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# Sown Diversity Effects on the C and N Cycle and Interactions with Fertilization

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**Abstract:** A better understanding of the role of plant composition and N cycle on agroecosystems is necessary, as these will be affected by future developments in agriculture intensification. To explore the effect of plant diversity on yield and carbon (C) and nitrogen (N) balances in forage mixtures, identifying potential co-benefits between functions. We analyzed results from a field experiment where plants of three forage species (a grass, a legume, and a non-legume forb) were cultivated in monocultures and mixtures. Three years after sward establishment, dry matter yield, together with $\delta^{15}N$, $\delta^{13}C$, and C and N content in plant and soil material were measured. In addition, we analyzed a second scenario to investigate the effect of fertigation with pig slurry ($\delta^{15}N$ = +8.4‰) on the C and N balances of forage species. Results support the hypothesis that C and N allocation is affected by plant diversity. Plant composition affected N source (% N derived from air, % N derived from soil, and % N transferred in mixtures). In addition, sown diversity increased yield and modulated C and N balances. The $\delta^{15}N$ of samples was affected by both plant composition and fertigation. These results are consistent with previous work showing strong plant composition effects on N-balances, and the potential role that legumes play in enhancing nitrogen sources (derived from the atmosphere) into forage mixture systems. This study contributes to the prediction of suitable sown plant community composition and N management for the optimum agriculture with increased productivity and at the same time reduced environmental impact.

**Keywords:** plant diversity; forage mixtures; carbon and nitrogen balance; stable isotope



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## 1. Introduction

Grassland systems cover large land areas; perform important ecosystem functions, including biomass production and nutrient cycling [1]; and are a major part of the global ter-

restrial systems, covering 40% of the Earth's terrestrial areas [2] and 69% of the agricultural land area [3].

Livestock production in agroecosystems have favored low diversity of high producing forage species and have been typically dominated by monocultures; nevertheless, diversity in plants in natural systems is common [4]. Despite the benefits of sown plant diversity, livestock production systems are reluctant to value polycropping to the full extent of its potential, as made evident by the persistent simplification of agroecological systems to maximize yields of crops and pastures [5,6].

Sustainable intensification of agriculture aims to raise productivity while at the same time reduce its environmental impacts, following integrated soil–plant system management [7–12]. The balance between C and N assimilation and emission may be ecosystem-dependent and is also highly dependent on N availability for plants [13–15]. In the face of the prevailing N limitation, rising costs of inorganic N fertilizers, and deleterious side effects of excessive N application [16,17], increased sustainability and improved N self-sufficiency can be gained through homegrown $N_2$-fixing crops [18]. Plant diversity may be crucial in the management of the agroecosystems [19,20], because plant diversity influences N input, uptake, recycling [21,22], natural N fertility [23–27], and the C/N ratio [19,28,29].

Enhanced sown plant diversity in agroecosystems, including legumes but also other plant functional groups, such as grasses and non-legume forbs, has received great attention in recent years because of the potential advantage it offers to improve crop resources [14,23,24]. Further, the inclusion of legumes provides a N source from symbiosis that is regulated by plant demand [28]. In fact, Malhi et al. [21] found that without N application, dry matter yield (DMY) was lowest in monoculture of brome grass and increased when this grass was grown in combination with legumes [23,29].

Legumes-based mixtures can provide major contributions to the challenges of agricultural systems being productive yet environmentally friendly [30]. Legume-based mixtures (systems) offer many benefits: (a) symbiotic $N_2$ fixation by legumes, which are able to utilize atmospheric $N_2$ for their requirements and thereby produce more protein with less N input [18]; (b) minor N losses by leaching and emissions [14,30]; and (c) increased soil inorganic N availability [31]. Legumes may influence the availability and isotopic signature of N in the surrounding soil modifying $\delta^{15}N$ signals in adjacent non-$N_2$-fixing plants by N transfer [32,33]. The majority of N (up to 71%) is transferred via rhizodeposition (decomposition of nodules and root tissue and exudation of soluble N compounds by roots) [19,34].

The stable isotope N composition in soils and plants is a powerful tool to assess ecosystem N dynamics [34] and can provide clues and insights into physiological [35] and ecological [36,37] processes. In particular, the natural abundance of $^{15}N$ in different compartments of the ecosystem is considered as an integrator of the applied management practices on N cycle processes [38]. Sown diversity is expected to interact with N uptake, modifying N dynamics and therefore N isotope composition ($\delta^{15}N$), because it (a) reflects the input and output $\delta^{15}N$ signatures [39], (b) modifies N plant composition [11,18,40,41], (c) modifies sink/source ratios of N [42,43], (d) alters N dynamic dependencies of spatial and temporal complementarities in resource use [44], and (e) may stimulate N uptake [11]. As an example, the presence of legumes in a sward may influence soil N availability for plants changing soil and plant isotopic signatures in adjacent non-N2-fixing plants (i.e., producing lower $\delta^{15}N$ signals due to $N_2$ fixation) [32,33]. However, despite its potential to study ecosystem N dynamics, $\delta^{15}N$ can also be obscured by a variety of factors such as mycorrhizal status and type, nodulation, and intra-plant isotope partitioning [36]. As isotope-discriminating processes depend on the availability of N, the type of applied fertilizer is expected to alter $\delta^{15}N$ values of plants and soils, especially as a result of long-term

land use patterns [45]. Therefore, the variability in $\delta^{15}N$ values among different $N_2$-fixing and non-$N_2$-fixing plant species within an ecosystem remains unclear [21,24,46,47]. Plant diversity may also affect the C balance of terrestrial ecosystems [48], modifying biomass production and soil organic matter (SOM) storage [49]. The carbon isotope composition ($\delta^{13}C$) may show differences between phenology [50], water or nutrient regimens [51,52], and shoot or root traits [53], among others.

The main objectives of the study presented were (a) to quantify the effect of the biodiversity–function relationship between legumes, forbs, and grass on C and N dynamics of a forage crop system and (b) to determine the effect of fertigation on this relationship. To address these objectives, we determined the isotopic signals in several compartments (i.e., soil, shoot, root) in a diversity–function experiment with different sown plant diversity (i.e., three monocultures versus mixtures) [14] according to the Agrodiversity design [13,40,54].

## 2. Materials and Methods

### *2.1. Site Description*

In spring 2008, we established a BEF (biodiversity–ecosystem functioning) experiment in which we manipulated plant species composition and relative proportions to study the effects of plant identity and evenness on several ecosystem functions, including forage production and quality; for more details, see [14,55].

The field experiment was established on a semi-arid irrigated agricultural area in Castellnoud'Ossó, Catalonia, north-eastern Iberian Peninsula (41°46′ N, 1°8′ E, 353 m.a.s.l.), with a mean annual precipitation of 429 mm and a mean annual temperature of 13.9 °C (data from the meteorological station located at Castellnou d'Ossó), in a soil with 25–30% carbonates, silt loam texture, high water retention capacity (Xerofluvent), and 4.5% organic matter content (top 15 cm of soil). Full information about the site is given in [14], where further details on the climate and soil are provided.

#### Rationale

The seed mass of the sown species was modified in different plant communities to obtain a gradient in their relative proportions, resulting in a range of evenness in the communities. The plant communities comprised three monocultures and a mixture (centroid, consisting of a sward mixture with an equally sown proportion of the three species) including a grass (*Festuca arundinacea*), a legume (*Medicago sativa*), and a non-legume forb (*Cichorium intybus*, hereafter referred to as forb); these species are important in forage mixtures and pasture crops in agroecosystems. The main crops grown in the area are cereal and forage monocultures, with *Festuca arundinacea* and *Medicago sativa* being the most important forages. *Medicago sativa* is a basophilic and deep-rooted legume; it is cultivated globally in countries with a temperate climate and is present throughout Spain; it resists drought well, can live on a wide variety of soils, is one of the most productive legumes in the world, and provides abundant forage [56]. *Festuca arundinacea* is a perennial grass. It is cultivated in all countries with a temperate climate. It is one of the most valuable pasture grasses in areas with a temperate or Mediterranean climate [57]. *Cichorium intybus* is a perennial herb, that has a long, thick tap root. This crop produces large amounts of high-quality forage during warm seasons. It is used in different parts of the world as animal feed [58]. The plant communities were arranged according to a simplex design [13,54]. These four combinations were sown in plots of 12 × 12 $m^2$. Seeding rates considered appropriate in the region for each species in monoculture are 40 kg $ha^{-1}$ (legume and the grass) and 25 kg $ha^{-1}$ (forb). The fertilization treatment started in 2011, when the detailed assessment of N and C cycles took place (see Section 2.4). The measurements presented

in this study were taken in 2011, during the fourth year after the establishment of the plant swards.

