

Species composition but not interspecific facilitation enhances zinc and selenium uptake in multispecies grasslands

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Abstract

Aims

To understand the variations among grassland species in their ability to take up zinc (Zn) and selenium (Se) when cultivated as monocultures and to assess the effect of species richness on Zn and Se uptake. Additionally, to examine whether interspecific facilitation or species composition of the mixtures contributes to the potential increase in Zn and Se uptake in mixtures.

Methods

We conducted a grassland diversity 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.

Results

The capacity of the monoculture species to take up Zn and Se varied significantly in the pot experiment. 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 the large aboveground biomass production and/or high tissue Zn and Se concentration of some of the component species in the mixtures. However, there was insufficient evidence supporting interspecific facilitation.

Conclusion

To optimize 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.

Introduction

The establishment of multispecies plant communities is considered an important approach for promoting sustainability in agricultural grasslands (Finn et al., 2013). Previous studies showed that more biomass can be produced from multispecies swards with lower nitrogen (N) fertilizer 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, improve N use efficiency (Abalos et al., 2021; Husse et al.,

2017), reduce N leaching and N₂O emission (Bracken et al., 2022; Carlton et al., 2019; Cummins et al., 2021). While these studies have provided more insights into the potential of diverse grassland communities in the cycling of N, there is little evidence on the role of multispecies swards in enhancing micronutrient uptake in grassland swards.

The importance of essential micronutrients in grasslands is not only limited to optimizing yield, but also important for forage quality (Gupta et al., 2001; Kao et al., 2020; Vojnovic et al., 2018). Forages from grassland production represent a significant part of livestock feedstuff in many countries (Gupta et al., 2008; Lindström et al., 2014a), hence, micronutrient concentration and its uptake in the harvested biomass of the forage species can affect livestock productivity and health. Given that multispecies swards comprise a variety of grassland species, they could therefore play a crucial role in further enhancing the uptake of micronutrients that are important for livestock health, particularly zinc (Zn) and selenium (Se).

Zinc plays a key role in regulating plant growth and metabolic activities in animals (Hill & Shannon, 2019). Sufficient supply of Zn to plants has shown to enhance yield of grassland species (Grewal, 2001) as well as fodder crops (Kumar et al., 2017; Tondy et al., 2021). In animals, Zn is important for boosting immunity, increasing disease resistance, promoting the activity of enzymes and crucial for vitamin A transport (Hill & Shannon, 2019). Severe Zn deficiency in plants could cause leaf chlorosis, reduction in plant growth, and increased susceptibility to certain infections (Sharma et al., 2013) and for livestock, it causes stunted growth, poor reproduction, and weak bones (Hill & Shannon, 2019). On the other hand, Se is more relevant for livestock health than plant growth but can also offer certain benefits to plants (Vojnovic 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 & Gupta, 2017; White, 2016). Selenium is important for improving productive efficiency, fertility and boosting the immune system of livestock (Hefnawy & Tórtora-Pérez, 2010). Its deficiency in cattle is associated with placental retention, reduced fertility (Mehdi & Dufrasne, 2016), poor seminal quality (Beckett & Arthur, 2005), smaller litter size, reduced weight gains and lower milk production (Hefnawy & Tórtora-Pérez, 2010).

There are different plausible mechanisms through which multispecies mixtures can potentially increase Zn and Se uptake. Some studies showed that the inclusion of specific species or functional groups with high shoot micronutrient concentrations and/or high biomass production enhanced micronutrient concentration and uptake in multispecies mixtures (Høgh-Jensen et al., 2006; Lindström et al., 2014b). For instance, Lindström et al. (2014b) reported higher micronutrient uptake in grassland mixtures which was mainly attributed to both high micronutrient concentration and biomass of legumes, particularly, red clover. The study by Høgh-Jensen and Søgaard (2006) also showed that forbs, especially, chicory accumulated higher concentrations of Zn, thereby improving the overall micronutrient concentration of the multispecies swards.

Another phenomenon that could underlie increase in Zn and Se uptake in multispecies mixtures is interspecific facilitation (Li et al., 2014; Xue et al., 2016). Facilitation occurs when a particular plant species and/or functional group within a diverse plant community, directly or indirectly provides benefit(s) to another plant species in the community (Callaway, 1995; Loreau and Hector, 2001; Xue et al., 2016). This may include promoting the availability of an otherwise limited soil resource and making it available to the neighbouring plant (Li et al., 2014). Under Zn-limited conditions, the presence of graminaceous species belonging to Poaceae family can facilitate uptake by a non-graminaceous species by releasing phytosiderophores that complexate and solubilize Zn (Zuo & Zhang, 2008). The study of Inal et al. (2007) also showed that intercropping graminaceous with a non-graminaceous crop increased the Zn concentration of the non-graminaceous species. Selenium uptake in mixtures can also be enhanced by co-cultivating species that are Se-hyperaccumulators with non-accumulators (El Mehdawi et al., 2012; Schiavon et al., 2020). The decomposition of litter produced by the hyperaccumulators as well as root exudation enriches the soil with Se, thereby making it available for uptake by the neighbouring plants (El Mehdawi et al., 2012; Schiavon et al., 2020). The facilitation by hyper-accumulators could also be due to reduced herbivory (El Mehdawi et al., 2011). However, most of these studies with evidence of Zn and Se facilitation were conducted in either arable cropping systems (Inal et al., 2007; Zuo & Zhang, 2008) or shrublands (El Mehdawi et al., 2011, 2012) with little or no evidence from grasslands. Hence, it is unknown whether this phenomenon is also applicable to grassland species.

It is therefore relevant to investigate the mechanism(s) that underlie the potential increase in Zn and Se uptake in multispecies mixtures in the context of grasslands systems. We hypothesized that there will be increased Zn and Se uptake in species-rich mixtures, and this will not only be as a result of the presence of certain species with inherently high Zn and Se concentration and high biomass, but also due to interspecific facilitation by grass species. To test this hypothesis, we conducted a grassland diversity experiment at both pot and field scales. The pot trial in the greenhouse enabled us to: 1) investigate how grassland species vary in their capacity to take up Zn and Se when grown as monocultures 2) investigate the effect of species-richness on Zn and Se uptake 3) test if there is evidence of interspecific facilitation as a potential mechanism underlying any increase in Zn and Se uptake in mixtures. The field experiment on the other hand, provided an opportunity to further elucidate on other potential mechanisms driving the increased uptake by mixtures using grassland sward types that are realistic from farmers' perspective and practicable in real world agricultural systems. Hence, in the field experiment, we compared Zn and Se uptake by two multispecies swards with a monoculture grass sward and analysed their component species to establish whether the biomass or tissue concentration of the component species in the mixture is responsible for the increased uptake.

Methods

2.1. Greenhouse experiment

2.1.1. Experimental design

A pot experiment was set up in the greenhouse of Wageningen University and Research from July – August 2021. We used low Zn and Se soils with $\text{CaCl}_2\text{-Zn}$ and $\text{CaCl}_2\text{-Se}$ concentration of 6.0 $\mu\text{g/kg}$ and 9.9 $\mu\text{g/kg}$ respectively and $\text{HNO}_3\text{-Zn}$ and $\text{HNO}_3\text{-Se}$ concentration of 7.4 mg/kg and 37.9 $\mu\text{g/kg}$ respectively. The soils were collected from 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 and the soil-quartz sand mixture was in a ratio of 3:2. 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 two 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.

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	<i>Lolium perenne</i> L.	35.0
	Timothy	<i>Phleum pratense</i> L.	20.0
	Cocksfoot	<i>Dactylis glomerata</i> L.	20.0
Legumes	White clover	<i>Trifolium repens</i> L.	12.0
	Red clover	<i>Trifolium pratense</i> L.	15.0
	Birdsfoot trefoil	<i>Lotus corniculatus</i> L.	12.5
	Sainfoin	<i>Onobrychis viciifolia</i> Scop.	87.5
Forbs	Chicory	<i>Cichorium intybus</i> L.	15.0
	Plantain	<i>Plantago lanceolata</i> L.	10.0
	Yarrow	<i>Achillea millefolium</i> L.	2.5
	Sheep's parsley	<i>Petroselinum sativum</i> Hoffm.	10.0
	Salad burnet	<i>Sanguisorba minor</i> L.	22.5

The treatments in the pot experiment comprised of twelve monocultures, six combinations of two-species mixtures, two combinations of three-species mixtures, two combinations of six species mixtures and one twelve-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 randomized 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).

