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Effect of Oversowing and Fertilization on Species Composition, Yield and Nutritional Quality of Forages on a Permanent Wet Meadow

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Abstract

The improvement of forage production and nutrition quality on native grasslands through plant species oversowing and fertilization (legumes in particular, coupled with phosphorus fertilization) is known to have been widely adopted worldwide. Less is known about this practice on the wet grasslands of the French Atlantic littoral marshes. The purpose of this study, conducted over a 3-year period (2012-2014) on the Saint Laurent de la Prée research farm, was to investigate the effects on the yield and nutritional quality of forage hay on a permanent wet meadow, of oversowing with different plant species and fertilization. We found that the success of oversowing was influenced by species or mixtures, and depended on their ability to develop and persist in the cover. In general, oversowing tended to provide benefits in terms of the total annual forage yield in 2013, with a slight increase in forage quality in 2012 and 2013. Fertilization provided no real benefit in terms of forage quality. There was no persistence of introduced species in the sward, as in 2014 almost all of them disappeared. In the conditions of this study, the benefits of oversowing and fertilizer applications were limited and short-lived. These results are discussed in relation to the conservation value of these wet grasslands and the need to pursue research on agroecology for their biodiversity-oriented management.

Keywords: commercial seed mixtures, hay meadows, legumes, natural grassland conservation, oversowing, trade-offs, P fertilization

1. Introduction

French Atlantic littoral marshes cover an area of roughly 300,000 ha. In ‘dry marshes’, lands are made of polders with controlled groundwater levels. They are mainly used to grow field crops and grasslands (Durant et al., 2014). These marshes combine the permanent grasslands and grazing livestock farms of the Atlantic coast. Society recognizes the role of these grasslands in the preservation of a rich biodiversity and landscape, as well as the multifunctionality of agriculture (Michelet et al., 2016). However, agricultural policy in marshlands is currently torn between extensive livestock farming favorable to biodiversity but poorly viable economically, and intensive farming that is economically efficient but detrimental for biodiversity and water quality (Texier & Chourré, 2008).

We focus here on the wet grasslands used for cutting and/or grazing, mainly by suckling cattle, dairy cattle and horses. They are characterized by a high but seasonal productivity as soil and climatic conditions induce specific constraints on grass production. As the fields are inaccessible in winter because they are flooded, the turnout date occurs in late March-early April. Grass growth is usually halted by water deficits in summer and by low temperatures in autumn. Rooting depth of plants is limited by the high water level in the soil during wet periods. A short growth period occurs from April to June and drought occurs from July to September (Durant et al., 2014). The peak biomass production thus appears in June with 5 t dry matter ha^{-1} on average (it varies from 2 to 8 t ha^{-1}). This forage is of medium nutritive value as feed for cattle: forage unit: 0.6 (1 FU being equivalent to the energy value of 1 kg of barley); crude protein content: 60 g kg^{-1} dry weight; data from INRAE). Plan communities have a majority of grasses, with some legumes (only 10-15% in volume) and a few dicotyledons. The improvement of the nutritional quality of forage from meadows would contribute to the feed self-sufficiency of farms—that is, better forage for the herd to reduce the use of feed concentrates—and would thus be favorable to the economic viability of these farms. Pasture quality can be improved by introducing legumes into the grass dominant sward, in other words, by oversowing. As farmers demanded relevant information about technical ways to improve the quality of their hay, it was decided to explore agricultural practices for maintaining perennial legumes in their meadows.

The improvement of native pastures by oversowing plant species and P fertilizer application has been widely adopted worldwide for beef systems (Gutteridge, 1985; Miller & Stockwell, 1991; MacLeod & Cook, 2004; Tothill et al., 2008; Ferreira et al., 2011). For mowing meadows, however, only a few results from such experiments are available. For instance, Hofmann and Isselstein (2005) showed that on a permanent lowland grassland in Germany, the introduction of forbs increased the average dry matter yield by 23% within four years of oversowing, compared to unsown grasslands, and that it also increased the forage quality (crude protein concentration in particular). It has also been reported in one multi-site experiment that diverse methods (including oversowing of grass/forb seed mixtures for increasing the species diversity of permanent grasslands in England and Wales) had little effect on herbage yield in the subsequent two years compared to a control that had not been oversown (Hopkins et al., 1999). To our knowledge, no studies have been conducted in wetlands, and more precisely in wet grasslands.

This study therefore assessed oversowing of legumes and commercial seed mixtures on a hay meadow on the Saint Laurent de la Prée research farm, an experimental site of the French National Institute for Agriculture, Food and Environment (INRAE). The aim of this 3-year study was first to investigate the extent to which the yield and forage quality of hays on permanent meadow could be improved by: (i) oversown legume species (or grass/legume mixtures), and (ii) various fertilization modalities; and second to determine the most favorable fertilization for the establishment and persistence of species introduced in the sward. We posited that the introduction of legumes can partly increase forage quality and production, especially in the spring, but can also enhance autumn regrowth, without upsetting the native plant community due to the ‘heritage nature’ of these wet grasslands. We discuss the implications of this study from an ecological conservation point of view.

2. Materials and Methods

2.1 Site of the Experiment

The experiment was set up on a meadow (10 ha; parcel called F4) on the Saint Laurent de la Prée research farm which is located on the French Atlantic coast, in the “dry marshes” of Rochefort-sur-Mer (45°58′52″ North, 0°02′28″ West). The buildup of fluvial and marine sediments in marine gulfs has produced hydromorphic soils with 60% clay that are waterlogged in winter but dry out in summer. Decades ago, dikes, levees and locks were built to disconnect them from the sea. To control the rainwater levels, the fields are criss-crossed by a network of freshwater ditches through which the water flows gravitationally down to the ocean. Mechanical pump field drainage systems are used in the cropped fields. The climate is mild Atlantic (oceanic), characterized by 780 mm of rainfall per year and a mean annual temperature of 13 °C. The rainfall distribution is quite uneven in the year, with a water surplus in winter and drought from June through summer until the rains return in autumn.