### *2.2. Irrigation and Fertilization*

Water and fertilizer were applied through an automatized sprinkler fertigation system. The water supplied during this study was obtained from the local river. Fertilizer was mixed with the irrigation water at a 2% volumetric ratio by injection between the first and second isotopic sampling. The organic fertilizer was filtered pig slurry that contained 2.2 kg of total N per $m^3$, with 48% and 52% corresponding to the organic and inorganic (ammonium) forms, respectively. Isotopic compositions of the pig slurry were $\delta^{13}C = -19.9 \pm 2.65‰$ and $\delta^{15}N = 8.3 \pm 0.1‰$. Fertilization started in June 2011, within the second regrowth (Table S1). A total of 23.3 kg N $ha^{-1}$ was applied in the second regrowth period, corresponding to an annual application of c. 100–150 kg $ha^{-1}$. In early spring, up to the first harvest, all plots received 50 mm irrigation, and during the second following regrowth, all plots were irrigated with c. 330 mm (Table S1). Nitrogen of the irrigation water resulted in an extra N input ($NO_3^-$). Full information about fertigation is given in [14].

### *2.3. Plant Yield Determination and Soil Sampling*

At each harvest, total yield (plant dry matter) was determined by harvesting 9.6 $m^2$ per plot and weighing the fresh plant material in situ. A subsample of the collected material was oven-dried at 60 °C and weighed to estimate individual species yield. In mixture plots, total yield was previously separated into species. Moisture content was determined through the difference between fresh and oven-dried weight.

Simultaneously with the sampling of plant individuals, six soil samples were extracted from the same plots, at the sampling times (May and June), before and after fertigation, using probes (3 cm diameter and 10 cm depth), and mixed into composite samples for mass and isotopic determinations (see Section 2.4).

A subsample of the collected material was then dried at 60 °C in an oven for 48 h. The first soil sampling took place some days after the 1st harvest and prior to the first fertigation. The second sampling took place following the 2nd harvest, some days after fertigation (Table S1).

### *2.4. Isotopic Composition of C and N*

For the determination of C and N content and isotopic signals of different ecosystem compartments, shoot and root plant biomass, soil, and emitted gas were collected in selected plots at two moments within the first 2011 regrowth, representing two different scenarios: before and after fertigation. Ten plots were sampled, and two subsamples were analyzed at each plot for plant material. The first sampling (May) took place following the first 2011 harvest (just after restarting irrigation) and before slurry application, and the second sampling (June) was carried out after slurry application. Table S1 summarizes the timing of relevant management and sampling activities during the 2011 growing season.

Pig slurry was considered as a $\delta^{15}N$ labelling signal and then used as a tracer to analyze dynamics of N and C and fluxes due to compositional/evenness changes in soil–plant–atmosphere of the forage crop system. Three samples of pig slurry were measured as reference ($\delta^{13}C = -19.9 \pm 2.65‰$; $\delta^{15}N = 8.3 \pm 0.1$). Values of $\delta^{13}C/^{12}C$, C content (mg/mg), $\delta^{15}N/^{14}N$, N content (mg/mg), and C/N in waterhole, pig slurry, rainwater, and river water are summarized in Table S2.

#### 2.4.1. Plant and Soil Sampling

Three plant individuals of the three sown species, including both shoot and root systems, were sampled in monocultures and in the centroid mixture (independently for

each species). The same plots were sampled twice, in May and June, representing the two described scenarios: before and after slurry application. Simultaneously with the sampling of plant individuals, soil samples were extracted from the same plots, at both moments (May and June), using probes (3 cm diameter and 10 cm depth), and mixed into composite samples for mass and isotopic determinations. Ten plots were sampled, and two subsamples were analyzed at each plot for plant material.

Soil, shoot, and root samples were dried in an oven at 60 °C for 48 h, grounded to a fine powder, and weighed in tin capsules. Carbon and nitrogen isotope composition and content were then determined using an Elemental Analyzer Flash 112 (Carbo Erba, Milan, Italy) coupled to an isotope ratio mass spectrometer IRMS Delta C Conflo III Interface (Termo Finnigan, Bremen, Germany) at the Scientific Technical Services of the University of Barcelona, Spain.

#### 2.4.2. Measurement of Greenhouse Gas C and N Concentration and C Isotope Signatures

In May and June 2011, we took gas samples for N and C mass and isotope determination of carbon dioxide ($CO_2$) in selected plant community types: the three monocultures and the mixture. The first fertilizer application took place just before the second gas sampling (June).

Within each month/event, we sampled over a 1–2 day period, with 2–3 repeated measurements per sampling period (once or twice per day) between 9:00 and 17:00 h. Consecutive measurements took place after each fertigation event to capture expected maximum gas emissions, starting from 1 to 12 h after slurry application (Table S1). The gas exchange measurement system consisted of opaque static chambers connected to a photoacoustic field gas monitor (INNOVA 1412, Luma Sense Technologies, Ballerup, Denmark). C emission of $CO_2$ and $CH_4$ was measured with a photoacoustic field gas monitor. PVC chambers (height: 60 cm, to allow for measuring during the vegetation growth period; internal diameter: 25 cm) were placed above the soil and vegetation and fitted with a rubber joint on top of PVC rings (height: 7 cm; diameter: 25 cm). The rings were inserted into the ground (3–4 cm deep) several days before the measurements to ensure reasonable sealing of the system and to limit soil disturbance during measurement. The chambers were connected to the gas monitoring equipment with Teflon tubing (2 mm internal diameter). To improve spatial resolution, chambers were connected in pairs in each sampled plot using Teflon tubing and three-way valves (surface sampled area = 0.098 $m^2$). Gas exchange measurements are presented in [14]. The recording duration for gas exchange measurements was 20–40 min with the chambers closed. A T-connexion allowed for taking a gas sample using gas syringes of 50 mL (SGE International Pty Ltd., Australia), which was transferred to 10 mL vacutainers for gas mass and isotopic analysis.

Regarding respired $\delta^{13}CO_2$ (‰), air samples were analyzed using gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) according to [59]. Six samples of the $\delta^{13}C$ of ambient air (ca. −12.5‰) were taken as reference point.

#### 2.4.3. Inputs Description

Three liquid samples (from precipitation, irrigation water, and pig slurry fertilizer) were collected and analyzed to determine the amount of C and N, and $\delta^{13}C$ and $\delta^{15}N$ in our samples. The liquid samples were pH-stabilized (water from precipitation and irrigation water to pH = 4, and pig slurry to pH = 5) to avoid N evaporation. Then, samples were evaporated and weighed in capsules. Carbon and nitrogen isotope composition and content were determined using an EA-IRMS (described Section 2.4.1).

2.4.4. Isotopic Signals Calculation

All the GC-MS, GC-C-IRMS, and EA/IRMS analyses were performed at the Scientific Technical Services of the University of Barcelona.

Stable isotope composition was expressed according to the following equation:

$$\delta X = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \quad (1)$$

where $\delta X$ represents either $\delta^{13}C$ and $\delta^{15}N$ and $R$ denote the abundance of the heavy to the light isotope ratio of samples and reference material.

Results of carbon isotope ratio analyses were reported in parts per mil (‰) as $\delta^{13}C$ and refer to the international standard V-PDB (Vienna Pee Dee Belemnite). Nitrogen results were also expressed in δ notation ($\delta^{15}N$) using the international secondary standards of known $^{15}N/^{14}N$ ratios (IAEA $N_1$ and IAEA $N_2$ ammonium sulphate and IAEA $NO_3$ potassium nitrate) with reference to $N_2$ in air [60].

2.4.5. Calculation of N Balances and N Isotope Balances

To assess the relative importance of the nitrogen source (symbiotic and non-symbiotic sources) for the different compartments, we used the approach of isotope labelling [61] using the natural differences in isotope enrichment of the N sources. The changes that occurred in nitrogen sources with pig slurry fertigation application were also studied. Fertigation with pig slurry (2% volumetric ratio, with a $\delta^{15}N = 8.3 \pm 0.1$‰) was considered as a $\delta^{15}N$ labelling of the studied systems and then used as a tool to assess possible changes in processes involved in the C and N exchanges (within a second scenario, after fertigation).

To calculate the N balance we used the "$\delta^{15}N$ natural abundance method" for estimating $N_2$ fixation, by which the $\delta^{15}N$ in a legume that completely depends on atmospheric $N_2$ should have $\delta^{15}N$ in shoots very close to that of its N source, that is, atmospheric $N_2$ (0‰); that a plant totally dependent on soil N should have $\delta^{15}N$ close to that of soil N; and that a plant able to utilize both soil and atmospheric $N_2$ should have a $\delta^{15}N$ level that reflects the relative contributions of these two N sources. But also, if the plant received N transferred from other plants, that plant should have a $\delta^{15}N$ level that reflects this third N source.