Table 2
Composition of mixtures in the greenhouse experiment

Mixture	Species composition	Functional group*
2-species	<i>L. perenne</i> + <i>T. repens</i> (2S1)	G + L
	<i>P. pratense</i> + <i>T. pratense</i> (2S2)	G + L
	<i>L. perenne</i> + <i>P. lanceolata</i> (2S3)	G + F
	<i>P. pratense</i> + <i>C. intybus</i> (2S4)	G + F
	<i>T. pratense</i> + <i>P. lanceolata</i> (2S5)	L + F
	<i>T. repens</i> + <i>C. intybus</i> (2S6)	L + F
3-species	<i>L. perenne</i> + <i>T. repens</i> + <i>P. lanceolata</i> (3S1)	G + L + F
	<i>P. pratense</i> + <i>T. pratense</i> + <i>C. intybus</i> (3S2)	G + L + F
six-species	<i>L. perenne</i> + <i>P. pratense</i> + <i>T. repens</i> + <i>T. pratense</i> + <i>P. lanceolata</i> + <i>C. intybus</i> (6S1)	2G + 2L + 2F
	<i>D. glomerata</i> + <i>L. corniculatus</i> + <i>O. viciifolia</i> + <i>A. millefolium</i> + <i>P. sativum</i> + <i>S. minor</i> (6S2)	2G + 2L + 2F
12 species	<i>L. perenne</i> + <i>P. pratense</i> + <i>T. repens</i> + <i>T. pratense</i> + <i>P. lanceolata</i> + <i>C. intybus</i> + <i>D. glomerata</i> + <i>L. corniculatus</i> + <i>O. viciifolia</i> + <i>A. millefolium</i> + <i>P. sativum</i> + <i>S. minor</i> (12S)	3G + 4L + 5F

A preliminary experiment was set up where the twelve species were grown as monocultures in the same soil under similar conditions, with or without Zn and Se fertilization in the form of Zn-EDTA (5 kg /ha) and Na₂SeO₄ (10 g/ha) salts. All species responded to fertilization with an increase in Zn and Se tissue concentration. Biomass did not respond, indicating that Zn and Se were not growth-limiting micronutrients (Fig. S1a, b & c).

2.1.2. Aboveground biomass sampling and analysis

All the plants were harvested after 5 weeks of growth. 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 14M HNO₃ and 0.3 mL of 12M HCl was added. Subsequently, 1.5 mL of H₂O₂ 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 total Zn and Se concentration of the samples. Some species were excluded from the data analyses either due to laboratory error during Zn and Se determination or insufficient sample or no biomass for digestion. Hence, we excluded ten out of two hundred and sixteen 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. The soil texture of the field ranged from sandy-loam to loam. The total soil carbon content ranged from 4.2–5.8% and the total soil nitrogen content ranged from 0.4–0.5%. Soil pH measured in 0.01 M CaCl₂ at 1:10 (w/v) ranged from 5.8 to 6.8, soil organic matter (SOM) determined using the loss on ignition method (550°C) was in the range of 9.5–13.3%.

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 x 10 m² and all treatments were established in July 2020 in a randomized complete block design. The three sward types investigated were: perennial ryegrass monoculture, six-species (6S) and twelve-species (12S) swards (Table 3). The PRG sward was sown as a monoculture of *L. perenne* while 6S and 12S were sown as multispecies mixture of grasses, legumes, and forbs species at a sowing proportion of 40:30:30 respectively (Table S2). In the 12S sward, five species did not persist, namely: *Lotus corniculatus*, *Onobrychis viciifolia*, *Achillea millefolium*, *Petroselinum sativum*, and *Sanguisorba minor*. Hence, the twelve-species sward was almost comparable to the six-species sward as the only difference was the presence of *Dactylis glomerata* in the twelve-species sward. The seeding rates used were similar to that of the greenhouse experiment (Table 1).

Table 3
Composition of mixtures in the field experiment

Mixture	Species composition	Functional group*
six-species	<i>L. perenne</i> + <i>P. pratense</i> + <i>T. repens</i> + <i>T. pratense</i> + <i>P. lanceolata</i> + <i>C. intybus</i> (6S)	2G + 2L + 2F
12 species	<i>L. perenne</i> + <i>P. pratense</i> + <i>T. repens</i> + <i>T. pratense</i> + <i>P. lanceolata</i> + <i>C. intybus</i> + <i>D. glomerata</i> + <i>L. corniculatus</i> + <i>O. viciifolia</i> + <i>A. millefolium</i> + <i>P. sativum</i> + <i>S. minor</i> (12S)	3G + 4L + 5F
*Functional group: G, grass, L, legume, F, forb species		

The N fertilizer treatment used was chemical N fertilizer applied as granulated protected urea. Total N fertilizer rate of 70 kg N/ha/yr was applied across all treatments except for PRG sward which received 170 kg N/ha/yr to compensate for the absence of N₂ 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 environmental conditions were optimal for all species at that moment. A 50 x 50 cm² 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.

2.3. Calculations and statistical analysis

Micronutrient uptake was calculated by multiplying the aboveground biomass of each species or sward by their corresponding Zn and Se concentration respectively and expressed in g/ha.

For the net effect analysis of the mixtures, the expected Zn and Se uptake values were calculated by multiplying the Zn and Se uptake of each species grown as monocultures in the greenhouse experiment

by their respective sowing proportion while the observed values were the cumulative Zn and Se uptake of all species in the mixture. To calculate expected Zn uptake for *T. pratense* in samples harvested in block three, we used the mean value of the other three blocks because *T. pratense* was part of the samples excluded from analyses due to laboratory error. The mixtures that had observed values that were significantly different from the expected values were partitioned into complementarity and selection effects following the additive partitioning method described by Loreau and Hector (2001).

All statistical analyses were performed in R version 4.2.2. The effect of plant species (greenhouse experiment) as well as sward type (field experiment) on aboveground biomass, Zn and Se concentration and uptake was tested with linear mixed effect model, lme from package “nlme” (Pinheiro et al., 2023), fitted with a type III SS ANOVA. Plant species was used as fixed factor and block as random factor in the greenhouse experiment while sward type and experimental year were used as fixed factors and block as random factor in the field experiment. When there was no significant interaction between the fixed factors, the model was re-analysed without the interaction term. Tukey HSD test was used to test differences between levels of each 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 (Lüdtke et al., 2021) in R. When the model residuals are not normal or heterogeneous, we either transformed the data using log- or square root-transformation or include a variance structure in the model using the function varIdent from package “nlme”. Student T-test was used to test the significant difference between the observed and expected Zn and Se uptake values for net effect analysis.

Results

3.1 Greenhouse experiment

Zinc and Se concentration as well as uptake varied among the twelve species after five weeks of growth as monoculture in the greenhouse ($P < 0.01$). *Onobrychis viciifolia* had the highest Zn concentration which was about 3.5 times higher than *P. sativum* which had the lowest Zn concentration (Fig. 1a). A different pattern was observed with Se concentration, in which *S. minor* had the highest tissue Se concentration and *L. perenne* had the lowest Se concentration (Fig. 1c). On the other hand, *D. glomerata* and *P. pratense* had the highest Zn and Se uptake and *S. minor* had the lowest (Fig. 1b & d). The twelve 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 (Fig. 1e). Based on the functional groups, grasses and legumes had the highest Zn concentration ($P < 0.001$) while legumes and forbs had the highest Se concentration. Grasses had the highest aboveground biomass as well as Zn uptake ($P < 0.001$) whereas, there was no significant difference in Se uptake among the functional groups (data not shown).

All mixtures had higher Zn uptake than the monocultures ($P < 0.01$). Selenium uptake by six- and twelve-species mixtures was larger than by the monocultures. The twelve-species mixtures had a 118% larger Zn uptake ($P < 0.01$) and 87% larger Se uptake ($P < 0.01$) than the monocultures (Fig. 2a & b). Although there

was an increase in Zn uptake along the species richness gradient, there was no statistically significant variation among the two-, three- and six-species mixtures. Similarly, there was no variation in Se uptake among the monocultures, two-, and three-species mixtures, despite the increasing trend in Se uptake with increasing species richness. The effect of species richness on Zn uptake was similar to its effect on aboveground biomass, which also increased along the species richness gradient. The twelve-species mixtures had a 111% larger aboveground biomass than the average of the monocultures ($P < 0.01$), with no difference between the two- and three-species mixtures (Fig. 2c).

