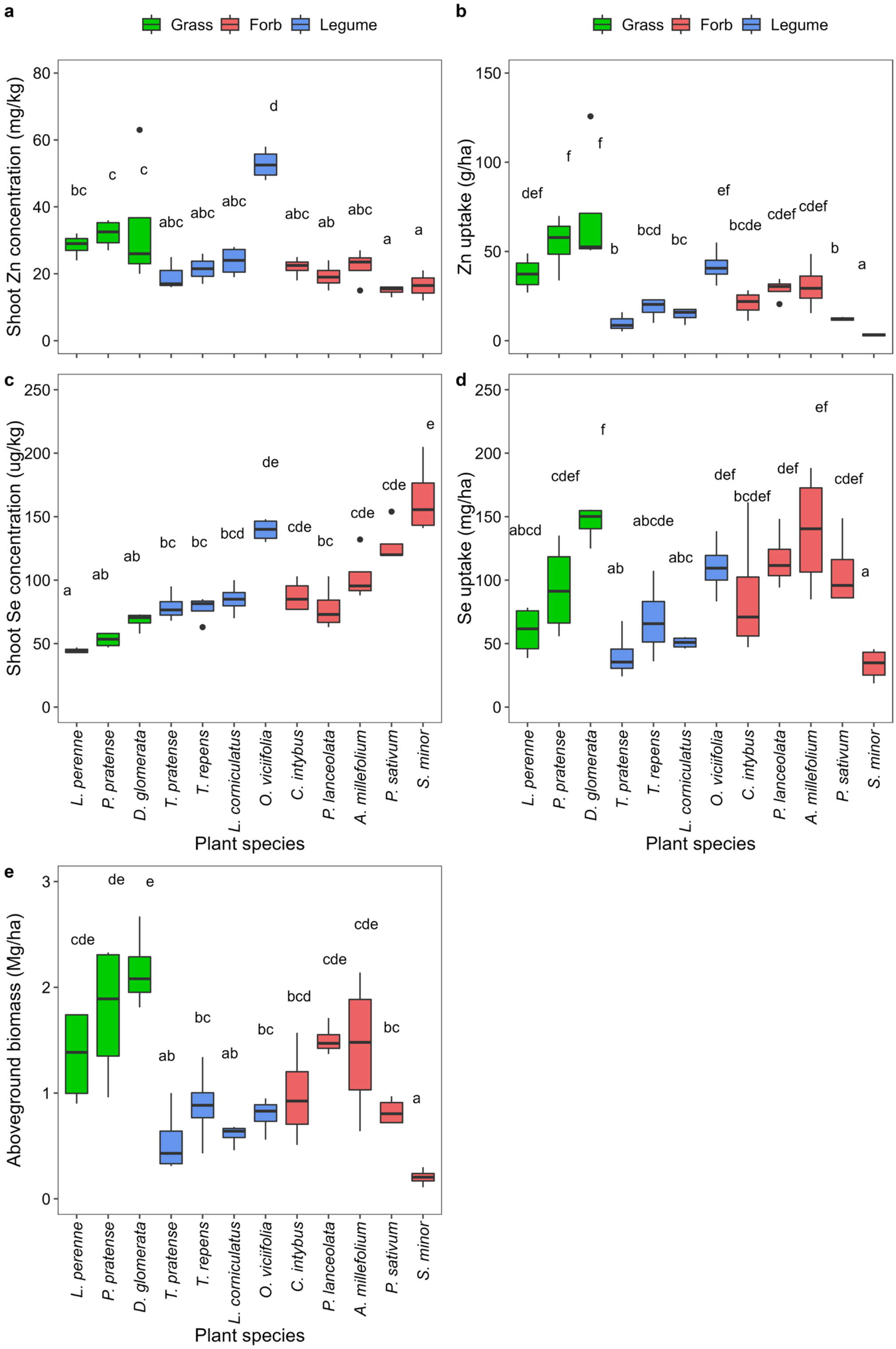

FIGURE 1 | (a) Shoot Zn concentration, (b) Zn uptake, (c) shoot Se concentration, (d) Se uptake, and (e) aboveground biomass of each plant species grown as monocultures in the greenhouse experiment. Boxes with the same letter are not significantly different (Tukey HSD after square root or log-transformation, $p = 0.05$). The borders on the boxplots from top to bottom represent the upper quartile, median and lower quartile, respectively, and the lines connect the maximum and minimum datapoints (without the outliers).