The field used for this study is a permanent grassland, a hay meadow in which the vegetation at the start of the experiment was predominantly *Alopecurus bulbosus*, *Hordeum secalinum*, *Carex divisa* and *Bromus comutatus*. It is however important to note that this parcel had previously been flooded by seawater in 2010 because of a storm (Durant et al., 2018). In 2011, the farm consisted of about 180 ha of cropland, with about 50 ha of non-irrigated arable land and 130 ha of fodder crops (of which 115 ha of permanent grasslands) used for cutting and/or for grazing by about 60 cows and replacement heifers of a local wetland breed called Maraîchine. The 4-7 cm soil depth in summer 2011 showed a pH of 7, an available soil phosphorus content of 0.072 g kg^{-1} dry soil (P_2O_5 Olsen), and an exchangeable potassium of 1.5 cmol kg^{-1} (K_2O). This field had a more than 12 year history of one annual hay cut and a fertilizer level of 50 nitrogen units (NU) ha^{-1}.

Meteorological data were collected using an automatic weather station located on the farm.

2.2 The Experimental Design, Methods and Materials

The effects of oversowing nine legumes species, a grass species and four mixtures in combination with five fertilization types were studied in a split plot design with 4 replicates and a total of 344 plots (*i.e.*, 14 species or mixtures × 5 fertilization types × 4 replicates = 336 plots + 8 plots without oversowing). A block (about 60 m wide by 70 m long) was composed of 14 strips, each spaced 3-4 m from one another, and in which 2 plots had no oversowing. Each strip had 6 plots (9 × 3 m) separated from each other by 2 m. Each plot annually received one of the 5 fertilization types or no fertilizer (F0):

(i) ammonitrate (33.5%) applied at 151 kg ha^{-1} (or 50 NU ha^{-1}),

(ii) 0-25-20, at 180 kg ha^{-1} (or 45 PU ha^{-1}),

(iii) 20-16-0-21, at 225 kg ha^{-1} (or 45 NU ha^{-1}),

(iv) 15-15-15 (called hereafter 3 × 15), at 320 kg ha^{-1} (or 48 NU ha^{-1}),

(v) potassium slags (N-P-K-S composition: 0-15-15-0), at 120 kg ha^{-1} (or 18 PU or KU ha^{-1}).

See Table 3 for the fertilization dates. We used P-based fertilizers, considering that they are the limiting factor for leguminous plants. We expected them to be the most efficient to increase the forage yield and quality. On the other hand, we hypothesized that the N-fertilization (ammonitrate 33.5% treatment in particular) would not be useful to legumes, but could significantly increase the total plant biomass.

On September 27, 2011, the 14 strips were oversown with one of the 9 legume species, 4 commercial seed mixtures (consisting of legumes and grasses or of grasses only), or tall fescue (*Festuca arundinacea*). The lists of individual species and the species composition of mixtures are reported in Tables 1 and 2, respectively. They were sown with a seeder (Aitchison Seeding Machinery, SimTech T-SEM model, New Zealand), and slits in the sward were closed with a following roller. The oversowing depth was 2-3 cm. Note that the seeding equipment was not passed over the plots that did not have oversowing. To enhance germination, a fertilizer called Combivert, 11.5 Zn and 50 Mn from AgroQualita, was added at 40 kg ha^{-1} with a windmill. Alfalfa was sown after inoculation with the appropriate rhizobia to improve nodulation. The plots received no irrigation.

Table 1. The common names, scientific names, varieties, seeding rates, and properties of the oversown legumes (9) and tall fescue grass

Common name	Scientific name	Variety	Seeding rate (kg ha^{-1})
Alfalfa	*Medicago* sp.	Galaxie	25 (+ inoculum)
Suitable for mowing; high potential for production of biomass; high protein production; perennial (3-4 years); without excessive moisture.			
White clovers	*Trifolium repens*	Aberdai	4
High quality forage; good resistance to drought and cold (but no resistance to heat > 25 °C).			
White clovers	*Trifolium repens*	Aran	4
Large-sized white clover (very competitive) and perennial; well suited for mowing; not resistant to very wet soils and lack of light.			
Micheli clover	*Trifolium michelianum*	Paradana	20
Aggressive growth in the spring; adapted to very humid conditions; high protein content; annual legume (but excellent potential of regenerating itself from the seed dispersion).			
Persian clover	*Trifolium resupinatum* L.	Cirrho	20
Very rapid growth; drought resistant; supplies highly palatable and nutritive hay; suitable for all types of soil (in particular wet ones); annual.			
Alsike clover	*Trifolium hybridum*	Aurora	15
Less aggressive than red clover; but better adapted to very humid conditions; resistance to cold; short-lived biennial.			
Red clover	*Trifolium pratense L.*	Diplo	20
Suitable for mowing; perennial (2-3 years); quick establishment, tolerant to cold soils, not as tolerant to wet conditions as other clovers such as white or alsike.			
Garden vetch	*Vicia sativa*	Pépite	50
Suitable for well-drained soils; annual.			
Hairy vetch	*Vicia villosa*	Savane	50
High yielding forage; annual.			
Tall fescue	*Festuca arundinacea*	Elodie	25
Long-lived, rhizomatous and tall perennial grass; well adapted to mowing, good growth in difficult habitats; tolerance to prolonged flooding.			

Table 2. Species composition and seeding rates of four mixtures. The name of the species variety is indicated in parenthesis

Mixture name	Species composition	Seeding rates (kg ha^{-1})
Proteïs Alliance	20% *Trifolium repens* (Aberdai), 20% *Trifolium pratense* L. (Diplo), 20% *Trifolium incarnatum* (Tambolo), 10% *Trifolium hybridum* L. (Dawn), 15% *Lotus corniculatus* L. (Léo), 15% *Medicago lupulina* (Virgo Pajberg)	10
Herba'Regarnir	70% *Lolium perenne* L. (Thémis), 20% *Lolium hybridum* (Plétor), 10% *Trifolium repens* (Aran)	15
Herbafibre-FE	60% *Festuca arundinacea* (Soni), 20% *Lolium perenne* L. (Thémis), 20% *Phleum pratense* (Climax)	10
Mélopro Trial	60% *Lolium hybridum* (Enduro), 27% *Trifolium pratense* L. (Diplo), 8% *Trifolium incarnatum* (Cegalo), 5% *Trifolium repens* (Aran)	15

To evaluate seedling emergence and then establishment, observations were carried out on November 9, 2011; November 17, 2011; and December 8, 2011. The number of seedlings per m² was estimated by counting seedlings/plants on a 50 cm-long seeding line on each of the four plots. The data were then averaged and success of the establishment was then classified according to 6 categories: < 10 seedlings; 10 seedlings; > 10 seedlings; < 50 seedlings; 50 seedlings; and > 50 seedlings. Then, in 2012, 2013 and 2014, each plot was examined in spring (in April, May or June; see Table 3) for its species composition. The presence or absence of oversown species was recorded on each plot in a 50 × 50 cm quadrat and its frequency was expressed as a percentage, corresponding to their volume proportion in the herbage. The percentage of native grasses, native legumes, and other species (dicotyledons), as well as the bare soil, were also recorded.