The net effect analysis (i.e., the performance of the mixtures relative to their performance when grown as monocultures) of Zn uptake showed positive net effect in four out of eleven mixtures ($P < 0.05$); the observed Zn uptake by these four mixtures was significantly greater than the expected values based on the weighted average of the Zn uptake in the corresponding monocultures. These mixtures are: 2S1, 2S5, 3S2, and 12S mixtures which represent grass-legume, legume-forb, grass-legume-forb, grass-legume-forb and grass-legume-forb combinations respectively (Fig. 3). With regards to Se uptake, there was a significant positive net effect in two out of the eleven mixtures, namely: 6S2 and 12S mixtures, which both represent grass-legume-forb combinations (Fig. 4).

Partitioning the net effect of mixing species into complementarity and selection effects for Zn uptake, the result was not consistent: the complementarity effect contributed more than the selection effect in the 2S1, 3S2 and 12S which are grass-containing mixtures, whereas the selection effect was dominant in the 2S5 mixture which is not a grass-containing mixture. For Se uptake, however, the complementarity effect was greater than the selection effect in the two mixtures with significant positive net effect, i.e., 6S2 and 12S mixtures.

When we compared two species-rich mixtures (i.e., 6S1 and 12S) with the monoculture of perennial ryegrass, we found that Zn uptake by the mixtures was marginally higher than the perennial ryegrass monoculture ($P = 0.06$) (Fig. 5b). Selenium uptake by the twelve-species mixtures was significantly higher than the perennial ryegrass monoculture ($P < 0.05$) (Fig. 5d). *Phleum pratense*, *P. lanceolata* and *D. glomerata* contributed the most to Zn and Se uptake (Fig. 6). These species also had the highest biomass even though *T. repens* and *O. viciifolia* had the highest Zn and Se concentration (Table 3).

3.2. Field experiment

There was no significant interaction of sward type and year on Zn concentration and Zn uptake in the swards ($P > 0.05$), so only the main effects are presented. Across the two experimental years, Zn concentration in the six- and twelve-species swards surpassed that of perennial ryegrass monoculture sward by 25% and 26%, respectively ($P < 0.01$) (Fig. 7a). Similar to Zn concentration, Zn uptake by the six- and twelve-species swards was higher than the perennial ryegrass monoculture sward by 35% and 21% respectively ($P < 0.05$) (Fig. 7b). Zinc concentration in the second experimental year (2022) was higher than in the first year (2021) by 34% ($P < 0.01$) (Fig. 7a) whereas Zn uptake in the second year was lower than in the first year by 21% ($P < 0.01$) (Fig. 7b). With regards to Se, there was an interaction ($P < 0.05$)

between sward type and year on both sward Se concentration and sward Se uptake. The six-species and twelve-species swards had higher Se concentration and Se uptake than perennial ryegrass monoculture sward in 2022 ($P < 0.01$), but there was no significant difference among the sward types in 2021 (Fig. 8a & b).

There was no significant interaction of sward type and year on aboveground biomass. All the three sward types (i.e., perennial ryegrass, six-species and twelve-species) produced a similar amount of biomass and this was lower in the second year (2022) compared to the first year (2021) ($P < 0.01$) (Fig. 9).

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 (Fig. 10). These species also had the highest biomass, although *P. lanceolata* had the highest Zn concentration and *C. intybus* had the highest Se concentration (Table 5).

Table 5

Mean biomass as well as Zn and Se concentration of all the component species in the 6S and 12S swards in the field experiment ($n = 4$). Values with the same letter in the same column are not significantly different ($P < 0.05$). Data were either log- or square root-transformed.

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

Discussion

We showed that both Zn and Se concentration as well as uptake were higher in species-rich mixtures than monocultures, regardless of the experimental scale. Forage Zn and Se concentrations varied inconsistently among the treatments but were never lower in the mixtures. In the greenhouse experiment, Zn concentration in the mixtures did not differ from the *L. perenne* monoculture (Fig. 5a) but Se concentration was substantially increased (Fig. 5c). In the field experiment, Zn concentrations were higher

in the mixtures (Fig. 7a), but for Se, the effect of mixtures was absent in one year and positive in the other year (Fig. 8).

In the greenhouse experiment, Zn and Se uptake increased with increasing richness with the most species-rich mixture having higher Zn and Se uptake than the average of the monocultures (Fig. 2). Moreover, when we compared two multispecies mixtures with a monoculture of perennial ryegrass in the greenhouse experiment, the multispecies mixtures had marginally higher Zn uptake ($P = 0.06$) and significantly higher Se uptake ($P < 0.05$) than the perennial ryegrass monoculture (Fig. 5b and c). In the field experiment, we further demonstrated that multispecies swards consistently had a higher Zn and Se uptake than the monoculture sward of perennial ryegrass across the two experimental years (Fig. 5b & d). This is in line with the findings of Høgh-Jensen and Sørensen (2012) who showed that, species-rich mixtures have higher micronutrient uptake than grass monocultures. The average Zn uptake values of multispecies mixtures found in our study were in the range of the values found in previous studies (Lindström et al., 2014b; Høgh-Jensen and Sørensen 2012). Although Zn uptake values reported in the study of Lindström et al. (2014b) varied from one site to the other, the average Zn uptake values found in our study were comparable to the values obtained in one of their experimental sites which had a similar soil texture as that of our field experiment.

The high Zn and Se uptake in the species-rich sward was as a result of the presence of specific plant species with inherently high biomass and/or shoot Zn and Se concentration. For instance, *P. pratense*, *P. lanceolata* and *D. glomerata* which had low Zn concentration, but high biomass (Table 4) contributed the most to Zn uptake in multispecies mixtures in the greenhouse experiment (Fig. 6). Moreover, *L. perenne* and *T. pratense* species which produced high biomass (Table 5) contributed the most to sward Zn uptake by the multispecies swards in the field experiment (Fig. 8). This is in consonance with findings of Brink et al. (2001) who showed that biomass of grassland species was well correlated with Zn uptake. The study by Pederson et al. (2002) also indicated that Zn uptake is more related to biomass. Biomass plays a key role in determining the overall Zn uptake by the swards, hence, plant species that dominate the mixture are most likely to influence Zn uptake (Lindström et al., 2014b). However, McLaughlin et al. (2004) showed that both biomass and Zn concentration were responsible for high Zn uptake in grass species, particularly, bermudagrass. On the other hand, shoot Se concentration was the main determinant of Se uptake in the field experiment. We found that *C. intybus* with the highest Se concentration (Table 5) contributed the most to the sward Se uptake even though it had relatively low aboveground biomass (Fig. 10).

Table 4

Mean biomass as well as Zn and Se concentration of all the component species in the 6S1 and 12S mixtures in the greenhouse experiment (n = 4). Values with the same letter in the same column for each sward type are not significantly different ($P < 0.05$). Data were either log- or square root-transformed and 6S is the 6S1 mixture.

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

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 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). Moreover, differences in environmental factors can exert huge influence on Zn and Se concentration and uptake by the species (Darch et al., 2020) as the greenhouse is a more stable environment. The differences in developmental stage and environmental conditions between greenhouse and field and related differences in competitive relations most likely explain that the species responsible

for the positive net effect by certain mixtures, differ. The fact that we found overyielding in both experiments, suggests a robust net effect of mixtures on Zn and Se uptake.