In the preliminary experiment where the 12 species were grown as monocultures with or without Zn and Se fertilisation, we found that Zn and Se shoot concentration in all the species was higher in the fertilised treatments than the unfertilised control ($p < 0.05$) (Figure S1a,b). However, there was no difference between the biomass of the species in the fertilised treatment and unfertilised control ($p < 0.05$) (Figure S1c). This indicated that Zn and Se were not growth-limiting micronutrients for any of the species on the soil we used but that concentration and uptake are responsive to the bioavailability of Zn and Se.

There was a positive effect of species richness on biomass, Zn and Se uptake. All mixtures had higher Zn uptake than the monocultures and Se uptake by six- and 12-species mixtures was higher than Se uptake by the monocultures ($p < 0.01$) (Figure 2a,b). There was an increase in Zn uptake along the species richness gradient, although there was no statistically significant difference among the two-, three- and six-species mixtures. Similarly, there was an increasing trend in Se uptake with increasing species richness, but there was no difference in Se uptake among the monocultures, two- and three-species mixtures. The 12-species mixtures had a 118% larger Zn uptake ($p < 0.01$) and 87% larger Se uptake ($p < 0.01$) than the average of the monocultures (Figure 2a,b) and performed transgressively better (13%) than the monoculture with the largest Se uptake. The effect of species richness on aboveground biomass was also positive, as biomass increased along the species richness gradient (Figure 2c). The 12-species mixtures demonstrated transgressive overyielding (6%) and had a 111% higher aboveground biomass than the average of the monocultures ($p < 0.01$), with no difference between the two- and three-species mixtures (Figure 2c).

The net effect analysis of Zn uptake showed a positive net effect in four out of 11 mixtures ($p < 0.05$), i.e., the difference between the observed Zn uptake by these four mixtures and the expected based on the weighted average of the Zn uptake in the corresponding monocultures was significantly greater than zero. These mixtures are: 2Sa, 2Se, 3Sb and 12S mixtures which represent grass-legume, legume-forb, grass-legume-forb and grass-legume-forb combinations, respectively. Notably, all these mixtures are legume-containing swards. The result of partitioning the net effect of mixing species showed complementarity and selection effects for Zn uptake. The complementarity effect contributed more than the selection effect in the grass-containing mixtures 2Sa (with *L. perenne*), 3Sb (with *P. pratense*) and 12S (with *L. perenne*, *P. pratense* and *D. glomerata*) (Figure 3a). However, six other combinations containing grasses (2Sb–d, 3Sa and 6Sa,b) did not show any net effect of mixtures. The selection effect was greater