Nitrogen from symbiotic sources (Nsym) comprises legume N derived from symbiotic N2 fixation directly from the atmosphere (Nsymfix) and grass N derived from apparent transfer (Nsymtrans) of Nsymfix, as follows:

$$\text{Nsym} = \text{Nsymfix} + \text{Nsymtrans} \quad (2)$$

N from non-symbiotic sources (Nnonsym) correspond to the total N minus Nsym and comprises N derived from unlabeled soil organic matter (Nsoil) and N from mineral fertilizer (Nfert), as follows:

$$\text{Nnonsym} = \text{Ntot} - \text{Nsym} = \text{Nsoil} + \text{Nfert} \quad (3)$$

The percentages of N in the legumes derived from direct symbiotic $N_2$ fixation (%Nsymfix), from apparent N transfer from legumes to grasses (%Nsymtrans), and N derived from fertilizer (%Nfert) were calculated from isotopic dilution ($^{15}N$) in plant samples harvested from monocultures.

$$\%\text{Ndfa} = \frac{(\delta^{15}\text{N reference} - \delta^{15}\text{N}_2 \text{ fixing legume})}{(\delta^{15}\text{N reference} - \delta^{15}\text{N of N}_2)} 100 \quad (4)$$

%Nsymfix was calculated following [61], assuming no direct N transfer from legumes to grasses or forbs during the measurement period:

$$\%\text{Nsymfix} = (1 - \frac{\delta^{15}\text{N legume}}{\delta^{15}\text{N legume reference}})100 \quad (5)$$

where $\delta^{15}$N is the atom percent excess $^{15}$N relative to atmospheric N. The subscript "legume" represents *Medicago sativa* in the mixture, and the subscript "legume reference" represents monoculture of *Medicago sativa* without N transference with other species that served as a reference plant. With the legume reference, Nsymfix quantifies symbiosis-N within the legume directly fixed from the atmosphere. However, it does not include symbiotically fixed N that would have been released to the soil and recovered again by the legume during the experiment. The resulting underestimation of Nsym was small in our study due to low proportion of Nsymtrans in monoculture as evident from the legume fraction and due to low levels of N uptake from the soil N pool by the legumes.

Apparent N transfer from legumes to grasses and forbs were calculated as follows [52]:

$$\text{Ntransfer Grass} = (1 - \frac{\delta^{15}\text{Ngrass mix}}{\delta^{15}\text{Ngrass mono}})100 \quad (6)$$

$$\text{Ntransfer Forb} = (1 - \frac{\delta^{15}\text{Nforb mix}}{\delta^{15}\text{Nforb mono}})100 \quad (7)$$

where $\delta^{15}$N is like in Equation (5), and where the subscripts "grass mix" and "forb mix" represent the grasses and forbs grown in mixture and "grass mono" and "forb mono" the grasses and forbs grown in monocultures.

The grasses grown in monocultures served as reference for grasses grown in mixtures with legumes and forbs, and the forbs grown in monocultures served as reference for forbs grown in mixtures with legumes and grasses [61]. Since a possible different mineralization rate of (unlabeled) soil organic matter under the mixture as compared to the grass monoculture (e.g., priming effect) could result in an overestimation of Nsymtrans, we will refer to the values derived from Equations (6) and (7) as "apparent N transfer".

### *2.5. Data Analysis*

Analyses of variance (ANOVAs) (Table S3) were used to test possible effects of the composition effects on the N and C dynamics. Four factors were taken into account: compartment (gas, plant shoot and root, and soil), sown species (grass, legume, forb) in monoculture vs. 3-species mixture, and scenario (pre-fertilized and post-fertilized). The statistical analysis was conducted with the SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA). The means ± standard errors (SE) were calculated for each variable. When a particular test was significant ($p < 0.05$), we compared the means using a Duncan multiple comparison test or LSD multiple comparison test.

## 3. Results

### *3.1. Carbon Dynamics*

#### 3.1.1. $\delta^{13}$C in Plant Material, Soil Organic Matter, and Respired $CO_2$ (‰)

Regarding plant shoot and root compartments, higher discrimination against $^{13}$C in shoot than in root ($p = 0.001$) was observed. No significant differences were obtained between monoculture and mixture values, either in plant shoot $\delta^{13}$C nor in root for any of the tested species (for the legume, $p = 0.7$; grass, $p = 0.7$; and forb, $p = 0.3$ components, respectively—from now on L, G, F).

Among species, the forb (i.e., −29.41 ± 0.8‰) showed the highest discrimination against $^{13}C$, while the grass (i.e., −26.06 ± 0.9‰) was the species that discriminated less.

Regarding scenarios, plant $\delta^{13}C$ showed differences between scenarios, with a higher discrimination against $^{13}C$ in scenario 2 (after fertigation) than in scenario 1 (before fertigation). That discrimination was lower in shoot than in root organs and higher in scenario 2 than scenario 1 (Figure 1). This effect was particularly important for the grass component in mixture and emerged only for the shoot compartment ($p < 0.000$).

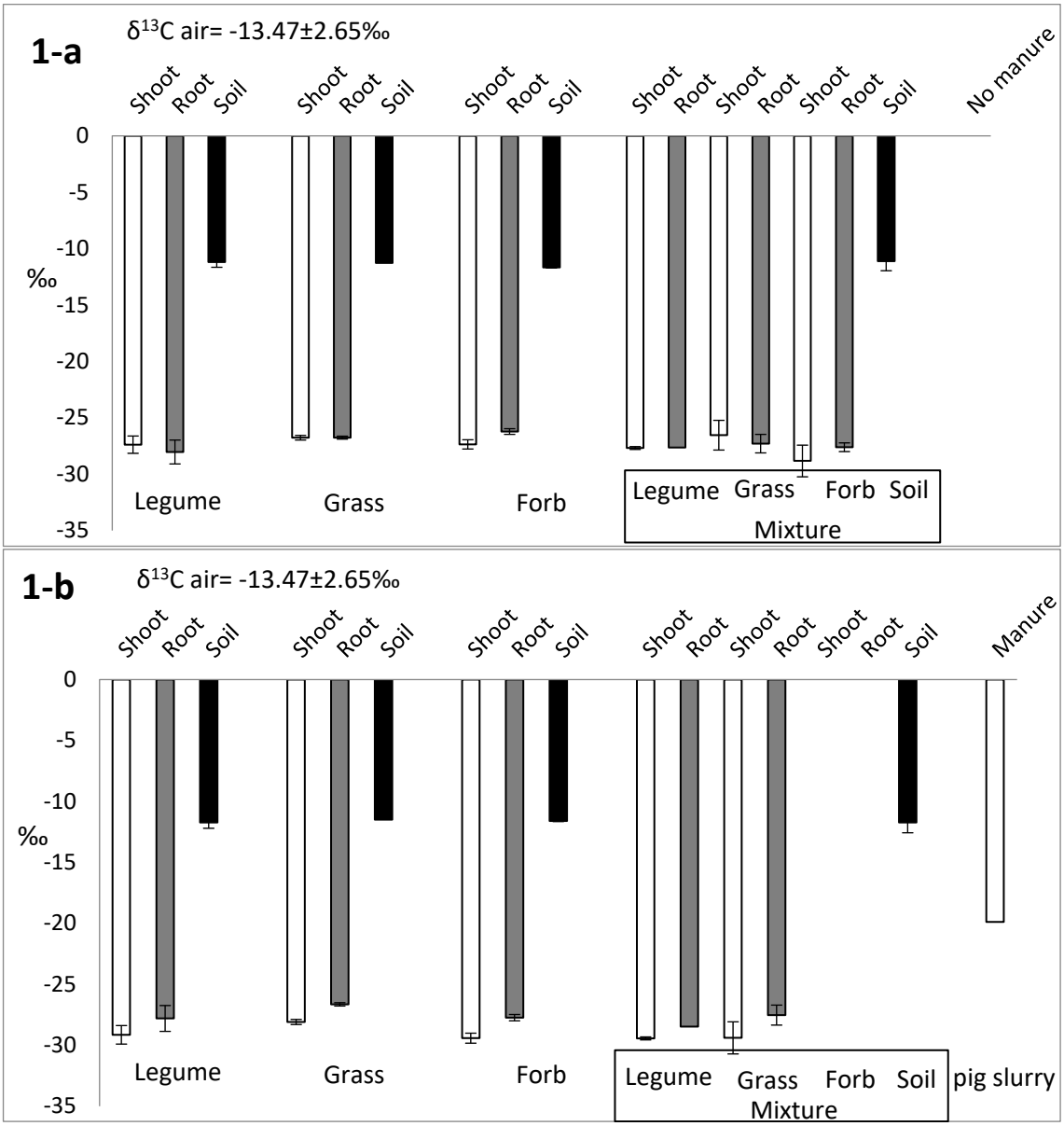


**Figure 1.** $\delta^{13}C$ (‰): shoot (open bars), root (grey bars), total organic matter (TOM), and total organic matter on soil (TOS) (black bars) in two different scenarios, (1-**a**) SC1 (before fertirrigation) and (1-**b**) SC2 (after fertirrigation), in the three monocultures (grass, legume, and forb—*Medicago sativa*, *Festuca arundinacea*, and *Cichorium intybus*, respectively) and in a mixture with the values separated by the three species (centroid). No data are available for the forb in mixture and scenario 2. The number of averaged samples is $n = 3$. Analyses of the variance of the composition effects showed that significant differences were obtained in $\delta^{13}C$ between species ($p < 0.001$) (monoculture vs. mixture), scenarios ($p < 0.01$), and compartments (shoot, root, and soil) ($p < 0.05$) Table S3.