In contrast to our hypothesis, we did not find consistent evidence for facilitation as a plausible mechanism driving the higher Zn and Se uptake in species mixtures with grasses. Partitioning the net effect of Zn uptake into complementarity and selection effect indicated that, increased Zn uptake by mixtures was mainly due to complementarity in only three grass-containing mixtures (i.e., 2S1, 3S2 and 12S) out of the four mixtures that showed significant difference between the observed and expected Zn uptake. Although, this could indicate facilitation of Zn acquisition by grasses in these mixtures (Li et al., 2014; Loreau & Hector, 2001) but there is no evidence of facilitation by grasses as a general mechanism as we found a positive net effect in a mixture with no grass (i.e., 2S5). The absence of grass species in the 2S5 mixture which still had a positive net effect makes it challenging to establish any direct evidence of facilitation by grass species. Higher complementarity effects observed in the grass-containing mixtures with positive net effects may merely connote positive interactions among the species which in turn may have resulted in higher biomass and ultimately, increased Zn uptake. With respect to Se uptake, we did not expect any complementarity effect, as none of the mixtures included a hyperaccumulator. Accordingly, only two out of eleven mixtures (i.e., 6S2 and 12S) resulted in a significant positive net effect on Se uptake, mainly caused by complementarity. Therefore, positive net effect on Zn and Se uptake are driven by factors other than the presence of grass species.

Conclusions

Our study showed that, multispecies mixtures have higher Zn and Se uptake than monoculture of perennial ryegrass across the two experimental scales. For Zn, the increase in uptake is mainly driven by aboveground biomass, whereas for Se, the Se concentration of the sward also played a role. 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. For instance, a grassland system that aims to boost Zn and Se uptake in the sward can increase the sowing proportion of *C. intybus* and *P. lanceolata* in the mixtures as these species have inherently high Zn and Se tissue concentration but have low biomass production. Given that we were unable to show whether there is evidence of facilitation by grass species in the mixtures based on our analysis of aboveground samples, we suggest that further research should be conducted on belowground interactions. This may provide more insight into root-root interaction of the component species and the potential role of root exudation on facilitation.

Declarations

Competing interests

The authors declare that they have no conflict of interests.

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Author contribution

All authors contributed to the study conception and design. Data collection was done by Omotola Obasoro, Jane Shackleton and Cornelia Grace and data analysis by Omotola Obasoro, Ron de Goede and Ellis Hoffland. The first draft of the manuscript was written by Omotola Obasoro and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

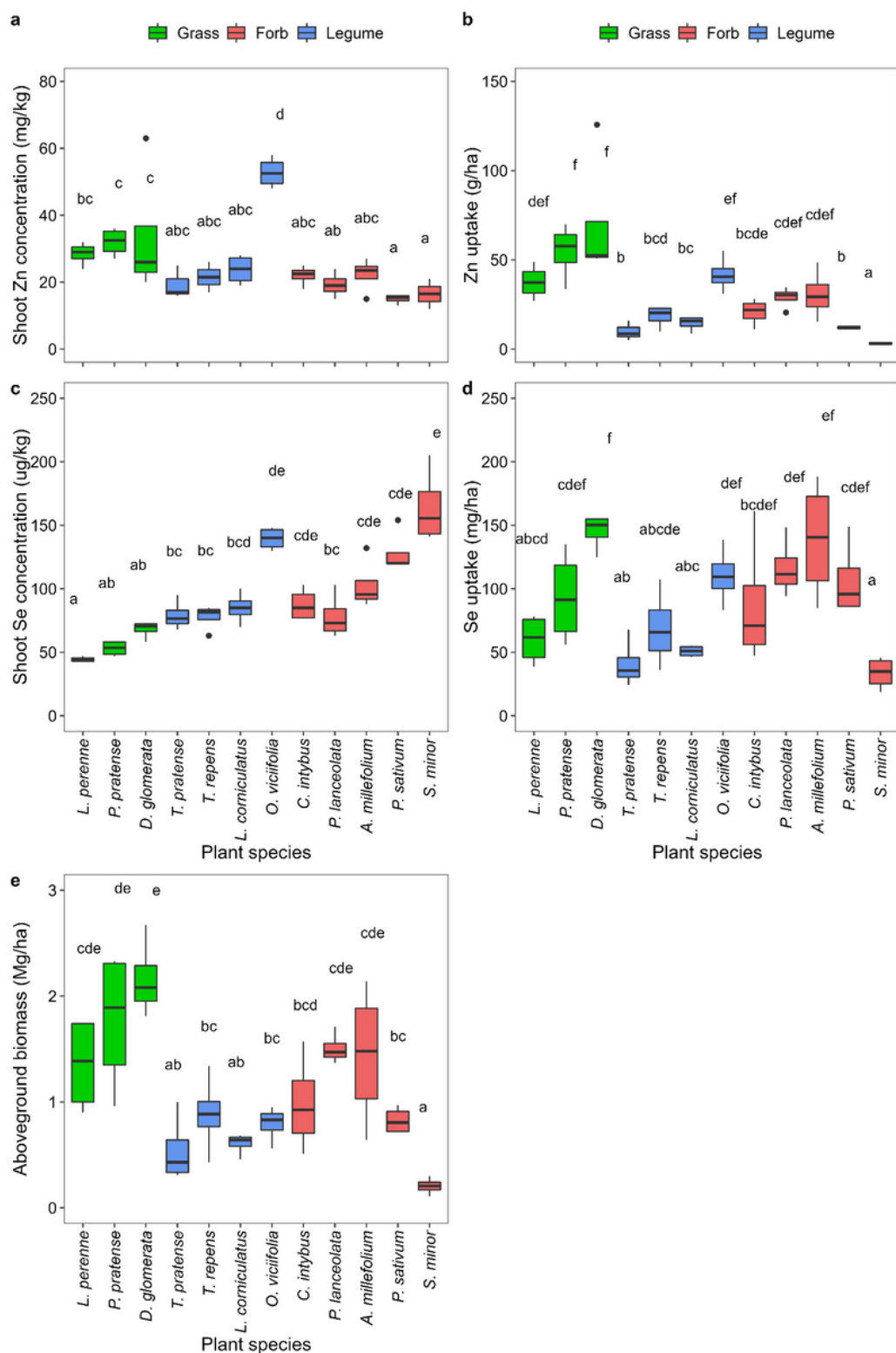


Figure 1

(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 box-plots 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)