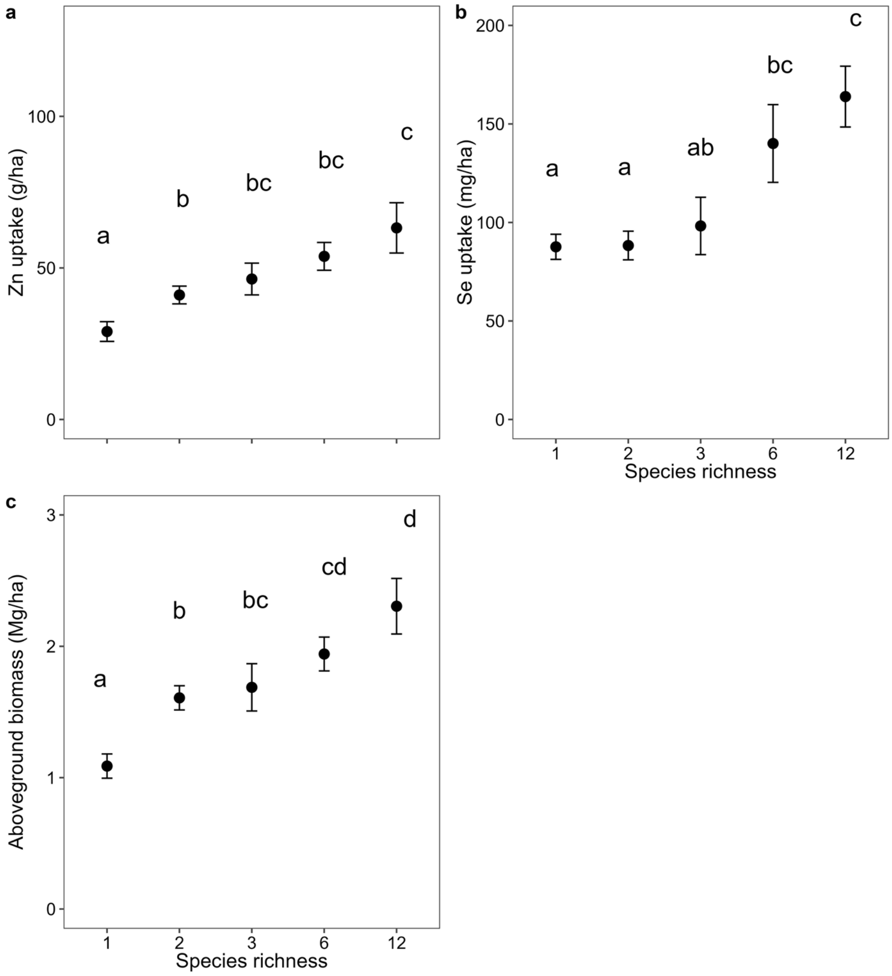

FIGURE 2 | Effect of species richness on (a) Zn uptake, (b) Se uptake and (c) aboveground biomass in the greenhouse experiment. Error bars indicate SEM. Points with the same letters are not significantly different (Tukey HSD, $p = 0.05$). Zn uptake values were log-transformed.

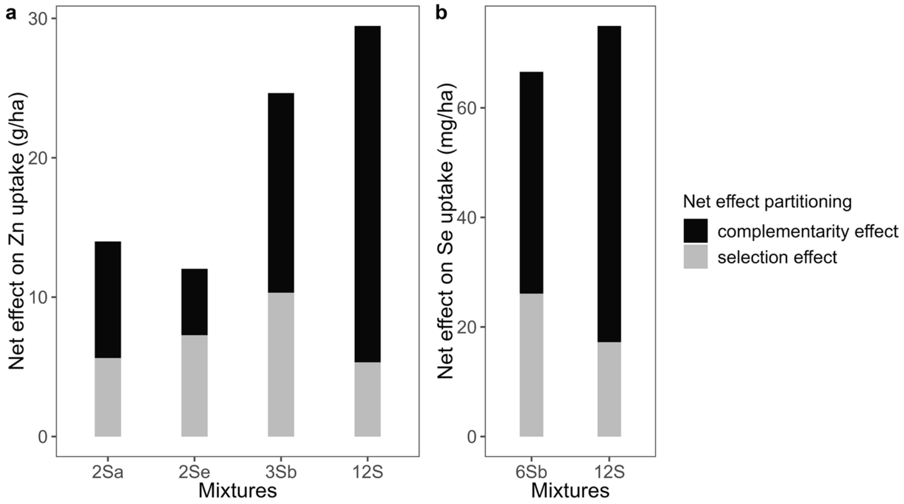

FIGURE 3 | Contribution of complementarity and selection effects to the net effect of species mixtures as compared to their monocultures on (a) Zn uptake and (b) Se uptake by mixtures with a significantly positive net effect in the greenhouse experiment.

in the 2Se mixture which did not contain any grass (Figure 3a). With regards to Se uptake, there was a significant positive net effect of mixing in two out of the 11 mixtures, namely: 6Sb and 12S mixtures, which represent grass-legume-forb combinations. Both selection and complementarity effects explained the overyielding; the complementarity effect being greater than the selection effect in these two mixtures (Figure 3b).

The mixtures with positive net effect in Zn and Se uptake were further analysed by comparing the shoot Zn and Se uptake by each species in the mixtures with their respective monocultures. Zinc uptake was higher in *L. perenne*, *P. lanceolata* and *P. pratense* in the 2Sa, 2Se and 3Sb mixtures, respectively, compared to their monocultures. Similarly, in the 12S mixture, *P. lanceolata*, *C. intybus*, *D. glomerata*, *A. millefolium*, *P. sativum* and *S. minor* exhibited increased Zn uptake relative to their monocultures (Table S3). For Se, *D. glomerata* and *A. millefolium* showed greater uptake in the 6Sb mixture than in monocultures, while *P. lanceolata*, *C. intybus*, *A. millefolium* and *S. minor* had enhanced Se uptake in the 12S mixture (Table S4). Notably, legumes such as *T. pratense* and *O. viciifolia* had lower Zn uptake while *L. corniculatus* had lower Se uptake in mixtures compared to their respective monocultures ($p < 0.05$).