Forage samples were collected with a two-wheel motor mower in a sub-area of 1.2 × 2 m on May 24, 2012 (or June 5, 2012 for grasses). The field was then mown. In 2013, we decided however to bring forward the mowing date to prevent the flowering and seeding of Micheli clover. Samples were dried at 60 °C to constant mass and reweighed. Dry matter (DM) yield was converted to t DM ha^{-1}.

Table 3. Dates of fertilizer applications, species observations and forages samples in 2012, 2013 and 2014

Years	Fertilizer application	Species composition	Forage samples
2012	March 8	May 21 and 22	May 24
2013	March 12	April 30	May 13
2014	March 12	June 6	June 11

To assess the nutritive value, forage samples were ground to pass through a 1-mm screen. As a large number of samples were collected, Near Infrared Reflectance Spectroscopy (NIRS) was used at the INRAE laboratory in Lusignan to reduce costs (Foley et al., 1998; Lila & Furtoss, 2000). A NIRSystems 6500 model was used to obtain spectra of each sample between 1100 and 2500 nm. To develop calibration curves for the NIRS data, 10% of the samples were analyzed by standard procedures for crude protein content (CPC, in g kg^{-1} of DM, with CPC = N × 6.25, Dumas method), and acid detergent fiber (ADF, in g kg^{-1} of DM—Van Soest method; Van Soest 1982). The absorption data and chemical analyses for these samples were used in developing partial-least squares (PLS) regression equations for CPC and ADF. We then predicted the value from NIRS wavelengths. Chemical analyses allowed digestibility prediction of the forage (OM digestibility, in % OM).

2.3 Statistical Analysis

As data from seedling emergence are expressed as the number of seedlings per m², they were analyzed separately for each species or mixture. As a first step, we tested for the effect of species (or mixtures) and fertilization types on the percentage of sown legumes or mixtures using a mixed procedure, specifically for cases of repeated measures in time (as plots were not independent, *i.e.*, the measures were made on the same plots over 3 years). The species (or mixtures) (SPE), fertilization types (FERT) were considered as fixed effects, and the blocks as a random effect. When treatment effects were significant ($p \leq 0.05$), the difference between means were tested with Tuckey tests. Data were not transformed as they followed normality conditions. Analyses were first carried out with the 14 oversown species and seed mixtures. But, as one of the experiment objectives was to explore the success of legume oversowing, we repeated the analysis to assess the development and persistence of the 9 legume species in the cover. We carried out analyses for each of the three YEARS separately. The differences in DM yield and % of legumes between years in control plots were tested using non-parametric tests (Wilcoxon tests).

The same statistical procedure was then applied to: i) the DM yield of sown legumes; and ii) three variables summarizing the forage quality: crude protein content (CPC), fiber content (ADF) and organic matter (OM) digestibility.

In addition, the examination of the oversowing effect (without fertilization) on forage yield and quality (in particular CPC) was investigated by calculating two ratios [mean values in oversown F0 plots/mean values in control plots (without oversowing)] for CPC ($ratio_{CPC}$) and yield ($ratio_{yield}$) for each species or mixture and each year. A ratio > 1 indicates a positive effect of oversowing. We considered that a gain of at least 20 g of CPC kg^{-1} DM would positively affect the quality of hay for animals. This corresponds to ratios of 1.25 in 2012, 1.23 in 2013 and 1.3 in 2014. We also tested for a gain of yield of 20% (*i.e.*, 200 g kg^{-1} DM). These data were subjected to a one-sample Wilcoxon signed rank test (Bertrand and Maumy 2011; http://www.sthda.com/english/wiki/

one-sample-wilcoxon-signed-rank-test-in-r) to determine whether the median of the sample was > a theoretical value (*e.g.*, 1.0). All statistical analyses were performed with R statistical software (version 3.5.3).

3. Results

3.1 Weather During the Years of the Experiment

The rainfall data showed that the species establishment phase was characterized by a drier 2011 autumn than normal (32 mm *vs.* 82 mm average in the period 1980-2009). It rained on October 28, 2011, *i.e.*, one month after oversowing. Although above-average rainfall occurred in December (155 mm of rainfall *vs.* 86 mm for 1980-2009 average), we had relatively mild and dry winter conditions. Low minimum temperatures, including frosts, severely reduced plant growth in the 2011-2012 winter, and probably killed some seedlings. Two cold periods occurred: the first one from January 12 to 17, 2012 (6 days with a mean daily temperature of 1.7 °C) and the second one from February 1 to 12, 2012 (12 days with negative daily temperatures: -3.6 °C average). This was followed by a spring with average rainfall (April was however wetter than normal: 130 mm of rainfall *vs.* 69 mm average).

Autumn and winter 2012-2013 were wetter than normal (123 *vs.* 82 mm autumn average, and 112 *vs.* 73 mm winter average), but the following growing season was normal. In 2014, the experimental site experienced moderate drought conditions in spring (rainfall received in March-April was only 71 mm *vs.* 124 mm average).

3.2 Establishment of Oversown Species

Observations carried out on three different dates, a few weeks after September 2011, showed that most of the species emerged within 3 months of oversowing (Table 4). During the last observation, there were still seedlings emerging in many species. Among legumes species, the most successful establishment was obtained with Persian clover and garden vetch (present at more than 50 plants m^{-2}), followed by alfalfa, red clover, alsike clover, and hairy vetch, with 50 plants m^{-2}. Seedling emergence of Micheli clover was comparatively lower (with > 10 plants m^{-2}). We found the lowest establishment rate with white clovers (Aran and Aberdai), which showed only 10 plants m^{-2}. For seed mixtures, Proteïs Alliance and Herba'Regarnir had the best establishment (with 50 plants m^{-2}), but the Herba'Regarnir had difficulties persisting over time. Mélopro Trial had low emergence (10 plants m^{-2}) results and Herbafibre-FE was hardly differentiable from the native species.