Figure 2 shows that higher $CO_2$ emissions (corresponding to respired $CO_2$) were obtained in the mixture treatment than what would be expected according to the monoculture exchange values. $\delta^{13}CO_2$_respired (‰) was affected by fertigation in all the sampled plots (monocultures and mixture). The $\delta^{13}CO_2$ respired values were lower in legume monoculture and mixture plots than in the forb and grass monoculture plots, and lower than the pig slurry $\delta^{13}C$ signal ($-19.9 \pm 2.65$‰). Regarding scenarios, plants showed a decrease in $\delta^{13}CO_2$_respired (‰) in scenario 2 (post-fertilized) compared to those of scenario 1 (pre-fertilized) plots ($p < 0.000$).

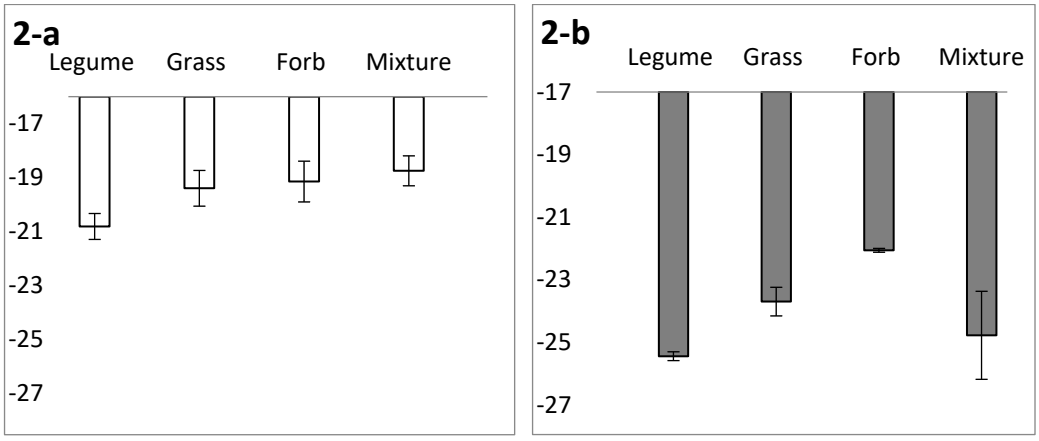


**Figure 2.** $\delta^{13}CO_2$ respired (‰) in the three species in monoculture (legume (*Medicago sativa*), grass (*Festuca arundinacea*), and forb (*Cichorium intybus*)) and in mixture with the three species (mixture or centroid) in two different scenarios: (2-**a**) before fertigation, scenario 1 (SC1) and (2-**b**) after fertigation, scenario 2 (SC2). The number of averaged samples is $n = 3$. Analyses of variance of the composition effects showed that significant differences were obtained in $\delta^{13}C$ $O_2$ respired between scenarios ($p < 0.000$) but not between species (monoculture vs. mixture) Table S3.

### 3.1.2. C Content (mg/mg)

Figure S1 shows the results for the C content (mg/mg) in gases, shoot, root, soil, and pig slurry in the two studied scenarios. The main differences in the C content of shoot and root samples were found between species ($p < 0.05$) and between scenarios (before and after fertigation $p < 0.05$). However, no differences were found between monoculture versus mixture for any of the species ($p = 0.5$; 0.7; 0.4 for G, L, and F, respectively). Regarding the differences in plant C content among species, forbs showed the lowest C content values in shoot, with 0.34 mg/mg in both monoculture and mixture compared to grasses ($p = 0.07$ in monoculture and $p = 0.02$ in mixture) and legumes ($p = 0.3$ in monoculture and $p = 0.7$ in mixture), and the highest C content in root, $0.69 \pm 0.054$ mg/mg in monoculture and $0.51 \pm 0.06$ mg/mg in mixture, compared to grass ($p = 0.013$ in monoculture and $p = 0.012$ in mixture) and legume ($p = 0.8$ in monoculture and $p = 0.1$ in mixture).

For the post fertigation moment, C content increased for legumes both in mono and mixtures swards in roots. A significant increase was also observed in the grass species grown in mixtures.

In the gas samples, legume monocultures showed the highest C content in the $CO_2$ and $CH_4$ emissions compared with the other two monocultures and mixture (Figure S1). For $CO_2$ values, the differences were 50% (in grass monoculture), 41.6% (in forb monoculture), and 13.3% (in the mixture) lower than legume monoculture, and for $CH_4$ values, the differences were 47.4% (in grass monoculture), 33.6% (in forb monoculture), and 81.3% (in the mixture) lower than the legume monoculture.

Although the C content of pig slurry was 0.064 mg/mg (double that of soil), for soil samples, we could not observe differences in C content between plots in both scenarios.

### *3.2. Nitrogen Dynamics*

#### 3.2.1. δ15N (‰)

Figure 3 and Table S4 show the results for the $\delta^{15}N$ (‰) in total organic matter (TOM) of plant material (shoot and root) with significant differences ($p < 0.002$) and soil, in both scenarios. In scenario 1, before fertigation, grass monoculture presented significant differences ($p < 0.05$) relative to the forbs and legumes in monoculture or mixture and showed the highest $\delta^{15}N$ (‰), with higher values (13.6 and 9.5‰; shoot and root, respectively) and percentage values in shoot (50.8 and 85.3%) and root (42.1 and 75.8%) than forb and legume monocultures, respectively. The legume presented significant differences with other species in monoculture ($p < 0.01$) but not in mixture ($p = 0.296$ and 0.514) and showed the lowest $\delta^{15}N$ (‰) values (2.0 and 0.9‰ in shoot monoculture and mixture, respectively, and 2.33 and 1.2‰ in root monoculture and mixture, respectively) compared with the grass and the forb in monoculture and mixture. However, the differences in the N signal among species decreased when grown in mixture compared to the signals in monoculture, in both scenarios. Forb monoculture presented statistical differences with grass in monoculture and legumes (in monoculture and mixture) ($p < 0.05$) but not in grass mixture ($p = 0.113$).

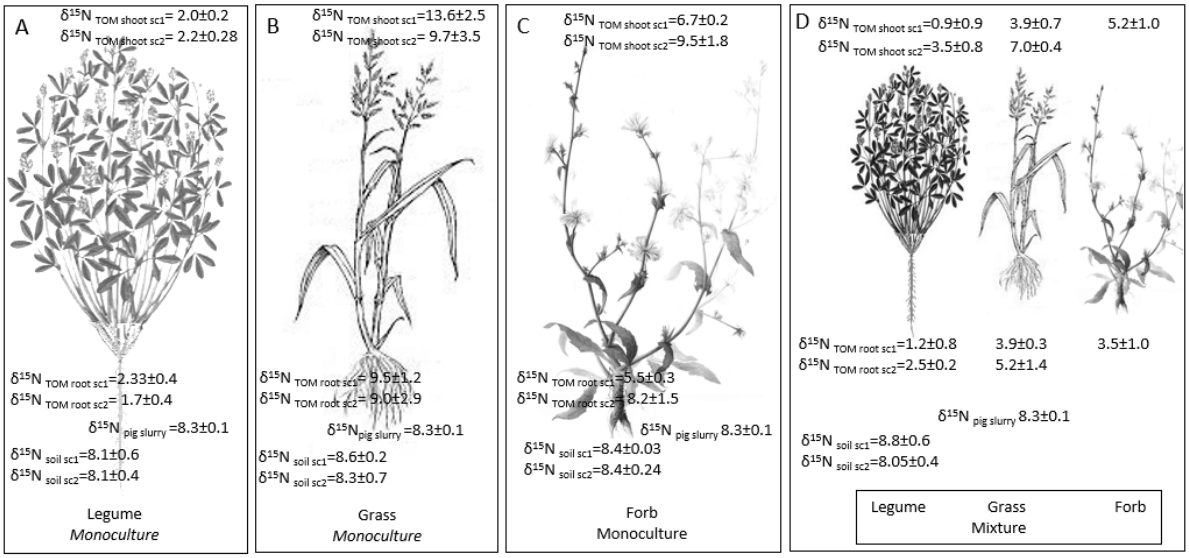


**Figure 3.** $\delta^{15}N$ (‰) signal in shoot and root biomass, total organic matter (TOM), soil, and slurry at two different scenarios: SC1 (unfertilized) and SC2 (fertilized), for the three species, grass (*Festuca arundinacea*), legume (*Medicago sativa*), and forb (*Cichorium intybus*), in monoculture (**A**–**C**) and in mixture (**D**). The number of averaged samples is $n = 3$. Analyses of variance of the composition effects showed that significant differences were obtained in $\delta^{15}N$ only between compartments (shoot, root, and soil) ($p < 0.000$) Table S3.

After the application of pig slurry fertilizer (the $\delta^{15}N$ was 8.3 ± 0.1), increases in $\delta^{15}N$ were observed in plant material for the legume and forb in monocultures as well as for legume and grass in mixtures. However, for the grass monoculture, we observed decreases in $\delta^{15}N$.

$\delta^{15}N$ values in the soil were stable in all the plots in both scenarios and no significant differences were found.