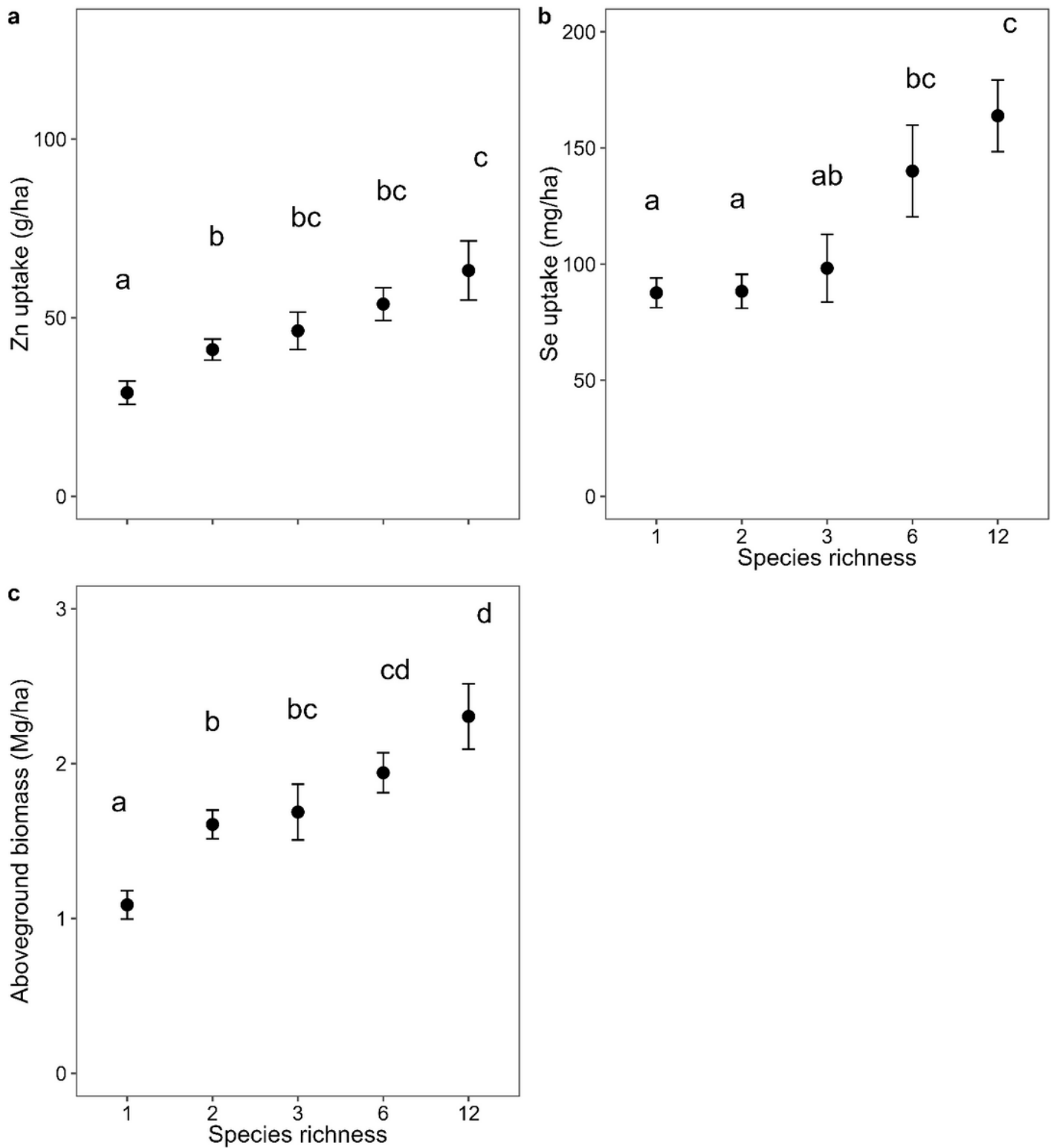


Figure 2

Effect of species richness on a) Zn uptake, b) Se uptake, and c) aboveground biomass in the greenhouse experiment. Error bars indicate SEM ($n = 4$). Points with the same letters are not significantly different (Tukey HSD, $P = 0.05$). Zn uptake values were log-transformed

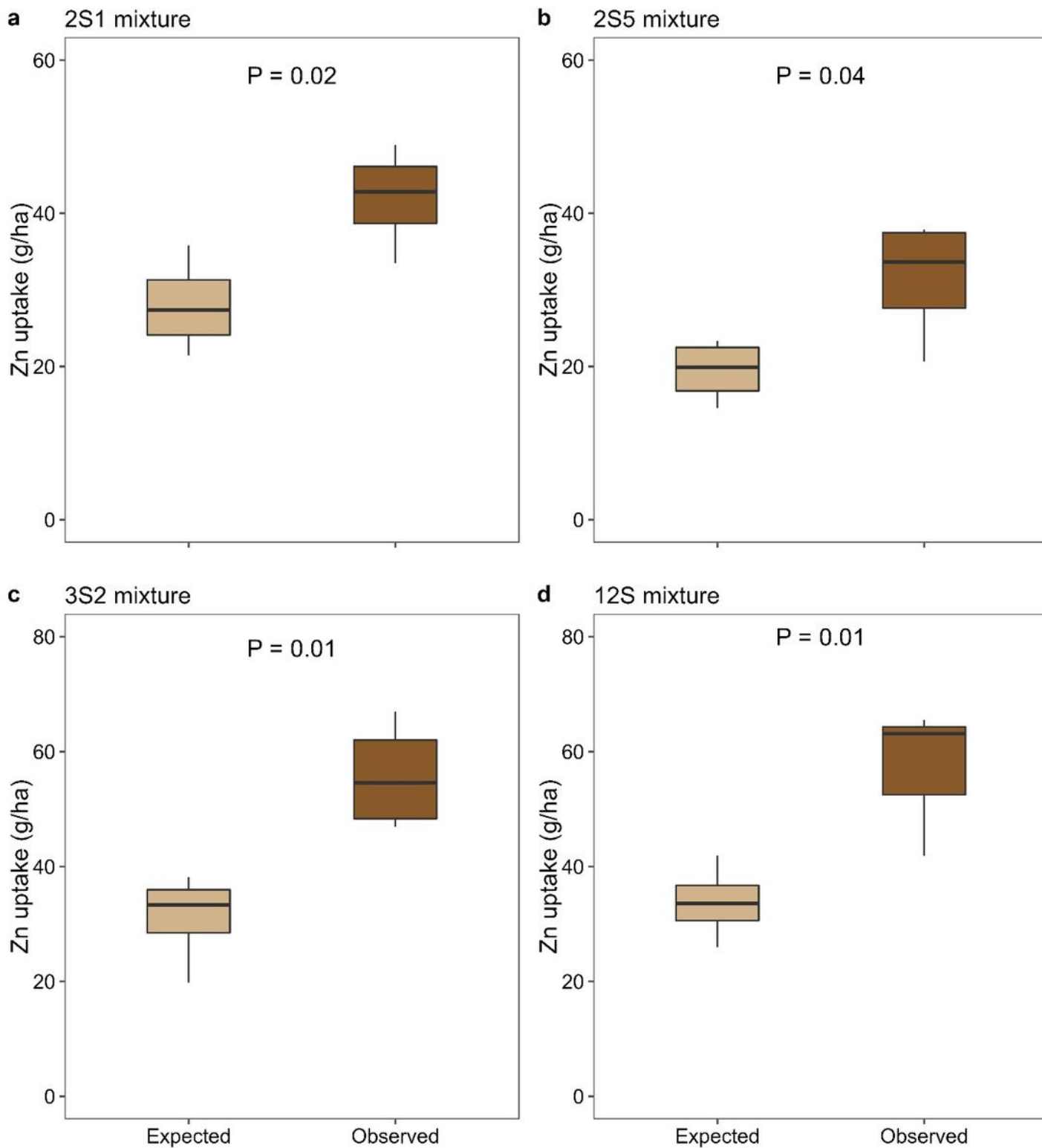


Figure 3

Net effect analysis of selected mixtures on Zn uptake in the greenhouse experiment. Zn uptake values were log-transformed