Table 4. Density (number of plants m^{-2}) of established legume species or seed mixtures at three dates after oversowing in 2011

Species/mixtures	Number of seedlings		
	November 09, 2011	November 17, 2011	December 08, 2011
Species			
Alfalfa Galaxie	50 3 leaves, 2 cm high	50 3-4 cm high	>50 quite sparse in average
White clover Aberdai	10 3 leaves, 0.5 cm high	10 2 cm high	10 2-3 cm high
White clover Aran	10 2 leaves, 0.5 cm high	10 1.5 cm high	10 quite sparse, 3 cm high
Micheli clover Paradana	> 10 3-4 leaves	> 10 1-2 cm high	> 10 present but not regular, 2-3 cm high
Persian clover Cirrho	> 50 3 leaves	> 50 2 definitive leaves, 2-3 cm high	> 50 3-4 cm high, the most abundant vegetation
Alsike clover Aurora	50 0.5 cm high	50 1 definitive leaf, 2 cm high	> 10 3 cm high, not regular
Red clover Diplo	50 3 leaves	> 50 2-3 cm high	50 3-5 cm high
Garden vetch Pépite	> 50 5-7 cm high	> 50 9-10 cm high	> 50 > 10 cm high
Hairy vetch Savane	50 5-7 cm high	50 6-8 cm high	< 50 10 cm high
Tall fescue Elodie	10 6 cm high, more advanced than *Dactylis* sp.	10 8-9 cm high	10 > 10 cm high, very weak presence; difficulties to differentiate *Dactylis sp.* from other species
Seeds mixtures			
Proteïs Alliance	50 *Trifolium incarnatum*: 3 leaves; red or white clovers less advanced	50 *Trifolium incarnatum*: 6 cm high; other species are present but smaller (2-3 cm high)	50 *Trifolium incarnatum* present; other clovers also present, but sparse
Herba'Regarnir	50 clovers present, *Lolium sp*. abundant	> 10 strong presence of *Lolium sp.* (6-7 cm high); weaker clovers presence (1.5 cm high)	> 10 strong local disparity in seedling growth
Herbafibre-FE	10 difficult to differentiate between species (still at the seedling stage)	10 3 cm high; except *Lolium perenne* (8 cm high)	- difficult to differentiate between native and oversown species
Mélopro Trial	10 weak presence of clovers	10 clovers: 2-3 cm high *Lolium hybridum*: 8-10 cm high	10 good presence of all species but sparse

3.3 Effect of Oversowing on Species Composition in the Vegetation Cover

The development of oversown species was considered on the basis of their percentage in the vegetation cover. In the 3-year period, the results of the ANOVA analysis for repeated measures in time (carried out with the 14 oversown species and mixtures) showed that the percentage of oversown legumes or seed mixtures varied considerably between years (Table 5). The effect was significant for YEAR as well as the YEAR × SPE interaction, showing that owersown species did not have the same development every year. This difference between years was confirmed by the dynamics of species composition of forage in the control plots (no oversowing and no fertilization; Figure 1) where the percentage of native legumes was different from one year to another (but only when comparing year 2012 to 2014 and year 2013 to 2014; Wilcoxon tests: $P \leq 0.05$). The year 2014 in particular was not favorable for legumes, as even native legumes were observed in the cover.

Table 5. Results of the ANOVA with repeated measures on percentage of oversown species during the three-year period

Fixed effects	F	df	*P*
(intercept)	7.74	1	**
YEAR	8.43	2	***
SPE	30.99	13	***
FERT	3.08	5	*
YEAR × SPE	28.82	26	***

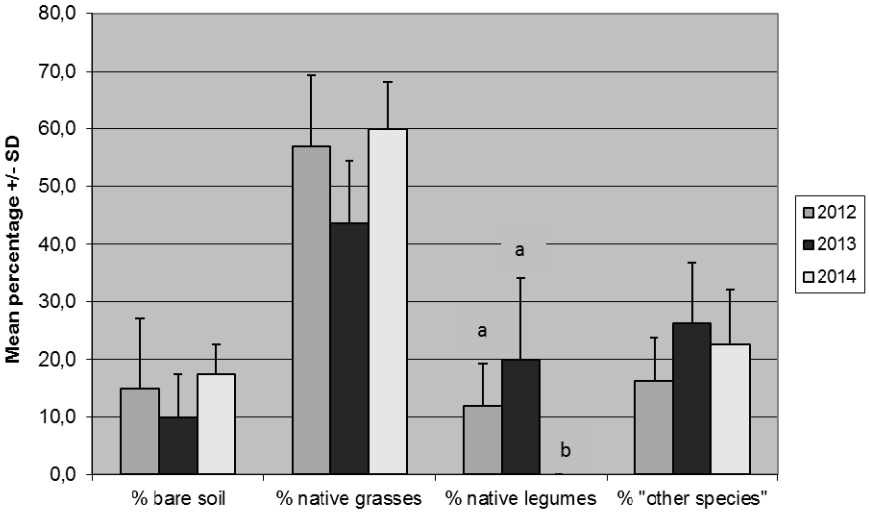

Figure 1. Dynamics of plants in plots not oversown, over three years (2012, 2013 and 2014) expressed in mean percentage±SD; Wilcoxon tests: different letters indicate a significant difference

We found a strong effect of the SPE factor (Figure 2). The proportion of Micheli clover in the cover (about 55% and 72% in average in 2012 and 2013) was about 4-5 times higher compared with all other legumes species (Tuckey test: all $P \leq 0.001$), despite a medium establishment rate in late 2011 (with > 10 plants m^{-2}). By contrast, three species with high establishment rates in late 2011, red clover, garden vetch and alfalfa, had the lowest contribution (≤ 5%) in the cover. For seed mixtures, the proportion of oversown species varied between 5% and 20%, depending on the year. The FERT effect was much less pronounced than the SPE factor. On average, only fertilization with slags resulted in a 5% higher establishment rate (significantly different from F0; $t = -3.85$, df = 15, $P \leq 0.05$; data not shown). We repeated the analysis by taking only the 9 legumes, and the results yielded very similar effects (YEAR × SPE interaction: $F = 34.63$, df = 16, $P \leq 0.001$; SPE effect: $F = 49.9$, df = 8, $P \leq 0.001$; FERT effect: $F = 3.35$, df = 5, $P \leq 0.05$).