#### 3.2.2. Estimation of the N Source

The $\delta^{15}N$ was used to estimate the N source (Figure 4), as described in Section 2. In the scenario 1 (Figure 4-a), grass and forb monocultures were totally dependent (100%) on soil N, and they had a $\delta^{15}N$ value close to that of soil N; however, the legume monoculture had two sources of N: soil N and atmospheric $N_2$. This $\delta^{15}N$ reflected a relative contribution

from these two N sources (26.6 and 74.4% soil and atmospheric $N_2$, respectively). The species in mixture showed a third N source, N transference between plant species, and as a result of the N transference, the contribution (%) from different sources changed. Grasses (29.6 and 70.4% from N soil and N transferred, respectively) and forbs (86.4 and 14.6% from N soil and N transferred, respectively) in mixture showed lower values from the soil source due to N transferred source than in monocultures (100% from N soil), and finally, legumes in mixture (10.7 and 89.3% soil and atmospheric $N_2$, respectively) with higher values from atmospheric source than in monoculture.

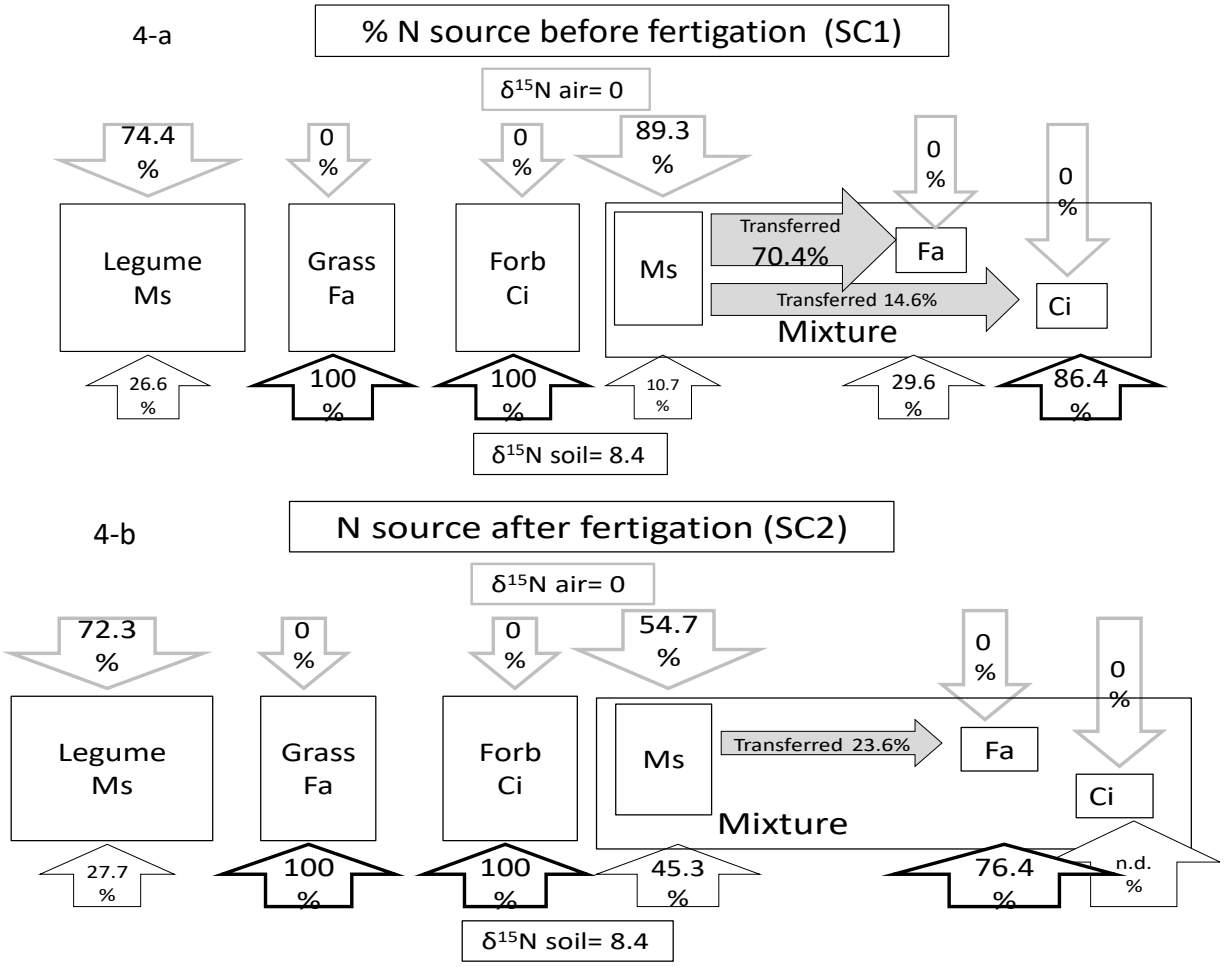


**Figure 4.** N source (%): N derived from air (fixed by symbiotic microorganisms in legumes and transferred to non-symbiotic plants) and N from soil in the three species in monoculture (*Medicago sativa* (Ms), *Festuca arundinacea* (Fa), *Cichorium intybus* (Ci)) and in mixture in two different scenarios: (4-**a**) before fertigation, scenario 1 (SC1) and (4-**b**) after fertigation, scenario 2 (SC2). Values are obtained from $\delta^{15}N$ signals in different compartments. $\delta^{15}N$ signals of the two N sources, air and soil, are indicated. The number of averaged samples is $n = 3$.

In scenario 2 (Figure 4-b), with non-legume monocultures (grass and forb) being totally dependent on soil and fertilizer N (100%), plants had $\delta^{15}N$ closer to the soil and slurry values. The N source used by the legume grown in monoculture did not change significantly after fertigation (27.7 and 72.3% soil and atmospheric $N_2$, respectively; Figure 4-b). However, in the mixture, the fertilizer application affected significantly the legume grown in mixture (45.3 and 54.7% soil and atmospheric $N_2$, respectively; Figure 4-b), with higher values of $\delta^{15}N$, closer from soil source (fertilizer) than in monoculture. Grass in mixture (76.4 and 23.6%, N soil and N transferred, respectively) showed lower values from the N soil source due to transferred N source than in monocultures.

3.2.3. N Content (mg/mg)

Figure 5 shows the results for the N content (mg/mg) in gases, shoot, root, soil, and manure in both scenarios (SC1 and SC2). N content (mg/mg) was affected by compartment (shoot, root; $p = 0.001$), diversity, and fertigation ($p < 0.0001$) (Table S3).

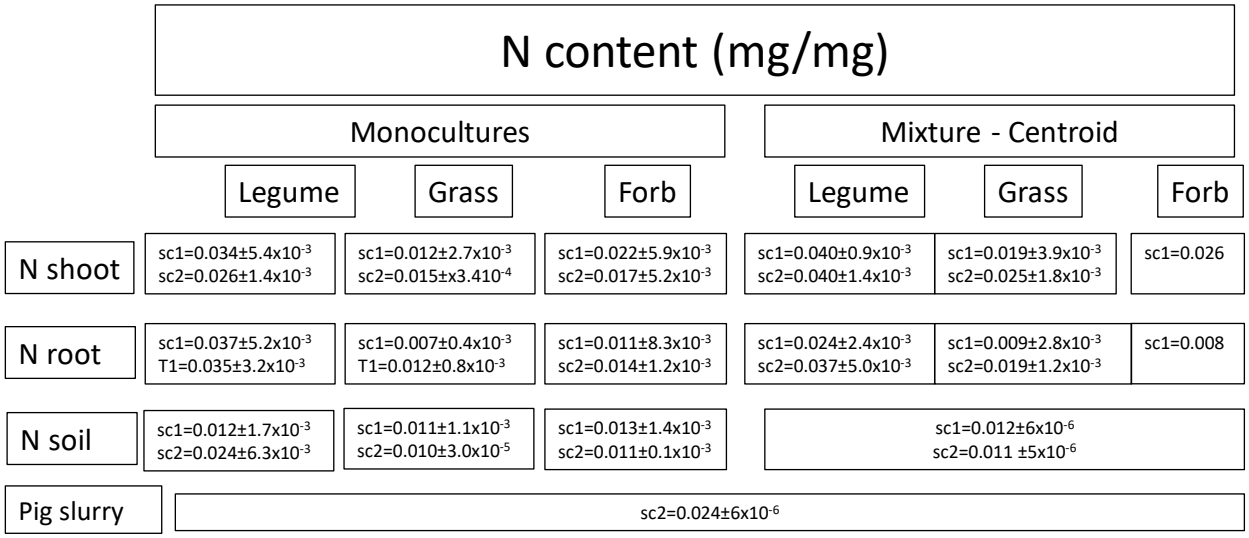


**Figure 5.** N content (mg/mg) in shoot, root, soil, and pig slurry in the three monocultures (*Medicago sativa* (Ms), *Festuca arundinacea* (Fa), *Cichorium intybus* (Forb, Ci)) and the mixture (mixed plot with the three species) at two different scenarios: before fertigation, Scenario 1 (SC1) and after fertigation, Scenario 2 (SC2). The number of averaged samples is $n = 3$.