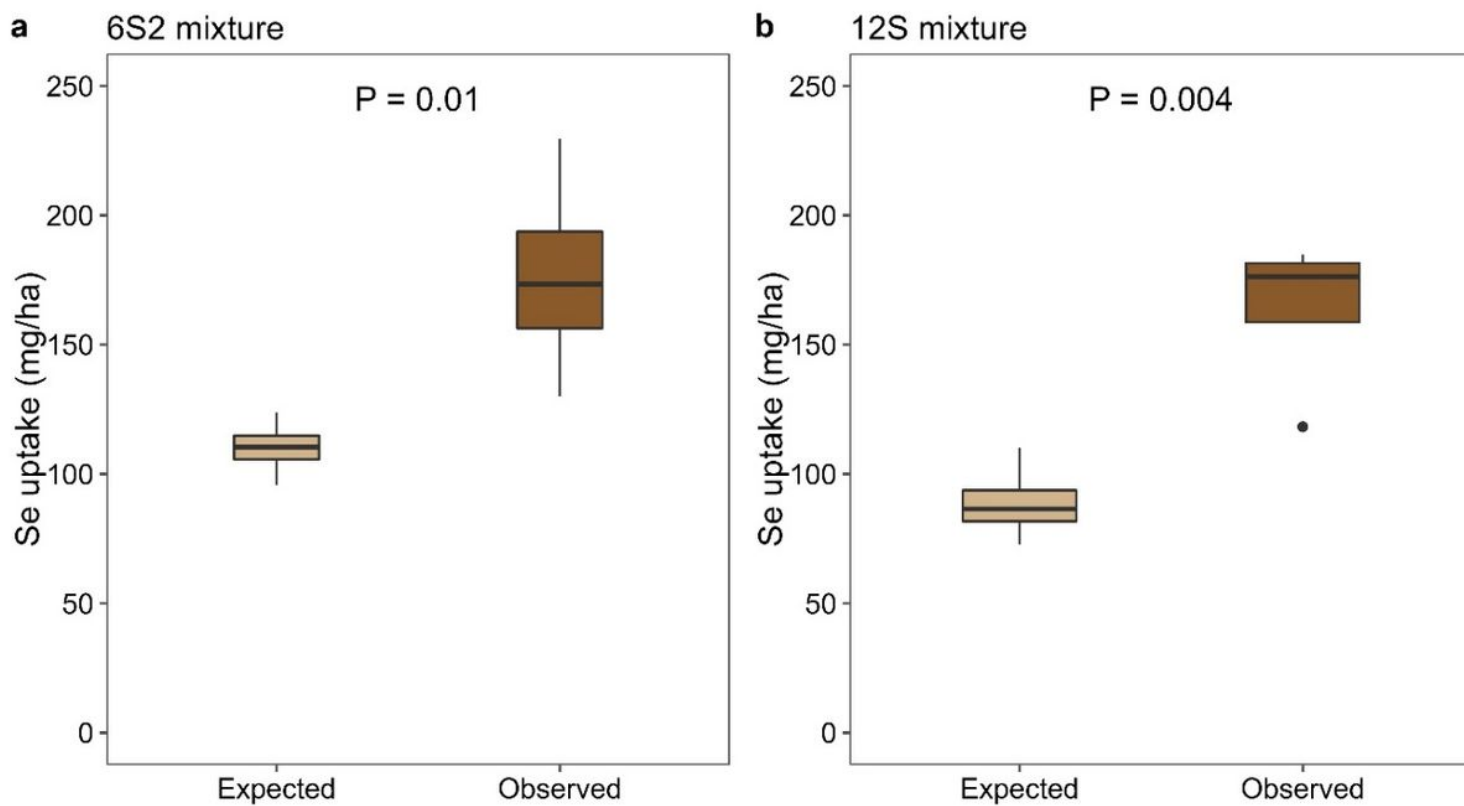


Figure 4

Net effect analysis of selected mixtures on Se uptake in the greenhouse experiment. Se uptake values were log-transformed

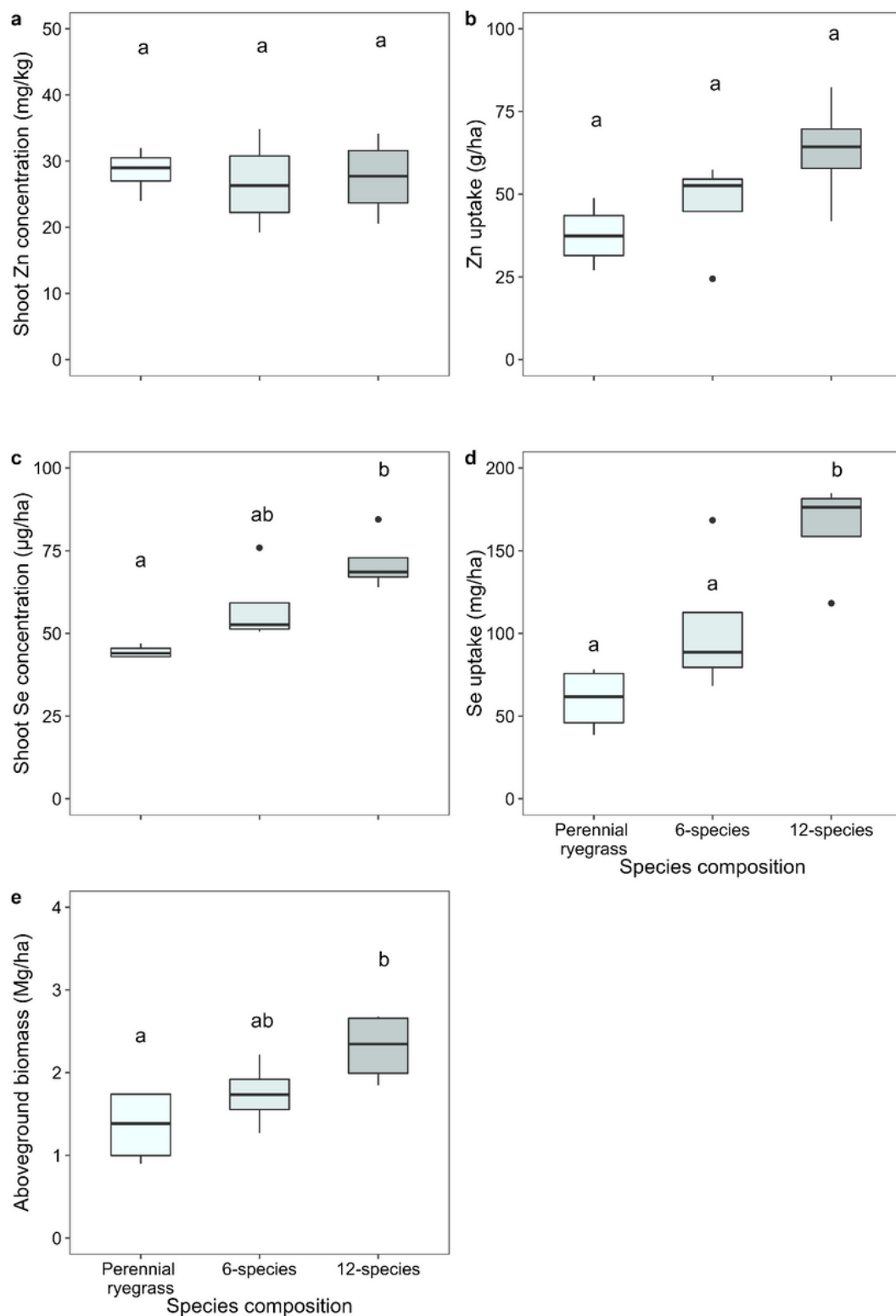


Figure 5

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

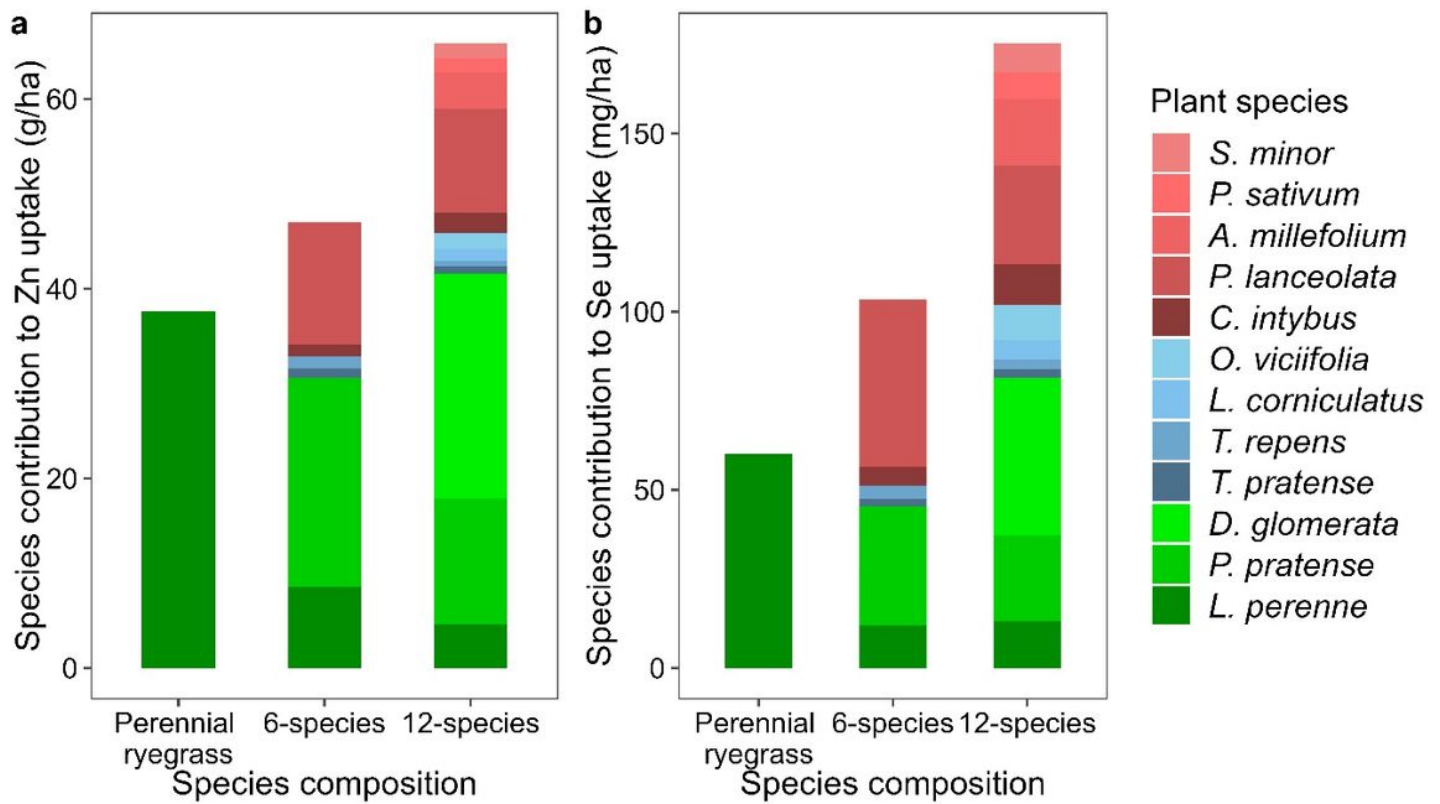


Figure 6

Contribution of each species to a) Zn uptake and b) Se uptake by the mixtures in the greenhouse experiment