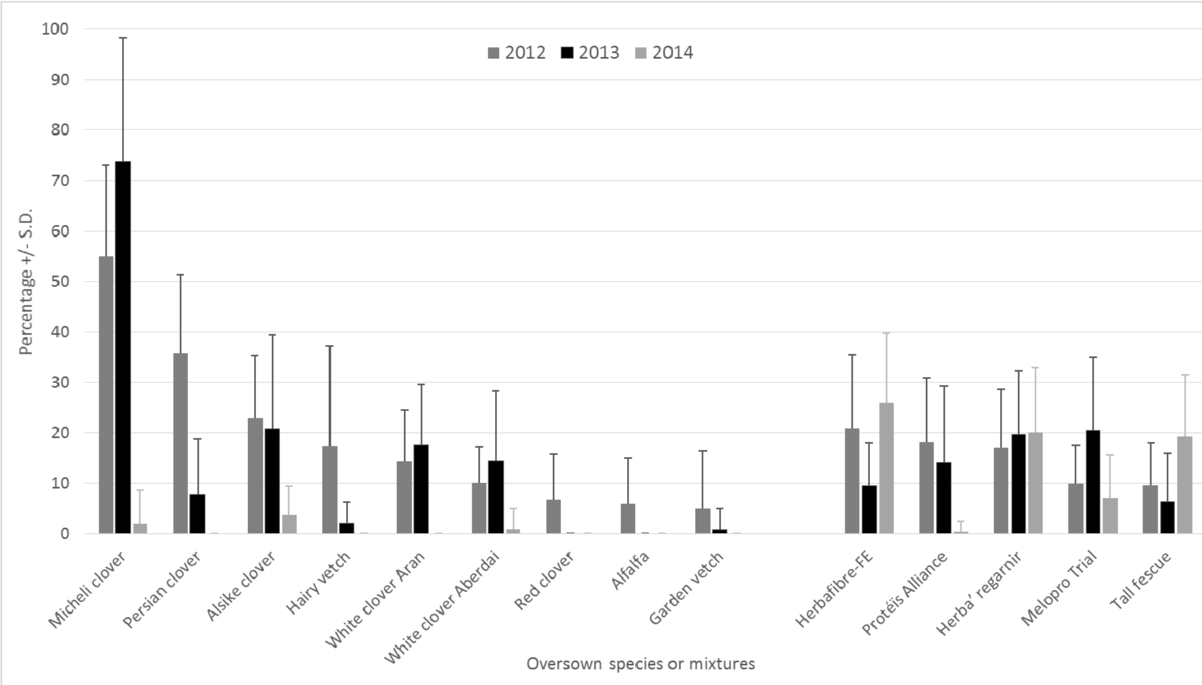

Figure 2. Mean percentages (±S.D.) of oversown species or mixtures over the three years (2012, 2013 and 2014)

The analyses carried out for each year separately showed that in 2012 there was a strong SPE effect ($F = 23.05$, $df = 13$, $P \leq 0.001$). Micheli clover, as well as Persian clover and alsike clover, had higher proportions than other species (with a difference of 50%, 31% and 18%, respectively, compared to garden vetch, for instance). Other species such as red clover, alfalfa and hairy vetch had the lowest percentages of abundance (6.7%, 5.8% and 5.0%, respectively).

Due to a high variability in the proportion of species within the 4 replicates, the FERT effect tended to be significant in 2012 only ($F = 3.55$, $df = 5$, $P = 0.049$). The proportions of oversown species with slags were significantly higher than those in F0 in 2012 ($t = -2.99$, $P = 0.037$). The SPE × FERT interaction was not significant.

In 2013, only the SPE effect was significant ($F = 25.30$, $df = 13$, $P \leq 0.001$). The Micheli clover still had the highest percentage of oversown species. It tended to be the most prevalent species in the cover; on certain plots the native grass was replaced by an almost 100% Micheli clover cover. By contrast, Persian clover progressively decreased. In 2014, analysis was not possible because there were no legume species in the cover anymore; many oversown legumes had disappeared.

We distinguished 3 categories of legumes according to their success in remaining in the cover over time (see Table 6 and Figure 3 for an illustration). The reference to evaluate the success or the failure of oversown legumes was being equivalent to 20% of native legumes obtained in control plots in 2013. In the first category, Micheli and Persian clovers were introduced successfully (> 20% in some or all of the oversown plots). Although Persian clover proportions decreased from 2012 to 2013, Micheli clover continued to progress (up to 80-90%). The second category was composed of the two white clovers, the alsike clover and the hairy vetch which showed a medium percentage frequency (between 10-20% in 2012). Their proportion in the cover remained stable in 2013, but they disappeared in 2014. In the last category, alfalfa, red clover and garden vetch were not introduced successfully (percentage frequency < 7%). They showed no increase in 2013 and disappeared in 2014.

Table 6. The 3 categories of legumes according to their success in establishment and persistence in the cover, averaged over the 3-year period

Successful establishment	Medium establishment	Poor establishment
Persian clover, Micheli clover	White clover Aberdai, white clover Aran, alsike clover, hairy vetch	Alfalfa, red clover, garden vetch
2012: > 20% 2013: proportion of Persian clover decreased, and of Micheli clover increased (up to 80-90%) 2014: Persian clover disappeared (Micheli clover was persistent but low (10-15%)	2012: between 10 and 20% 2013: remain at the same level, or a slight increase 2014: the species disappeared	2012: between 0 and 7% 2013: 0% or < 5% 2014: the species disappeared

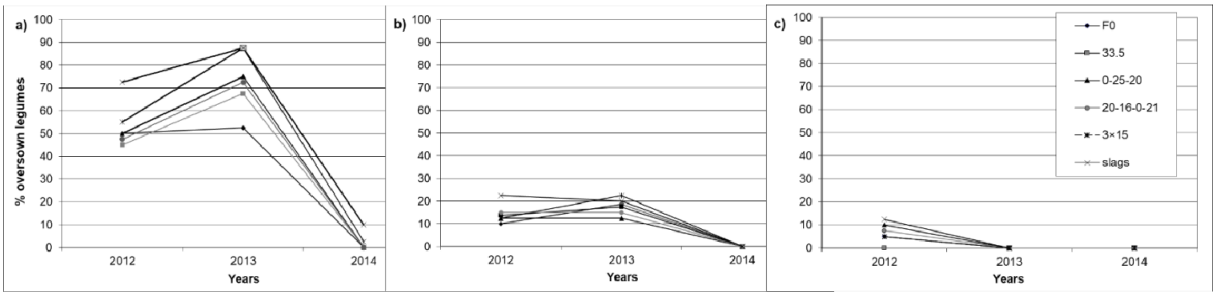

Figure 3. Dynamics of three legume species, a) Micheli clover, b) white clover Aran, c) red clover, illustrating the three categories of legumes (according to their presence each year)