In scenario 1, legumes showed the highest N content, both grown in monoculture and in mixture, higher than those found for the grass (64.7%in shoot and 81.08% in root) and for the forb (35.3% in shoot, and 70.3% in root) monocultures. In scenario 2, legumes in monoculture and in mixture also showed higher N content than the grass (42.3% in shoot and 65.7% in root), although differences decreased, particularly for the grass and the forb (34.6% in shoot and 60% in root). Also, in scenario 2, we observed significant increases in N content in the shoot and root of grasses both in monoculture and in mixture, and also in the root of legume in mixture. For soil samples, we observed a 50% increase in N content and no increase in root in the legume monoculture from scenario 1 to 2; however, there was no N increase in soil in mixture, but there was a N increase in root in mixture.

*3.3. Carbon/Nitrogen (C/N)*

Table 1 shows the results for the C/N values in plant shoot and root, soil, and slurry samples in both scenarios. Regarding TOM, C/N values were usually higher in root than shoot samples in all the plots and species sampled except the legume grown in mixture. In scenario 1, grass and forb in monocultures and mixture presented higher C/N than legume (75.4 and 77% in monoculture and 78.7 and 82.1% in mixture, respectively). However, in scenario 2 (SC2), those differences in C/N values were not so big, and values were closer to the value of the pig slurry (C/N = 3.56 $\pm$ 0.2).

**Table 1.** C/N ratio in shoot, root, and soil in two different scenarios: before fertigation, scenario1 (SC1), and after fertigation, scenario 2 (SC2), in three species monocultures (legume: *Medicago sativa*; grass: *Festuca arundinacea*; forb: *Cichorium intybus*) and a mixture with the three species. C/N pig slurry = 3.56 ±0.2. Data from forb in scenario 2 is not available. The number of averaged samples is $n = 3$.

| Scenario 1 C/N | Monocultures | | | Mixture | | |
|---|---|---|---|---|---|---|
| | Legume | Grass | Forb | Legume | Grass | Forb |
| Shoot | 3.2 ± 0.2 | 8.1 ± 1.0 | 4.1 ± 0.7 | 5.4 ± 0.04 | 5.6 ± 0.7 | 3.5 ± 0.02 |
| Root | 4.5 ± 0.3 | 18.3 ± 0.7 | 19.5 ± 5.9 | 2.7 ± 0.4 | 12.7 ± 4.2 | 15.1 ± 0.9 |
| Soil | 6.4 ± 0.6 | 6.8 ± 0.4 | 6.1 ± 0.4 | | 6.3 ± 0.1 | |
| Scenario 2 C/N | Monocultures | | | Mixture | | |
| | Legume | Grass | Forb | Legume | Grass | Forb |
| Shoot | 3.6 ± 0.5 | 5.6 ± 0.5 | 5.4 ± 0.8 | 4.5 ± 0.05 | 3.2 ± 0.02 | - |
| Root | 4.9 ± 0.1 | 9.9 ± 2.0 | 12.6 ± 4.2 | 2.8 ± 0.04 | 7.3 ± 0.3 | - |
| Soil | 5.8 ± 0.01 | 6.6 ± 0.01 | 6.1 ± 0.2 | | 6.1 ± 0.2 | |

Total organic matter in soil (TOS) did not show differences between monoculture and mixture, but C/N values decreased after pig slurry application.

### *3.4. Dry Matter Yield*

Figure S2 shows the results for the dry matter yield (DMY). Diversity significantly increased DMY ($p = 0.001$). In scenario 1, the mixture (3069.9 kgha$^{-1}$) presented the highest DMY, being 27.8% higher than that obtained for the legume monoculture. Among monocultures, the grass showed the highest DMY, followed by the legume and finally by forb monocultures (2663.4; 2592.2 and 780.25 kgha$^{-1}$, respectively).

DMY was significantly ($p = 0.01$) affected by fertigation and sown diversity. Averaging all harvests after fertigation (SC2), the legume monoculture (3444.2 kgha$^{-1}$) presented higher DMY (9.7%) than the mixture (3013.7 kgha$^{-1}$), followed by the forb monoculture (1307.3 kgha$^{-1}$), and the grass monocultures, which showed the lowest DMY (379.7 kgha$^{-1}$).

In both scenarios (before and after slurry fertigation), we observed that the mixture was not affected by fertigation, and the yield was 2.9% lower after fertigation than before fertigation. Legume and forb presented higher yield production with the fertigation (32.0%, $p < 0.5$ and 67.5%, $p < 0.01$, respectively). However, grass reduced the 86.5% DMY after the fertigation.

## 4. Discussion

In this paper, we explore how plant composition and fertigation modulate the C and N dynamics in a forage agroecosystem. Plant composition may prompt differences in plant interactions, and species may not interact equally [62] and may be associated with environmental conditions and species traits [13].

### *4.1. Carbon Dynamics*

$\delta^{13}$C isotopic composition presented an inherent differential isotope composition that could be mainly ascribed to (i) species' identity effects [55], (ii) their seasonal fluctuations due to phenological stages [35], and (iii) environmental fluctuations (including water or nutrient availability) [63]

In general, plants discriminated more against $^{13}C$ in mixture than in monocultures, suggesting that there is an interaction between plant diversity and water resources in mixtures. The presence of particular functional groups, and specially the use of different functional groups in a mixed sward, could be a good agricultural strategy to avoid water stress [64] because (1) water use and water maintenance in mixtures is more efficient [65]; (2) soil C content is increased in mixtures, resulting in an increase in soil water retention capacity [66]]; and (3) our three functional groups could have affected the soil water distribution by characteristic shifts of root water uptake depth, because the root morphology is different, and the response of water uptake depends on the plant functional type [67].

Carbon dynamics seemed to be driven by environmental conditions. In our second scenario, fertigation produced a general decrease in $\delta^{13}C$ total organic matter (TOM). We attribute this to two facts: (1) the fertilization and (2) the water supply. Marshall et al. [68,69] showed that nitrogen and water are critical elements limiting plant growth in natural ecosystems, and both can affect to plant physiology and $\delta^{13}C$. The magnitude of these responses differs among photosynthetic capacity and the species diversity. After fertigation, the higher water availability allows for the plant to keep the stomata open, so there is more $^{12}C$ available for the Rubisco enzyme and therefore the organic matter has less $^{13}C$. Response to fertigation impacted the isotopic signature of plant tissue, and as a result, discrimination against $^{13}C$ stable isotope was higher in plants, with lower $\delta^{13}C$ because of open plant stomata and greater $^{12}C$ availability, as in Goldman, [70]; for example, legume showed the lowest $\delta^{13}C$ values in TOM but also increased the C content in the tissues. $\delta^{13}CO_2$_respired was also affected after fertigation, showing lower values of $\delta^{13}CO_2$_respired than before the fertigation [35], both in legume monoculture [71] and mixture. This fact evidences that the effect of fertigation is modulated by functional group or species and the physiology of the plant can also affect the $\delta^{13}CO_2$_respired. The enrichment of $\delta^{13}C$ in roots is in agreement with the isotopic fractionation between both compartments [39,72–76]. The compartment effect in plant material (shoot, root) on $\delta^{13}C$ was significant ($p < 0.05$) and showed significant variation in $\delta^{13}C$ values across different plant compartments (mostly around 1‰). Substantial variation in $\delta^{13}C$ in different plants parts has been extensively reported in previous studies [33,35,72,77–80]. Hence, plant compartment type would add up to 1.0–3.0‰ of differences from means for different organs, less negative $\delta^{13}C$ values in roots compared to shoot. However, the grass presented less enrichment in shoot than root and higher C content in shoot than in root because C allocation to grain due to grain filling. That difference between shoot and root was also observed in C content. Roots are often a reservoir of C in herbaceous plants [81] and C content is also influenced by the physiology, the plant structure, and type of roots that the plants have [82]. Some parts of this C are sequestered by adding C to soil and increasing soil organic matter that promotes mitigation and adaptation to climate change [11,83].

### *4.2. Nitrogen Dynamics*

Nitrogen dynamics seemed to be driven by plant diversity and therefore fits nicely into the context of previous works [18,23,40,43,83–85] linking plant diversity to increasing yield and using reactive N more thoroughly.

We found differences in N uptake and plant $\delta^{15}N$ values across the plant species in monocultures versus mixtures, suggesting that $\delta^{15}N$ in soil and plant material may be used as an indicator for the N cycle [86,87]. Shoot and root $\delta^{15}N$ values can reflect the $\delta^{15}N$ signatures of the plant's specific N sources [88] revealing information on the plant's N uptake patterns [32,34,89].

The $\delta^{15}N$ values for all plant species and compartments (shoot, root, and soil) were positive in both monoculture and mixture. The $\delta^{15}N$ natural abundance method was

used for estimating $N_2$ fixation, highlighting that the $\delta^{15}N$ of a legume in monoculture is dependent on atmospheric $N_2$, but also depends on soil N; they had $\delta^{15}N$, which reflects the relative contributions from these two N sources [90]; so, they should have $\delta^{15}N$ in shoots and roots mixed between both sources, but closer to that of its main N source, atmospheric $N_2$ (0‰); conversely, the grass and forbs monocultures are totally dependent on soil N and had $\delta^{15}N$ close to that of soil N [34].