When the two species-rich mixtures that were also used in the field experiment (i.e., 6Sa and 12S) were compared with the monoculture of perennial ryegrass, we found that both Zn concentration and Zn uptake by the mixtures did not significantly differ from the perennial ryegrass, although Zn uptake by the 12-species mixture was more than 1.5-fold higher than Zn uptake by the perennial ryegrass monoculture ($p = 0.06$) (Figure 4a,b). Shoot Se concentration as well as Se uptake was highest in the 12-species mixture and Se uptake by the 12-species mixtures was more than two-fold higher than Se uptake by the perennial ryegrass monoculture ($p < 0.05$) (Figure 4d,e). Aboveground biomass was also highest in the 12-species mixture which was more than 1.5-fold higher than the perennial ryegrass monoculture (Figure 4c). Analyses of the component species in the mixtures showed that *P. pratense*, *P. lanceolata* and *D. glomerata* contributed the most to Zn and Se uptake (Figure S2a,b) because they had high biomass even though they do not have high Zn and Se concentrations (Table 3). *Trifolium repens* and *O. viciifolia* had the highest Zn and Se concentration in the 6Sa and 12S mixtures, respectively (Table 3).

3.2 | Field Experiment

After 2 years of the field experiment, Zn uptake by the six- and 12-species swards was higher than that of the perennial ryegrass monoculture sward by 45% and 33%, respectively ($p < 0.05$) (Figure 5b), although there was no difference in the sward Zn concentration among all the swards (Figure 5a). With regards to Se, both six- and 12-species swards had higher Se concentration and Se uptake than the perennial ryegrass monoculture sward ($p < 0.05$) (Figure 5d,e). The sward Se concentration and uptake in both multispecies swards were more than twofold greater compared to the perennial ryegrass sward. All the three sward types (i.e., perennial ryegrass, six-species and 12-species) produced a comparable biomass ($p < 0.05$) (Figure 5c). In the first year of the experiment, both Zn uptake and aboveground biomass did not differ among the sward types, but Se uptake was higher in the two multispecies swards than in the perennial ryegrass monoculture.

In contrast to the greenhouse experiment, *L. perenne*, *T. pratense* and *C. intybus* contributed the most to the sward Zn and Se uptake by the multispecies swards (Figure S3a,b) and these species also had the highest biomass (Table 4). In terms of concentration, *P. lanceolata* had the highest Zn concentration and *C. intybus* had the highest Se concentration (Table 4).

4 | Discussion

To the best of our knowledge, our study is the first to investigate the effect of species richness on Zn and Se uptake. We showed that Zn and Se uptake increased with increasing species richness, with the most species-rich mixture having higher Zn and Se uptake than the average Zn and Se uptake