3.4 Dry Matter Yield

In spring 2012, *i.e.*, the establishment year, oversowing without any fertilization led to a decline in the herbage DM yield. There was an average of 13% less herbage DM yield harvested from the oversown plots (Mann-Whitney U test however not significant, $P > 0.05$, because wide variability in yields, from one plot to another) compared with the controls without oversowing.

In the 3-year period, the ANOVA carried out with the 14 oversown species or mixtures showed a marked influence of YEAR on yield (Table 7). The forage production averaged 5.0±1.2 t DM ha^{-1} in 2012, whereas it was significantly higher in year 2013 (6.2±1.8 t ha^{-1}; Tuckey tests: $P \leq 0.01$). It then decreased significantly to 4.3±1.05 t ha^{-1} in 2014 (compared to 2012; $P \leq 0.05$). As regards the percentage of oversown species in the previous section, weather conditions caused the year-to-year variation in DM yields, also found in control plots (2012: 4.7 t DM ha^{-1}; 2013: 5.0 t ha^{-1}; 2014: 4.2 t ha^{-1}). In the conditions of the experiment, 2012 was a year with a medium production of forage, whereas 2013 and 2014 were, respectively, the most and the least productive of the three years.

Table 7. Results of the ANOVA with repeated measures on dry matter yield during the three-year period

Fixed effects	F	df	*P*
(intercept)	54.95	1	***
YEAR	26.29	2	***
SPE	2.22	13	**
FERT	1.71	5	ns
YEAR × FERT	6.23	10	***
SPE × FERT	1.34	65	*

There was a significant SPE effect (Figure 4), with the plots oversown with Micheli clover producing significantly (Tuckey tests: $P \leq 0.01$) more dry matter than white clover Aberdai (+13%), Proteïs Alliance (+12%), and Herba'Regarnir (+15%). There were no significant differences with other species or mixtures.

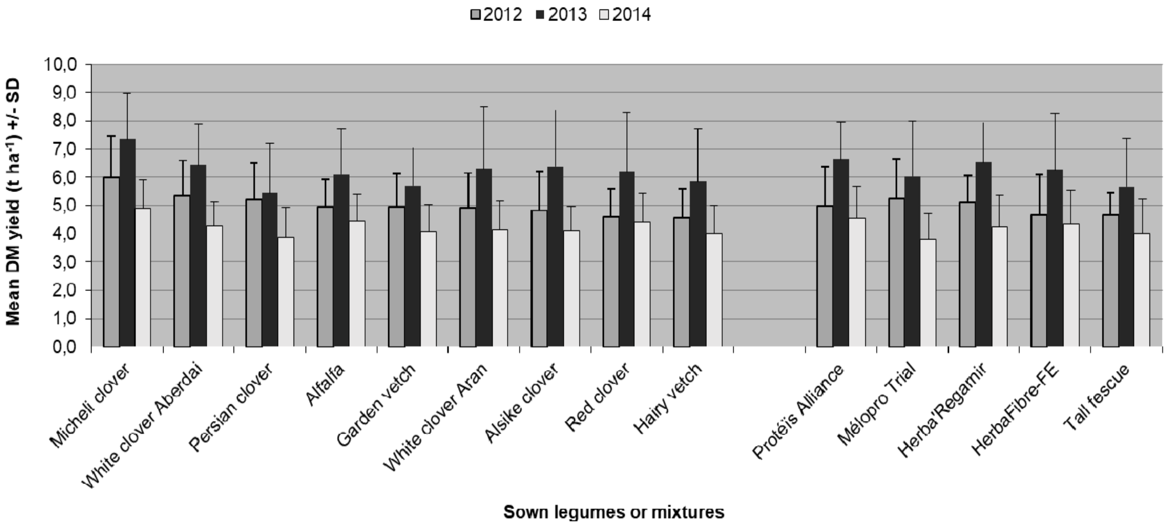

Figure 4. Mean yield (t DM ha^{-1})±S.D. over the 3-year period

There was no FERT effect (Figure 5), but the YEAR × FERT interaction was significant, showing that fertilizer inputs did not have the same effect on yield in different years. The significant SPE × FERT effect showed that the response to fertilization was different from one species to another. When the analysis was repeated with only the 9 legumes, the results again showed similar effects (YEAR effect: F = 19.11, df = 2, P ≤ 0.001; SPE effect: F = 3.71, df = 8, P ≤ 0.01; FERT effect: F = 6.38, df = 5, P ≤ 0.001; YEAR × FERT interaction: F = 3.40, df = 10, P ≤ 0.001) with Micheli clover having the highest yield of the 9 oversown legumes.

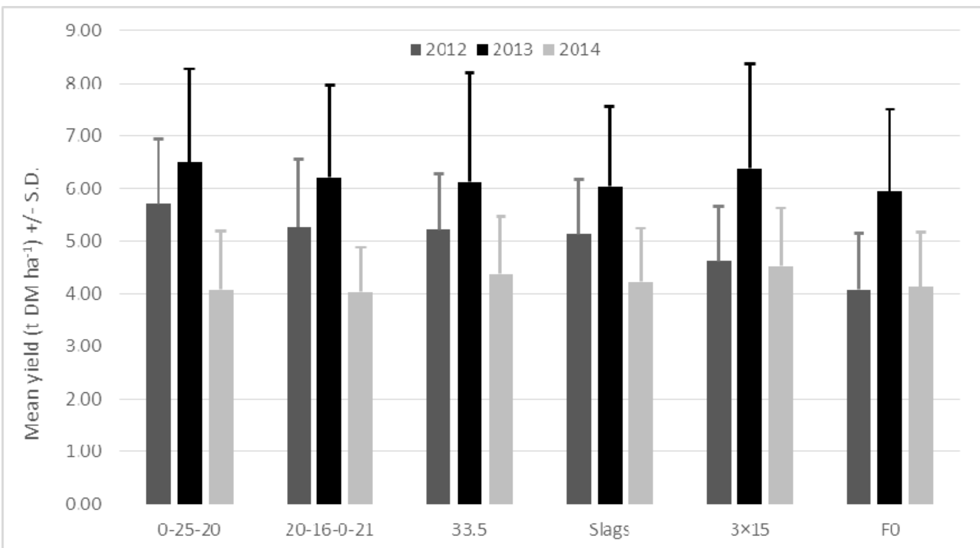

Figure 5. Mean yield (t DM ha^{-1})±S.D. according to fertilization types over three years