We found a stimulating effect of plant diversity on fixation and N uptake by legumes in mixture [91]. Mixing grasses, forbs, and legumes in grassland systems yielded benefits to nitrogen over monocultures [43,92]. In our study, a mixture with only one-third proportion of sown legumes had higher N content than monocultures, indicating a substantial N yield gain in mixture and the potential of grass mixtures for sustainable agriculture. As a result of the mixing of species, we found that the grass and the forbs (non-N-fixing plants) presented higher N content in shoot and root (Figure 5) and lower $\delta^{15}N$ (Figure 2) than monoculture, because of the incorporation of atmospheric N in the system by legumes and the transference of fixed N from legumes to the others functional groups, as reported previously [62,91,93]. Legumes may influence the availability and isotopic signature of N in the surrounding soil modifying $\delta^{15}N$ signals in adjacent non-N-fixing plants by N transfer [32,33]. The majority of N (up to 71%) is transferred via rhizodeposition (decomposition of nodules and root tissue and exudation of soluble N compounds by roots) [20,94]. This N transferred by rhizodeposition has an isotopic signal more similar to that of symbiosis fixation that modifies the signal of adjacent non-N-fixing plants. For grass in mixture, another N source was observed, the N transference between plant species, and as a result, different contributions from the N sources. The effects of legumes were usually considered to be a direct result of higher N availability for non-legumes since legumes relied more on symbiotically fixed $N_2$, because the amount of symbiotic $N_2$ fixation by legumes can be substantial and ranges from 100 to 380 kg $ha^{-1}$ $yr^{-1}$ in northern temperate/boreal regions [42,95]. Those benefits of functional groups, with a relative availability of soil N increases for grasses and forbs, have been shown in previous research [14,18,44,91,93].

Plant diversity favored both the biomass and protein content. This can be explained by the N transference of legume to others in mixture (Figure 4), and consequently, the biomass yield of forbs and grass in mixture increased more than in monoculture (Figure S2). Such an additional N source can explain higher biomass yields of mixture compared to monocultures of non-legumes but not compared to monocultures of legumes [13,44]. It can be explained because legume in mixture introduces N in the system and it is used by grass and forbs; however, legume in monoculture did not share the fixed N with other plants and can use the resource for his own growth. Furthermore, Legesse et al. [96] also found that plants in mixture contained more protein compared to plants in monoculture, except for legumes [97]. We also found that legumes have higher protein content than forbs and grasses; as a result, legumes showed the highest N content in shoot and root, followed by forbs (who presented better feed quality as forage than grass), and finally grass; this is constitutive of each species. Even in the mixture, legume showed higher N content than forbs and grasses, indicating that the legume improves feed quality as forage, especially in mixture, as in Foster et al. [26]. Furthermore, the mixture has another advantage than the monoculture, since, as was just said, the three species benefited from an increase in the N content in the mixture, both in shoot and root compartments. This distribution of N was not homogeneous in the different compartments of the plant; there was a greater increase in N content in the shoot compared to the root in the three species in the mixture, especially in legume. However, legume root reduces the N content in scenario 1 due to the transfer of that nitrogen to the other species in the mixture. This does not occur in scenario 2, where the extra nitrogen contribution from the fertilizer reduces the transfer of N and therefore

increases the N in the root of the legume. The authors of [53] calculated that in fertilized sward, the species interaction effect dropped at about 80%.

Additionally, Nyfeler et al. [43] explains other beneficial effects of mixing plants with legumes derived from niche differentiation due to differences in their root depth, root structure or promoting nodulation [82,91], and differences in their growth pattern across the season [11,41,98] or among years [44]. All of them benefit from resource-taking strategies, such as, for example, N acquisition. Differences in resource uptake strategies among plant species can drive enhanced community-level resource uptake of N and thus biomass production when species are grown in mixtures compared to monocultures. One classic example of resource partitioning is differentiation in rooting depth between species [99]. Root biomass production increases with plant species richness [100]. However, grass tends to have a denser and more superficial root system than forbs or legumes [99]. Forbs and legumes are more deep-rooting species; they can take up N and other resources from deeper soil layers and increase the quantity of soil resources available in the top soil for shallow-rooted species [101].

Another beneficial effect of mixed species found in our experiment is that the different phenology favors plant needs for the same resource in different moments. Eisenhauer et al. [102] suggested that $\delta^{15}N$, N allocation, and net shoot and root productivity can be modified with community composition, plant functional group identity, phenology, or decomposer diversity [103]. Kahmen et al. [34] showed that $\delta^{15}N$ from forbs and grasses were all depleted in $\delta^{15}N$ compared to soils. However, we found depleted $\delta^{15}N$ in forbs, but not in grasses (Figure 3). It could be explained because grasses are in grain filling, and they need a higher amount of N. For that reason, the discrimination against $^{15}N$ is minor, and the $\delta^{15}N$ and N content is surely higher in shoot than root because the N allocation is in the reproductive organs [35].

In our second scenario, after fertigation, we observed increases in $\delta^{15}N$ in all species and even higher increases in mixture than monoculture (Figure 3). Watzka and Wanek [86] found a strong correlation between $\delta^{15}N$ values of plant and soil points to fast incorporation of fertilizer N into N pools that explained the fast increases in $\delta^{15}N$ in our plants. Even if some studies have shown that plants can discriminate against $^{15}N$ during N acquisition [39,104–106], we considered this process of discrimination against $^{15}N$ not to be relevant in our ecosystem because (1) N is a critical and limiting resource [107]; (2) we did not apply fertilization from the beginning of the experiment in 2008 until June 2011; and (3) the plants acquired nitrogen from symbiosis or transference from legumes that is known to alter the $\delta^{15}N$ of plants [37,104,105]; however, legumes also showed increased $\delta^{15}N$, which was reflected in the system.

However, N fixation by legumes is reduced by fertilizer addition (Figure 4-b; [108]), and consequently, there was an increase in $\delta^{15}N$ (Figure 3; [109]) in the TOM of legume due to fertigation and diversity interaction in mixtures and a negative fertilizer effect on fixation by legumes [30,43,84], which may indicate low fertilizer N requirements in mixtures based on legume [62]. Although the N content was modified with fertilizer and mixture, fertilizer did not increase the N content in soil but increased it in root in all species (monoculture and mixture), except in legumes under monoculture, which showed the opposite response. This can be explained by the legumes in monoculture not needing more N; thus, the excess N applied by the fertilizer stays in soil. Nevertheless, as the N level in the fertilizer was low, we consider that our system is mainly managed by the species and not by the fertilizer, as we can observe in the legume monoculture, which can hardly modify the percentage between N uptake from soil or air [43]. It has been shown that under low N fertilizer supply, legumes acquire most of their N nutrition from symbiotic $N_2$ fixation [110–112]. In

contrast, other studies have shown that under elevated N fertilizer supply, legumes decline in N acquisition from symbiosis in field experiments [18,108,111,112].

The other plant species (grass and forbs) also showed a new source of N in the soil derived from the fertilizer, and increased $\delta^{15}N$ in shoot and root. As evidence of this new N source in the soil, grass and forb monoculture and legume in mixture increased N content after fertilization. As we observed in the first scenario, before fertilization, grass and forb monocultures had a N source from the soil without transference from the legume and also without the N from the fertilizer. However, in the second scenario, after the fertilization, grass and forb monocultures had a new N source in soil from fertigation, which is reflected in the increase in N content (Figure 5) and $\delta^{15}N$ (Figure 3), for example, roots of legume increased $\delta^{15}N$. Fertilization is also reflected in the N transference in the mixture (Figure 4-a), the source of N in grass from legume transference was reduced from 70.4% to 23.6%, and the source of N from soil was increased from 29.6% to 76.4% because the plants found a new source of N in the soil from the fertilizer. Legume also changed the % of N source from the soil, with increases from 10.7% to 45.3%, before and after fertilizer application, respectively, indicating the importance of plant community composition and plant functional group [102].