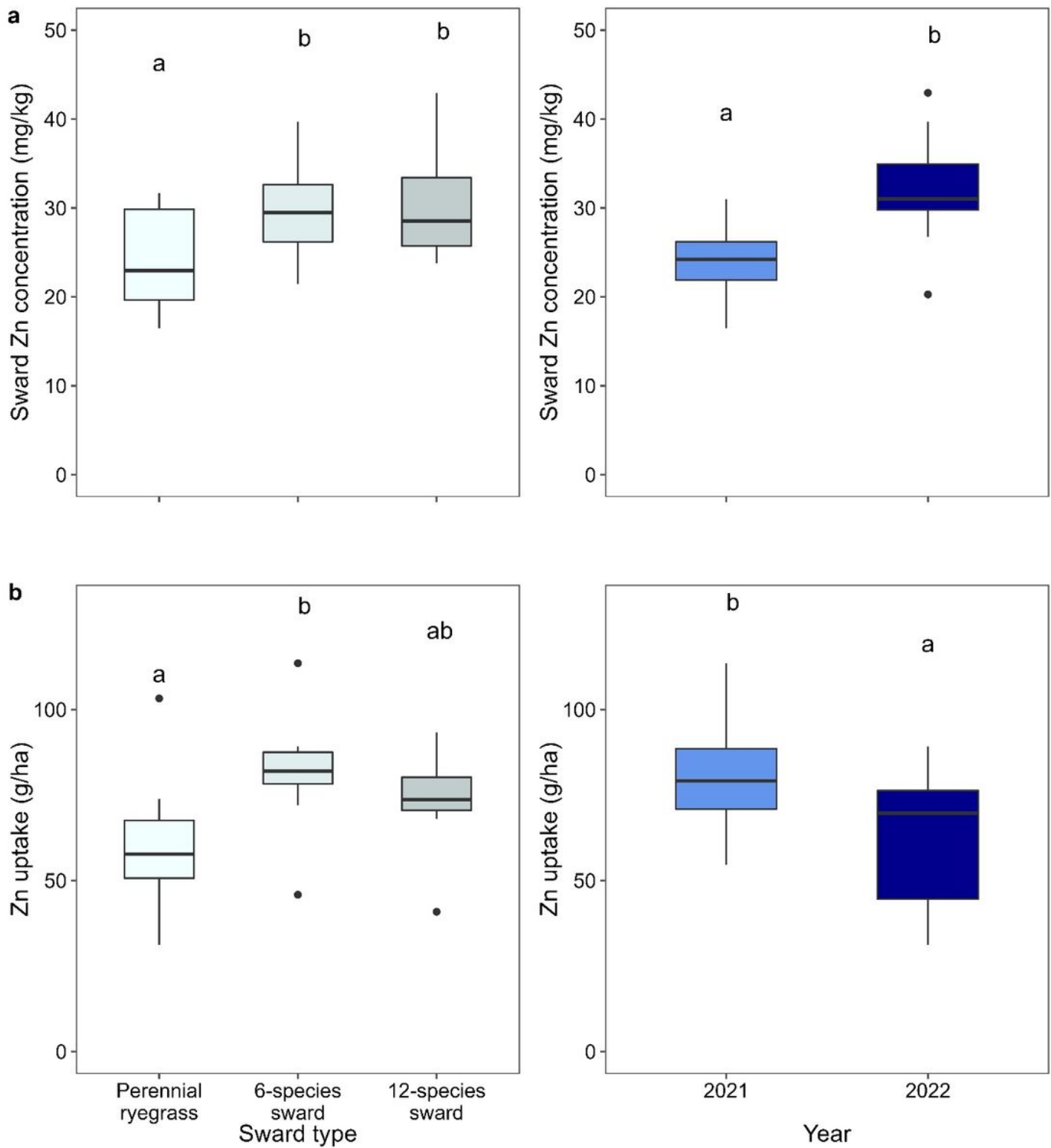


Figure 7

Effect of sward type and year on sward a) Zn concentration, b) Zn uptake in the field experiment. Boxes with the same letters are not significantly different (Tukey HSD, $n = 4$, $P = 0.05$)

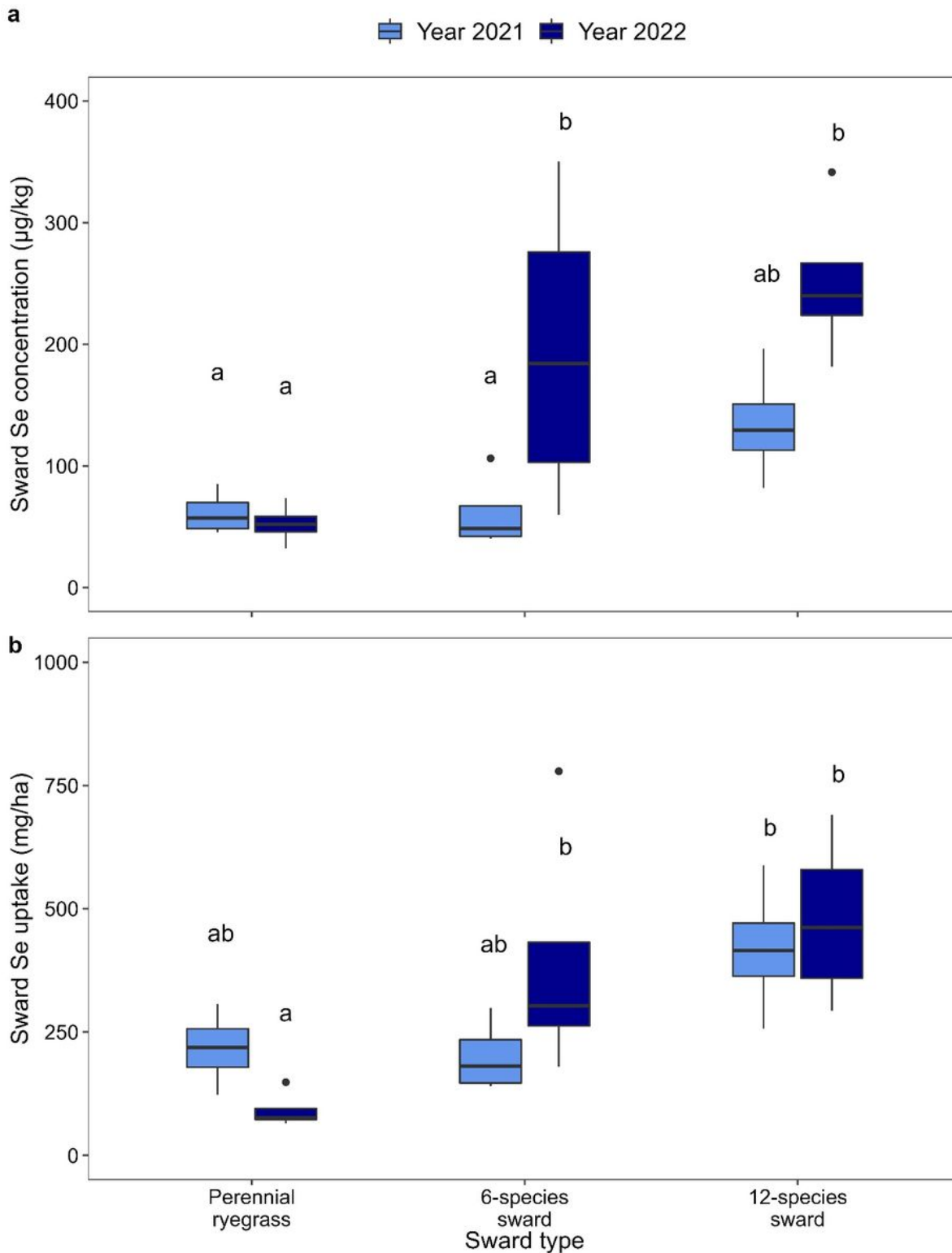


Figure 8

Effect of sward type and year on sward a) Se concentration, b) Se uptake in the field experiment. Boxes with the same letters are not significantly different (Tukey HSD, $n = 4$, $P = 0.05$). Se concentration and Se uptake values were log transformed