In 2012, there was no significant SPE effect (F = 1.27; df = 13; P = 0.23) nor FERT effect (F = 1.25; df = 5; P = 0.29). Although not significant, there was a kind of DM yield benefit from fertilization, as the mean DM yield was 5.7 t ha^{-1} for 0-25-20, 5.2 t ha^{-1} for 20-16-0-21, 33.5 and slags, and 4.6 t ha^{-1} for 3 × 15, with lowest DM yield of 4.1 t ha^{-1} for F0. The SPE × FERT interaction was significant (F = 1.43; df = 65; P ≤ 0.05), showing that oversown species/mixtures did not show the same response to the different fertilization types. The 0-25-20, 20-16-0-21 and to a lesser extent 33.5 and slags generally resulted in significantly higher DM yield compared to F0 (Figure 6).

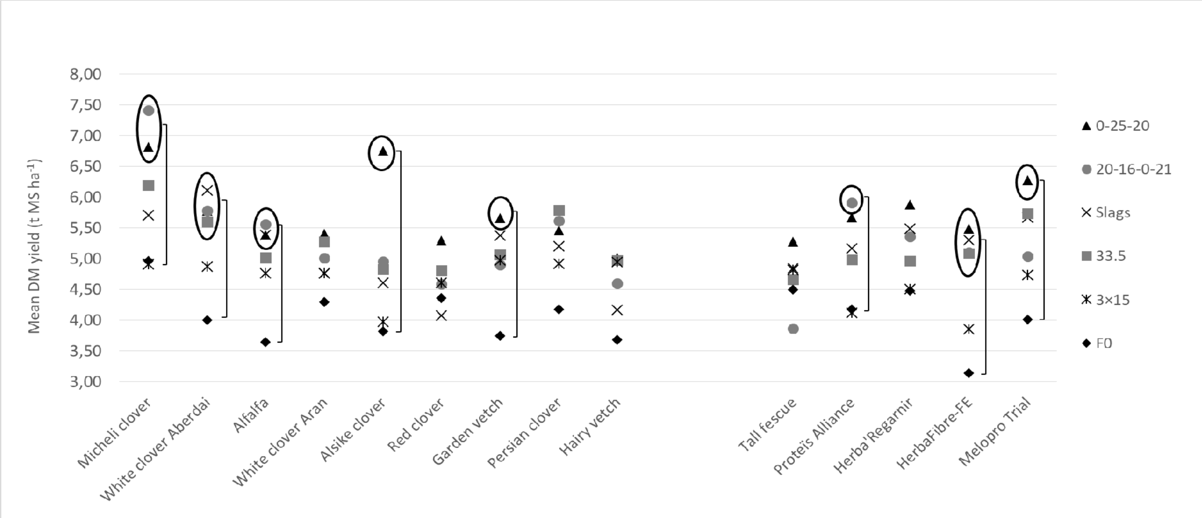

Figure 6. Mean DM yield in 2012 according to fertilization modalities for each legume species or mixtures. Points marked with an accolade are significantly different (Tuckey tests: all P ≤ 0.001)

The year 2013 was very productive, with higher DM for most species than in 2012. In 2013, there was no significant SPE effect (F = 1.26, df = 13; P = 0.24). Micheli clover completely dominated the vegetation, with the highest DM yield of 7.3 t DM ha^{-1}. Fertilization also had no effect (F = 0.55; df = 5; P = 0.74), nor did the SPE × FERT interaction (F = 1.09; df = 65; P = 0.31) on any of the sown species' yields. Here again, the 0-25-20 tended however to produce more DM yield than F0 (6.5±1.8 t ha^{-1} *vs.* 5.9 ±1.6 t ha^{-1}). In 2014, we found no effect at all from any treatment variable.

3.5 Effect on Forage Quality

Mean effects over the three year-period on the three components of forage quality, CPC, ADF and OM digestibility, are summarized in Table 8 (see Figure 7). We found a marked influence of the YEAR, with year 2013 having a higher CPC than others. Significant SPE and YEAR × SPE interaction effects were also found on forage quality, showing that three quality factors did not respond in the same way every year. The FERT and YEAR × FERT interaction effects were also significant, showing that the effects of fertilization modalities depended on the year. With the 9 legume species alone, the results were almost the same as with 14 species and mixtures.

Table 8. Results of the ANOVA with repeated measures on crude protein content (CPC), ADF and OM digestibility during the three-year period

Fixed effects	CPC			ADF			OM digestibility		
	F	df	*P*	F	df	*P*	F	df	*P*
(intercept)	393.54	1	***	4687.30	1	***	8260.73	1	***
YEAR	31.26	2	***	10.05	2	***	16.39	2	***
SPE	10.74	13	***	6.59	13	***	8.64	13	***
FERT	3.78	5	**	11.88	5	***	10.63	5	***
YEAR × FERT	2.56	10	**	5.42	10	***	5.09	10	***
YEAR × SPE	4.51	26	***	6.85	26	***	6.64	26	***

In 2012, we found a significant SPE effect on CPC (F = 10.18, df = 13, P ≤ 0.001), ADF (F = 9.17, df = 13, P ≤ 0.001) and OM digestibility (F = 11.92, df = 13, P ≤ 0.001). The highest CPC increase occurred with Micheli or Persian clovers (P ≤ 0.001), compared to hairy vetch (P ≤ 0.01), Herba'Regarnir, alfalfa, white clover Aberdai, alsike clover and garden vetch (P ≤ 0.05). The two most contrasting species were Micheli clover and tall fescue, 116.9±13.3 g kg^{-1} DM *vs.* 84.0±12.7 g kg^{-1} DM of CPC; 320.2±13.2 g kg^{-1} *vs.* 356.4±12.0 g kg^{-1} of ADF and 67.2±1.7% *vs.* 61.2±1.7% of OM digestibility. The FERT effect was significant for ADF (F = 21.4, df = 5, P ≤ 0.001) and OM digestibility (F = 15.1, df = 5, P ≤ 0.001), but was not the first factor to enhance the forage quality (a slight decrease of 20 g kg^{-1} DM in ADF; and a slight increase of 2.8% in OM digestibility).