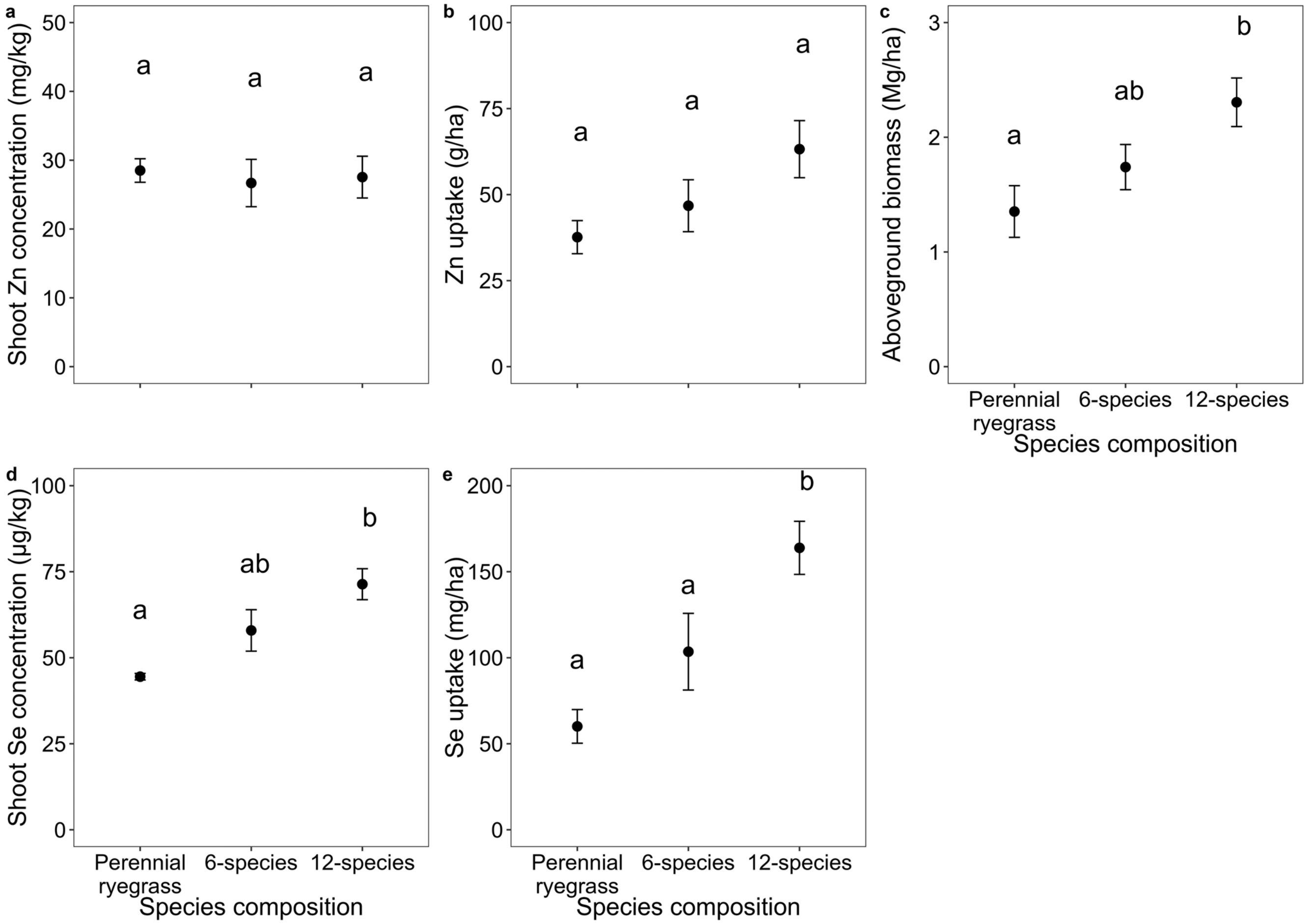

FIGURE 4 | (a) Shoot Zn concentration, (b) Zn uptake, (c) aboveground biomass, (d) shoot Se concentration and (e) Se uptake of the mixtures and monoculture of perennial ryegrass in the greenhouse experiment. Points with the same letters are not significantly different (Tukey HSD, $n = 4$, $p = 0.05$). Shoot Se concentration was log-transformed.

TABLE 3 | Mean biomass as well as Zn and Se concentration of all the component species in the 6Sa and 12S mixtures in the greenhouse experiment ($n = 4$).

	6Sa mixture			12S mixture		
Component species	**Biomass (kg/ha)**	**Zn (mg/kg)**	**Se (µg/kg)**	**Biomass (kg/ha)**	**Zn (mg/kg)**	**Se (µg/kg)**
L. perenne	232.0 ^{b}	34.5 ^{b}	56.0 ^{ab}	179.0 ^{abc}	26.5 ^{ab}	75.2 ^{ab}
P. pratense	693.0 ^{b}	30.8 ^{ab}	47.0 ^{a}	494.0 ^{cd}	26.5 ^{ab}	50.2 ^{a}
T. repens	43.1 ^{a}	37.0 ^{b}	84.8 ^{c}	26.9 ^{a}	19.0 ^{ab}	95.5 ^{ab}
T. pratense	38.2 ^{a}	21.3 ^{ab}	57.0 ^{abc}	21.4 ^{a}	28.0 ^{ab}	93.0 ^{ab}
C. intybus	64.9 ^{a}	20.8 ^{ab}	78.0 ^{bc}	118.0 ^{abc}	18.8 ^{ab}	97.0 ^{ab}
P. lanceolata	669.0 ^{b}	19.2 ^{a}	67.8 ^{abc}	352.0 ^{bcd}	26.8 ^{ab}	81.5 ^{ab}
D. glomerata	—	—	—	745.0 ^{d}	34.8 ^{ab}	60.0 ^{ab}
L. corniculatus	—	—	—	48.4 ^{a}	24.0 ^{ab}	102.0 ^{ab}
O. viciifolia	—	—	—	47.9 ^{a}	37.5 ^{b}	177.0 ^{b}
A. millefolium	—	—	—	152.0 ^{abc}	19.0 ^{ab}	93.0 ^{ab}
P. sativum	—	—	—	96.1 ^{ab}	15.8 ^{a}	79.8 ^{ab}
S. minor	—	—	—	22.9 ^{a}	18.0 ^{ab}	93.0 ^{ab}

Note: Values with the same letter in the same column for each mixture are not significantly different ($p < 0.05$). Data were either log- or square root-transformed.