If more N yield can be expected in a mixture for a given amount of nitrogen fertilizer applied, as in this study with a low N level in the fertilizer, we can say that mixtures with legumes have the potential to reduce fertilizer N application for environmental reasons, without necessarily compromising the N yield or total harvested biomass [8,18,44,83,108]. Growing legumes in mixture with grass and forbs can increase soil C sequestration, reduce GHG, add N (by atmospheric N fixation) and other nutrients to the soil, and improve soil fertility or increase soil water retention [113]. This information may be of use as an important strategy in grassland management for synthetic N fertilizer reduction applications, the reduction in GHG emissions from agriculture, and mitigation of the negative effects of climate change effects [16,114–116]

Finally, the homogeneity found in soil $\delta^{15}N$ values can be explained by net N mineralization and historical fertilization of the land where the experiment was made. The soil $\delta^{15}N$ values observed are often found in areas of nitrogen accumulation and mineralization, e.g., in areas where slurry or inorganic compounds have been used as fertilizer [117,118]. Additionally, the amount of N applied in the fertigation was not very high (N = 25 kg N $ha^{-1}$), and soil $\delta^{15}N$ is a very stable variable. Previous studies have suggested that increasing bulk soil $\delta^{15}N$ values may reflect increasing rates of soil N cycling, which are associated with losses of $^{15}N$-depleted mineral N that lead to a gradual $^{15}N$ enrichment of the remaining bulk soil N [119,120]. Although the plants investigated here had not been fertigated for at least three years prior to the experiment, those lands were traditionally cultivated with grasses and fertigated with pig slurry and inorganic fertilizer over decades. Ribas et al. [14] have shown that $NO_3^-$ in such areas is prone to losses, either via leaching or via microbial denitrification to $N_2O$ and $N_2$ [30,121]. However, an increase in soil N after fertilization was observed in legume monoculture, but not in the mixture, indicating that the mixtures have higher N use from the soil, and for that, there were no increases in $N_2O$. The mixture of plants can potentially reduce $N_2O$ emissions, contributing to the sustainability of grassland production [122], because the reduction on $N_2O$ emissions is due to the species identity effects and the interspecific interaction. But, $N_2O$ emissions of mixture grasslands can be also affected in several ways [123]: $N_2O$ losses during soil nitrification and denitrification; differential niche occupation of the rhizosphere, which can affect plant water uptake and soil gas diffusivity; C availability; root exudates; or pH changes. However, whereas the N content of $N_2O$ in mixture and legumes is similar, in grasses and forbs, it is about half of the value. Furthermore, nitrogen fertilization did not affect soil $NO^{3-}$ and $NH^{4+}$ contents,

or significantly increase GHG emissions, which may be the result of the fractionation, dilution, and N form (around 50% organic) of the applied fertilization. Furthermore, as Ribas et al. [14] showed, the pig slurry application was not followed by ammonia volatilization peaks, which may be due to the dilution of slurry in the irrigation water. This fertilization technique can therefore reduce $NH_3$ emission and increase infiltration [124]

Our material reflects the $\delta^{15}N$ signature of the plant compartment, available soil N, and symbiotic N. Consequently, we conclude that $\delta^{15}N$ values of plant material in our study are in fact driven by the uptake of N and plant composition.

### *4.3. Carbon and Nitrogen Balance (C/N)*

The C/N ratio typically reflects their sources and sinks. We also observed the importance of legumes in terms of the response of plants to the C and N balance. No differences were found in C content between monoculture and mixture, but we observed that legumes presented the highest C content in shoot and the lowest C content in root (because C is transferred to symbiotic microorganism) compared to grass and forbs. However, as legumes in monoculture obtain N easily, due to symbiosis, grasses or forbs in monocultures presented lower C/N ratio than legumes monoculture in TOM. However, in the mixture, legumes fixed $N_2$ by symbiosis but transferred part of this $N_2$ to other species. Grass and forbs in the mixture can benefit from the transference of this N from legumes as, for example, we observed higher protein content and lower C/N ratio in forbs and grasses in mixture than monoculture, while the C/N ratio of legumes increased when they were in mixture, confirming higher N availability by N transference from legumes to other functional types, and the benefits of plant mixtures with legumes [14], overall in areas where N was a critical and limiting resource, such as in this study.

However, C/N ratio can be modified with N supply from fertilizer. Legumes can reduced the N fixed by symbiosis by downregulation and increase the use of soil nitrogen after fertigation because of the energetic cost of supplying C to their symbiotic microorganism [125]. In the mixture, legume showed an increased N source from soil or fertilizer and decreased N source by symbiosis; grass increased the N source from soil or fertilizer and decreased the N source transferred from symbiotic to non-symbiotic plants. These changes in nitrogen source resulted in increases in the C/N ratio after fertigation. This is a mechanism that explains the positive interactions and effects of grass, forbs, and legumes mixtures, as the plants increased fertilizer utilization and soil N resources through temporal and spatial niche complementarity between species [18].

Additionally, the C/N ratio was higher in grass in the first scenario (early spring before fertilization) than in the second scenario (late spring after fertilization) because it is a winter grass and the nutrient allocation was sent to grain filling [35,74,126].

Grassland mixture plant systems with legumes, grass, and forbs modify C/N balance, sequester soil C by adding grass biomass, increase soil organic matter, increase forage production, and reduce/replace N fertilizer. This plant mixture mitigates and promotes adaptation to climate change [41,98].

### *4.4. Dry Matter Yield (DMY)*

Some studies reported that plant mixtures with legumes had greater yield than monocultures [21,24,46,47] because of the provision of benefits from one species to the other (e.g., transfer of nitrogen fixed by legumes via biological nitrogen fixation to non-legumes) [42,127]. They reported increased yields as mixture complexity increased. However, in our first scenario, the mixture presented higher DMY than monocultures, with little decreases in DMY in grass and legume monocultures. But, in our second scenario, after fertigation, the legume monoculture showed a higher DMY than the mixture and the

other monocultures. Some studies have concluded that plant mixtures with legumes offer little yield advantage over legume monocultures when harvested [23,128,129]. It has been suggested that the positive relationship between forage yield and species richness may be a result of the strong influence of one or two species, while other species may contribute minimally [23,130]. The yield benefits observed by Nyfeler et al. [44] were reduced due to grasses becoming more dominant and reduced mixture evenness, generally decreasing over time. We suggest that this may have been the case in our study, with low contribution from forbs to others.

Also, in scenario 2, grass reduced the DMY. *Festuca arundinacea* is a winter grass with the highest production in spring [131], and for that, the greatest grass production is in early spring, before the first harvest (scenario 1), and that phenology can explain the minor DMY in late spring, the second harvest (scenario 2) [132]. Our mixture could benefit from species split resource allocation in time or space (e.g., development phases are different during the vegetation period or for different rooting or canopy systems) [79].

Moreover, the mixture effect on yield suggests that diversity can substitute fertilization [14,44].

Studies with plant mixtures showed some disadvantages because they are more difficult to manage than a monoculture [23,133]. For example: (1) The instability of the botanical composition of the plant mixtures they seeded, suggesting that more frequent reestablishment of these pastures would be necessary to maintain the mixture complexity [1,23]. (2) Competition during the establishment period may result in the plant number of some of the participating species being reduced. (3) Plant mixtures can change the botanical composition due to ecological factors, for example, animal predation, crop pests, or unusual weather periods. But, like in our case, simple mixtures of three species may offer the best means to provide plant diversity and yet limit seedling competition, providing substantial information [134].

## 5. Conclusions

This study provides insight for understanding the mechanisms that drive the interaction between plant functional groups in N acquisition. Because species do not interact equally, further investigation is needed to make more certain predictions regarding better N management in grassland and the distribution of functional groups of plants. Stable isotopes are a good tool to study the diversity effects on the C and N dynamics and their interaction with fertigation.

Our study shows that the C and N dynamics, $\delta^{15}N$, and $\delta^{13}C$ were modified by species. Positive relations and effects between species mixtures with the $N_2$ fixated by symbiosis and N transference from legume to forb and grass were observed. Diversifying forage with legume-based systems could contribute to the mitigation of climate change while improving ecosystem productivity with less inorganic fertilizer input.

In addition, fertilizer can modify the balance of C/N and the source of N in the mixture with different functional types of plants (legume, forb, and grass). This can potentially be achieved by the benefit of mixtures used as an important strategy in grassland management for reducing N application, fertilizer use, and GHG emissions from agriculture, as well as mitigating climate change effects.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy15020287/s1. Figure S1: C content (mg/mg) in grass, shoot, root, soil and pig slurry in two different scenarios: SC1 (before fertirrigation) and SC2 (after fertirrigation). Three species monocultures (*Medicago sativa*, *Ms*; *Festuca arundinacea*, *Fa*; *Cichorium intybus*, *Ci*) and a mixture with the three plant species (mixture centroid Cent.). Figure S2: Dry matter yield (estimates ± SE) averaged per harvest in 2011 in two scenarios: before (SC1, open bars) and after pig slurry application (SC2, closed bars). Values correspond to the three monocultures (*Medicago sativa* L., *Festuca arundinacea* G. and *Cichorium intybus* F.) and the centroid. The number of averaged harvests is $n = 6$. Statistically significant differences are indicated as * for $p < 0.05$, ** for $p < 0.01$, and*** for $p < 0.001$. Table S1: Dates of harvest (H1, H2), fertilization (F), soil sampling (S1, S2), and emission measurements (E1, E2) along the growing period of 2011. Table S2: $\delta\ ^{13}C/^{12}C$, C content (mg/mg), $\delta\ ^{15}N/^{14}N$, N content (mg/mg), and C/N in waterhole, pig slurry, rainwater and river water. Table S3: Analyses of the variance of the composition effects on $\delta^{13}C$, $\delta\ ^{15}N$, C content (mg/mg), N content (mg/mg), and C/N at two different scenarios: SC1 (before fertigation) and SC2 (after fertigation). Three species monocultures (*Medicago sativa*, Ms; *Festuca arundinacea*, Fa; *Cichorium intybus*, Ci) and a mixture with the three plant species (centroid). Table S4. $\delta^{15}N$ (‰) signal in shoot and root biomass, soil and pig slurry at two different scenarios: SC1 (unfertilized) and SC2 (fertilized), for the three species, grass (*Festuca arundinacea*), legume (*Medicago sativa*), and forb (*Cichorium intybus*), in monoculture and in mixture.

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