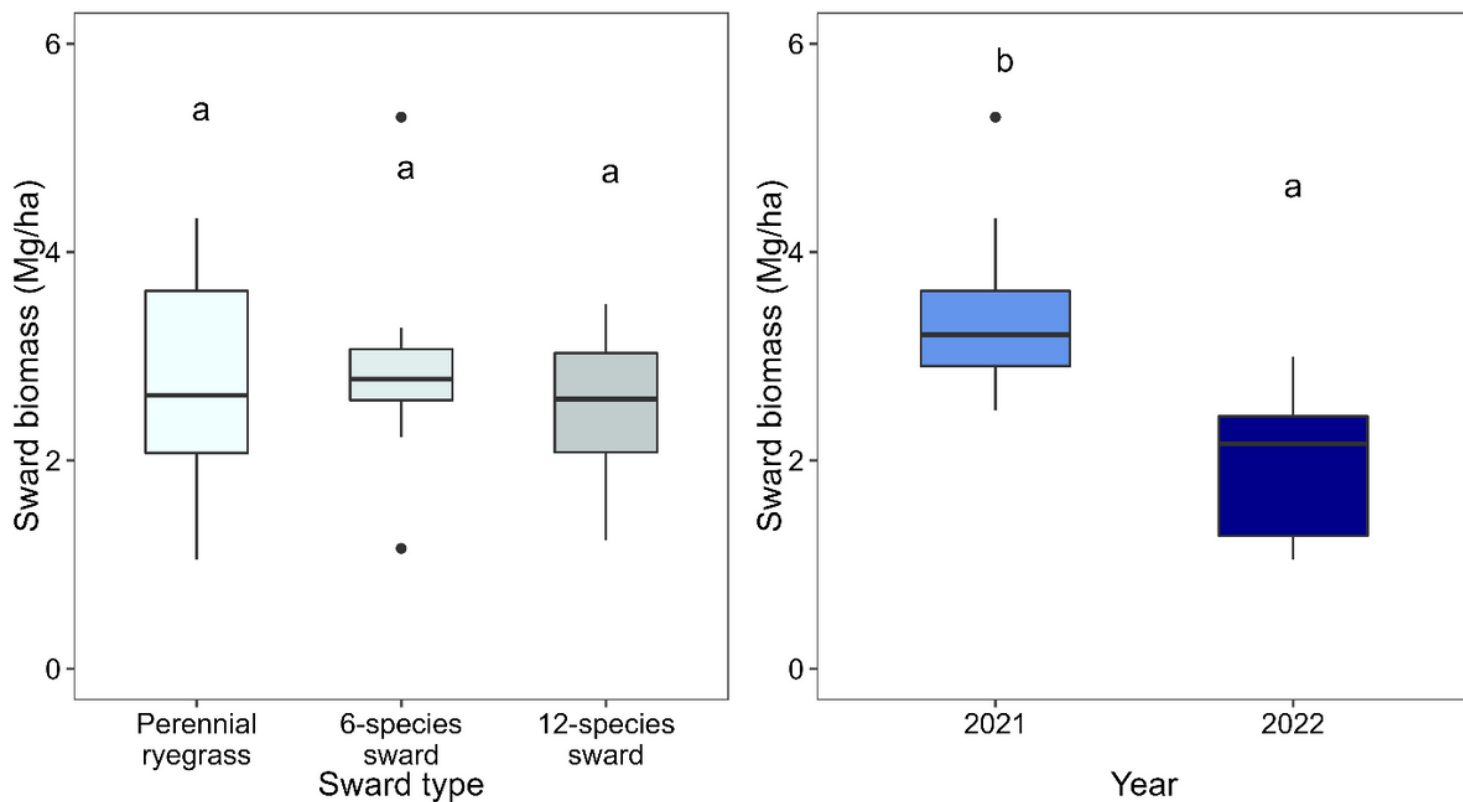


Figure 9

Effect of sward type and year on sward biomass in the field experiment. Boxes with the same letters are not significantly different (Tukey HSD, $n = 4$, $P = 0.05$)

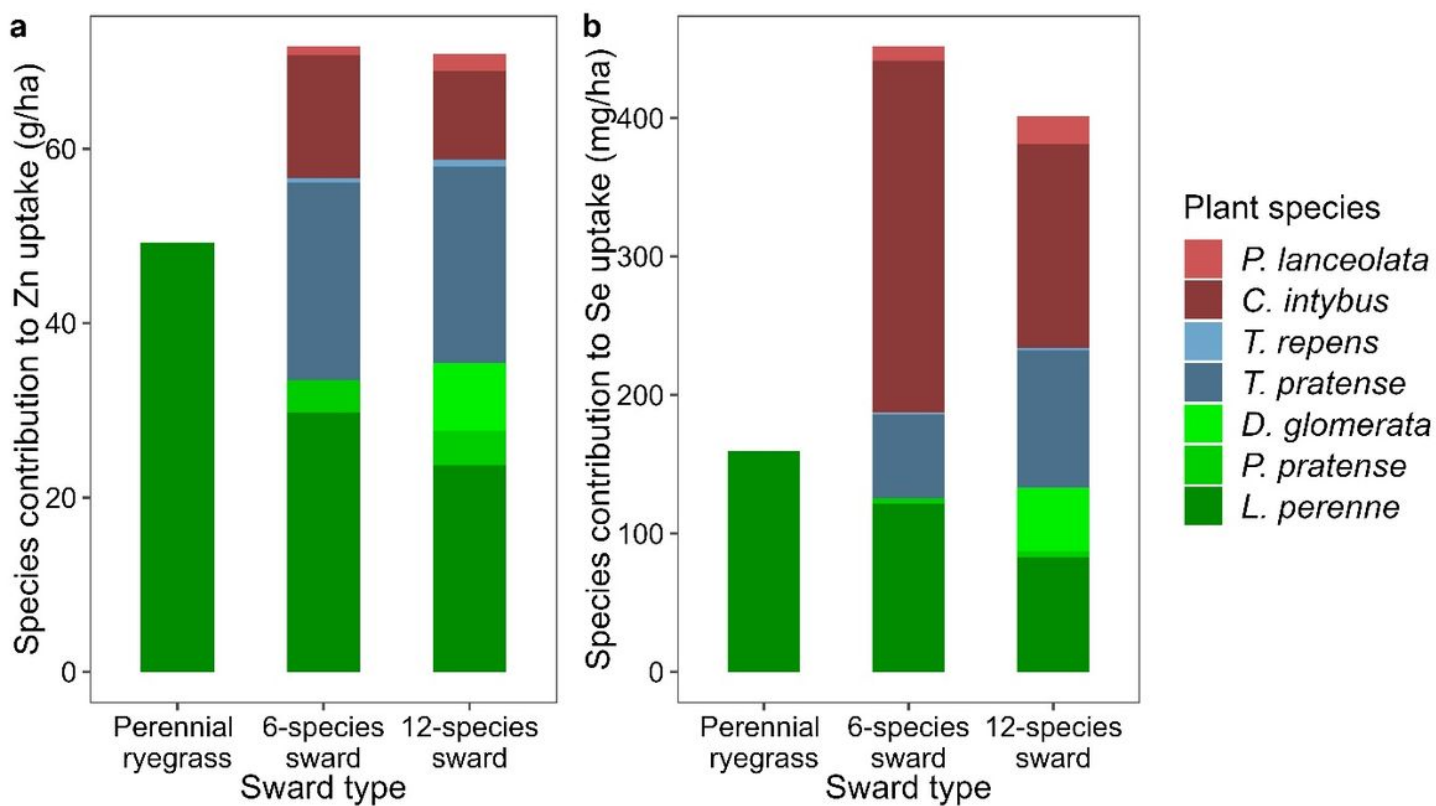


Figure 10

Contribution of each species to a) Zn uptake and b) Se uptake by the swards in the field experiment

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