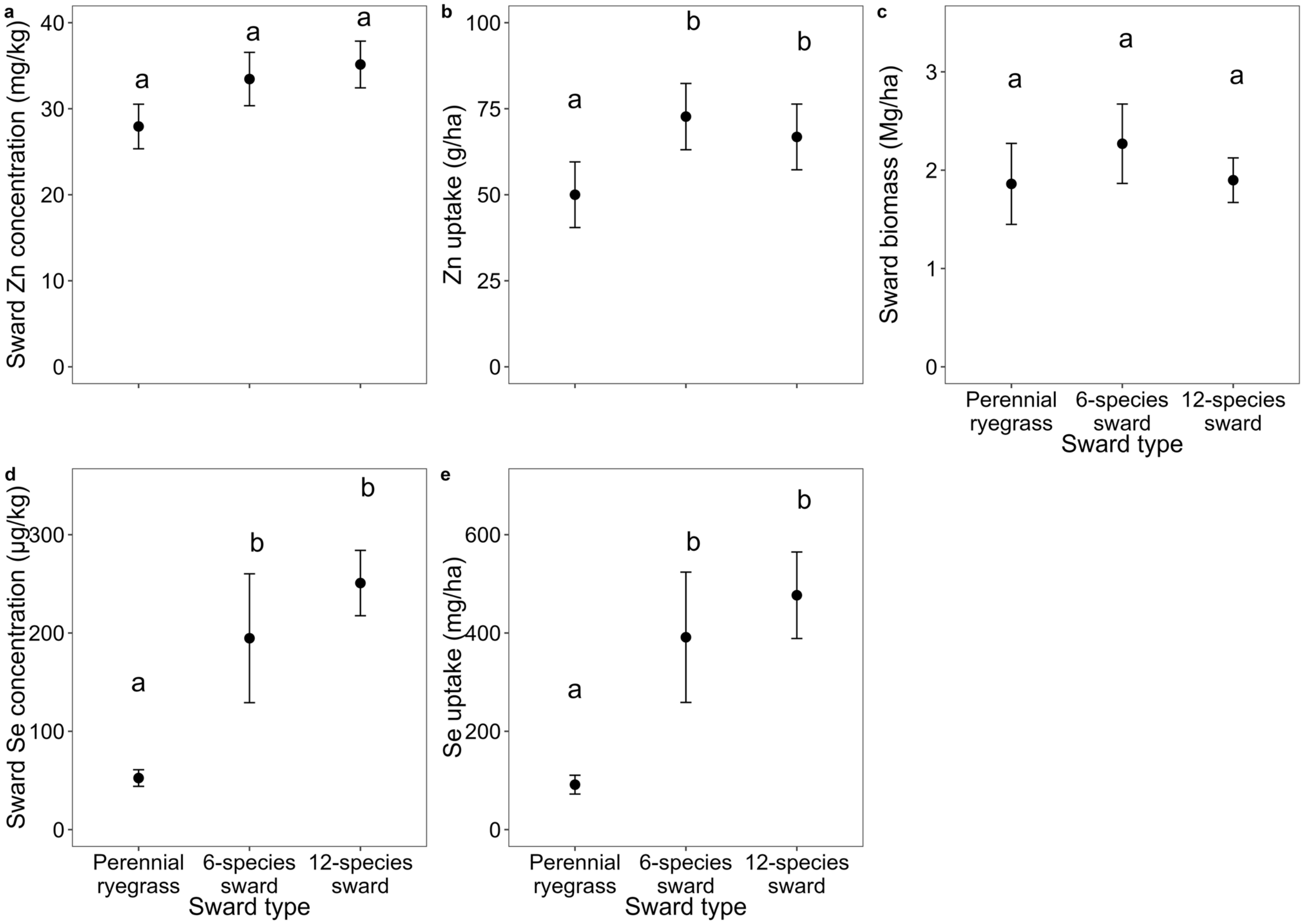

FIGURE 5 | (a) Sward Zn concentration, (b) Zn uptake, (c) aboveground biomass, (d) sward Se concentration and (e) Se uptake of the mixtures and monoculture of perennial ryegrass after 2 years of the field experiment. Points with the same letters are not significantly different (Tukey HSD, $n = 4$, $p = 0.05$). Se concentration and Se uptake values were log-transformed.

TABLE 4 | Mean biomass as well as Zn and Se concentration of all the component species in the 6S and 12S swards in the field experiment ($n = 4$).

	6S sward			12S sward		
Component species	**Biomass (kg/ha)**	**Zn (mg/kg)**	**Se (µg/kg)**	**Biomass (kg/ha)**	**Zn (mg/kg)**	**Se (µg/kg)**
L. perenne	1058.0 ^{d}	30.2 ^{ab}	100.2 ^{a}	685.0 ^{b}	34.4 ^{b}	117.7 ^{ab}
P. pratense	92.4 ^{bc}	40.7 ^{bc}	39.2 ^{a}	97.1 ^{a}	39.4 ^{b}	43.6 ^{a}
T. repens	20.6 ^{ab}	22.8 ^{a}	39.2 ^{a}	34.4 ^{a}	22.9 ^{a}	72.2 ^{a}
T. pratense	789.0 ^{cd}	30.3 ^{ab}	62.1 ^{a}	682.0 ^{b}	34.0 ^{b}	141.0 ^{ab}
C. intybus	260.0 ^{cd}	62.1 ^{cd}	832.7 ^{b}	169.0 ^{a}	63.1 ^{cd}	900.1 ^{c}
P. lanceolata	14.7 ^{a}	65.2 ^{d}	610.7 ^{b}	29.8 ^{a}	65.1 ^{d}	568.2 ^{bc}
D. glomerata	—	—	—	180.0 ^{a}	46.1 ^{bc}	287.7 ^{ab}

Note: Values with the same letter in the same column are not significantly different ($p < 0.05$). Data were either log- or square root-transformed.

of the monocultures in the greenhouse experiment. Moreover, when the two multispecies mixtures were compared with a monoculture of perennial ryegrass in the greenhouse experiment, the most species-rich mixture had higher Zn uptake ($p = 0.06$) and Se uptake ($p < 0.05$) than the perennial ryegrass monoculture (Figure 4b,e). This result from the greenhouse experiment was validated in the field experiment: the multispecies swards consistently had a higher Zn and Se uptake than the monoculture sward of perennial ryegrass (Figure 5b,e), although the contribution of the component species to this result differed between the two experiments. This confirmed and extended the findings of Høgh-Jensen and Søegaard (2012) who showed that a mixture of red cover and ryegrass had a higher Zn uptake than ryegrass monoculture.

In terms of forage quality, shoot Zn and Se concentrations of the swards are relevant. Shoot Se concentration was higher in the multispecies swards compared to the perennial ryegrass monoculture across the two experimental scales, but shoot Zn concentration did not differ among the sward types. Hence, the positive mixture effect on shoot micronutrient concentration was limited to Se. Although our preliminary experiment confirmed the responsiveness of the plant species to soil Se availability, the mixtures still exhibited increased Se concentration in the main experiment even under low Se soil. This suggests that the higher Se concentration in the mixtures was unlikely due to soil Se bioavailability.

The higher Zn and Se uptake in the multispecies mixtures was a result of the contribution of the component species with high biomass production and inherently high Zn and Se concentrations. This was confirmed by the selection effect which contributed to the overyielding in terms of Zn and Se uptake. It is known that plant species that dominate the mixtures are most likely to influence Zn uptake (Lindström, Frankow-Lindberg, Dahlin, Watson, et al. 2014). Hence, in our study, *P. pratense*, *P. lanceolata* and *D. glomerata* with high biomass contributed the most to Zn uptake in multispecies mixtures in the greenhouse experiment, whereas *L. perenne* and *T. pratense* species contributed to higher sward Zn uptake in the field experiment (Tables 3 and 4). Analysis of the monocultures in the greenhouse experiment further substantiated the role of biomass production in enhancing Zn and Se uptake. Despite the high Zn concentration of *O. viciifolia*, its Zn uptake was comparable to those of grasses and certain forbs. Similarly, *S. minor* with the highest Se concentration had the lowest biomass and consequently the lowest Se uptake (Figure 1).

Out of the 11 mixtures investigated in the greenhouse experiment, positive net effects of mixtures on Zn and Se uptake were observed in only four (2Sa, 2Se, 3Sb and 12S) and two (6Sb and 12S) mixtures, respectively. Partitioning the net effect revealed that complementarity effects exceeded selection effects in the 2Sa, 3Sb and 12S mixtures for Zn uptake and in the 6Sb and 12S mixtures for Se uptake. Since complementarity effects encompass niche differentiation and facilitation (Loreau and Hector 2001), higher complementarity effects in the mixtures may be attributed to these two mechanisms. However, we posit that niche differentiation is unlikely to be the primary driver, because all species were sown and harvested at the same time and root differentiation is minimal considering the size of the experimental pots. This excludes the potential of spatial and temporal niche differentiation as the underlying mechanisms and hints at facilitation as the main driver of enhanced Zn and Se uptake. Contrary to our hypothesis, there was no consistent evidence that grasses facilitated Zn and Se uptake across the mixtures, because not all the mixtures with positive net effects contained grass species; rather, they all contained legume species. This suggests that enhanced Zn uptake may be driven by facilitation by legumes. Further analysis comparing Zn and Se uptake in mixtures versus monocultures supports the notion of facilitation by legumes, as Zn and Se uptake in neighbouring grasses and forbs was higher in legume-containing mixtures than in their respective monocultures (Tables S3 and S4). Legumes typically nodulate more effectively when grown with non-N_2 fixing species, increasing atmospheric N_2 fixation. This process often leads to enhanced cation uptake and proton release, which solubilises Zn and improves its availability for neighbouring species (Tang et al. 1999; Van Eynde et al. 2022).

Unexpectedly, Zn as well as Se uptake by some of the legume species in the mixtures (i.e., *T. pratense* in 2Se; *O. viciifolia* in 12S; *L. corniculatus* in 6Sb) was consistently lower than in their monocultures. This suggests that legumes may have enhanced Zn uptake for neighbouring grasses and forbs at their own expense—a mechanism known as asymmetrical facilitation (Li et al. 2003). Asymmetrical facilitation has been reported in intercropping systems, where phosphorus uptake was lower in cassava/peanut intercrops compared to peanut monocultures (Mason et al. 1986) and lower in maize/mung bean intercrops than sole crops of mung beans (Chowdhury and Rosario 1992). The reduced Zn/Se uptake by legume species in the mixtures may be an indication that interspecific competition between legumes and the neighbouring species for available Zn and Se was greater than the facilitation of these micronutrients by legumes.

It is noteworthy that the average values of Zn and Se concentrations of the component species of the multispecies swards in the field experiment were generally higher than those in the greenhouse experiment. This may be due to the fact that the plant species in the greenhouse experiment were harvested at a younger vegetative stage while those in the field experiment were harvested in the mature, flowering and/or seeding stage. Plant growth stage is known to influence nutrient uptake by plants (Souri et al. 2019). Differences in environmental factors can also exert a huge influence on Zn and Se concentration and uptake by the species (Darch et al. 2020). Moreover, the field experiment spanned a 2-year period, whereas plant species in the greenhouse experiment were harvested after five weeks. While the 5-week duration of the greenhouse experiment aligns with the ~35-day harvesting interval in the field experiment, it may not be sufficient to fully replicate the outcomes observed at the field scale.

Biomass production in the multispecies mixtures was higher in the greenhouse experiment (Figure 2c) but not in the field experiment (Figure 4c). This is most likely because the comparisons with monocultures were different. In the field experiment, 170 kg N/ha/year was applied to the high productive monoculture of *L. perenne* and 70 kg N/ha/year was applied to the multispecies swards. In contrast, a uniform N fertiliser rate of 70 kg N/ha/year was applied to all treatments in the greenhouse experiment. The higher N fertiliser rate applied to the perennial ryegrass sward in the field likely enhanced its productivity, resulting in biomass production comparable to that of the multispecies swards. Moreover, complementary N use among species in the mixtures may be more pronounced under the shallow soil conditions of the greenhouse pots, where closer root interactions likely enhanced belowground resource use (von Felten and Schmid 2008).

5 | Conclusion

Multispecies mixtures had higher Zn and Se uptake than monoculture of perennial ryegrass in the field experiment. The increase in uptake was driven by aboveground biomass production of the component species with inherently higher Zn or Se concentrations—an indication of selection effect. This implies that, depending on the primary objective of the grassland system, an informed decision on the choice of species to be included in the multispecies mixtures can be made based on the biomass production potential as well as Zn and Se concentration of the component species. The greenhouse experiment also highlighted complementarity effect as an important mechanism, with evidence suggesting potential facilitation by legumes rather than grass species. Since our findings were based on aboveground analysis, further research should investigate belowground interactions over a longer duration to better understand root-root interactions, root exudation and rhizosphere pH effects on facilitation. Future studies should also explore other aspects of facilitation including asymmetrical facilitation by legumes and its underlying mechanisms to gain deeper insights into species interactions in multispecies grasslands that enhance Zn and Se uptake.

Acknowledgements

We appreciate the staff of Unifarm, Wageningen University for their support during the greenhouse experiment. We also thank Dr. Natalie Oram for the useful discussions and guidance during the study. We appreciate Dr. Segun Obasoro, Jason Reilly and Robyn Hutchinson for their help during the lab and field work. This project was funded by the European Union's Horizon 2020 Research and Innovation Programme (Marie Skłodowska-Curie Actions) under grant agreement No. 814030 and Devenish Nutrition.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Research data are not shared.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.