In 2013, the SPE effect was significant only for ADF ($F = 5.2$, $df = 13$, $P \leq 0.001$) and OM digestibility ($F = 11.9$, $df = 13$, $P \leq 0.001$). The FERT had no effect on the forage quality. In 2014, no SPE effect was found and the only FERT effect on CPC ($F = 4.01$, $df = 13$, $P \leq 0.05$) was due to 20-16-0-21, showing significant but moderately higher CPC (73.0±6.8 g kg^{-1}) than F0 (69.3±6.1 g kg^{-1}).

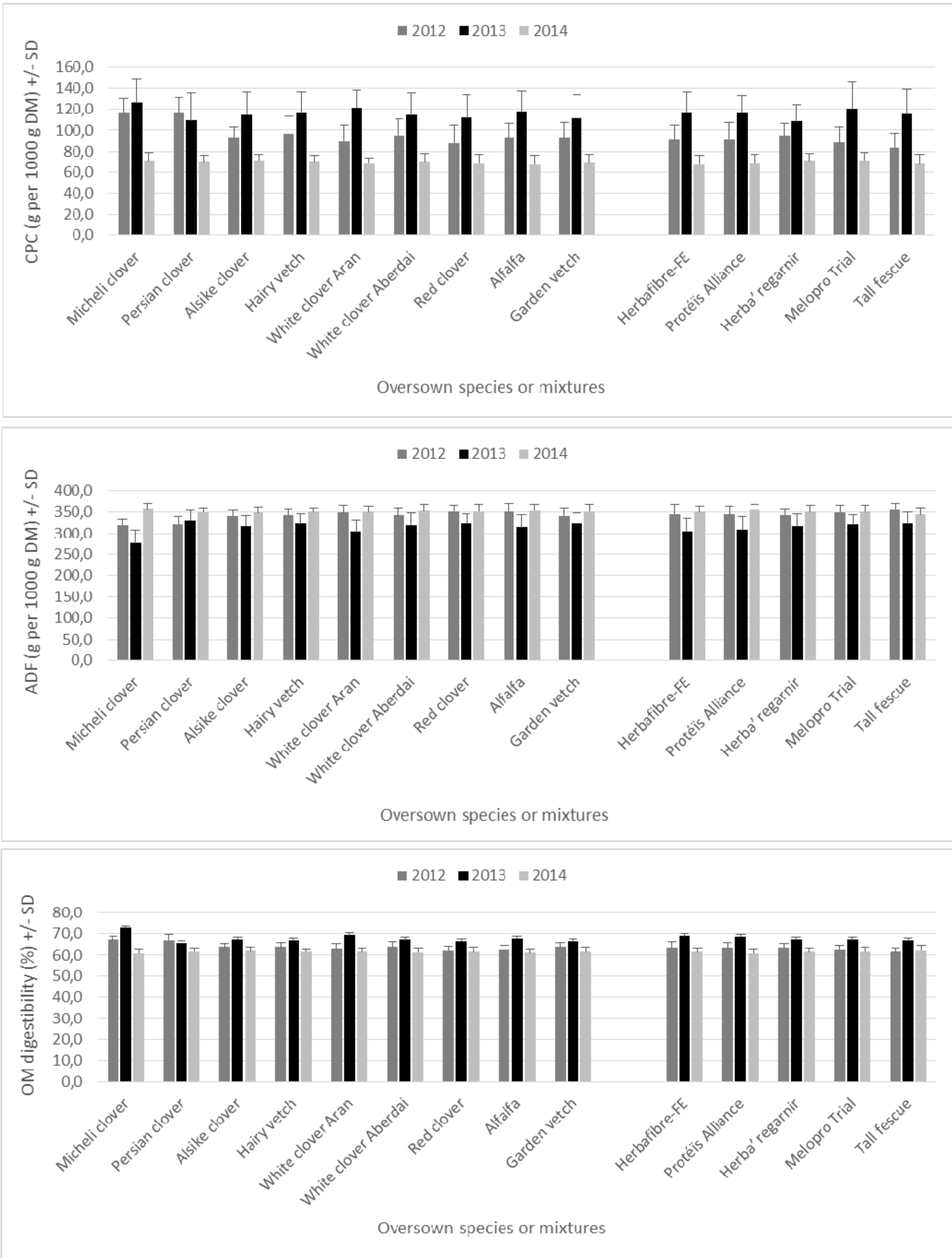

Figure 7. Mean CPC (g per kg DM), ADF (g per kg DM) and OM digestibility (%)±S.D. during the 3-year period

3.6 Effect on Forage Dry Matter Yield and Quality Ratios

To assess the overall benefit of oversowing legume species (or mixtures) and fertilization treatments, it was necessary to consider both forage quality and DM yield. The examination of the ratios [mean values in oversown F0/mean values in control plots], for $ratio_{CPC}$ and $ratio_{yield}$, showed that there was a slight depressive effect of oversowing on yield in 2012 (see the "Dry matter yield" section where we reported on average 13% less herbage DM yield harvested from the oversown plots; mean $ratio_{yield}$ = 0.90±0.29 for all species/mixtures; see also Figure 8). No oversown plot presented significantly higher CPC compared to the controls in 2012 (mean $ratio_{CPC}$ = 1.27±0.30 for all species/mixtures), even though there was a tendency for Micheli and Persian clover ratios to be > 1 (Wilcoxon signed rank test: V = 10, P = 0.063 for a $ratio_{CPC}$ = 1.57) and Persian clover (V = 10, P = 0.063 for a $ratio_{CPC}$ = 1.59). Testing the ratio > 1.25 (*i.e.*, a gain of at least 20 g CPC/kg DM), we found no significant effect (all P ≥ 0.05).

In 2013, oversowing generally had a positive effect on both CPC and yield (mean $ratio_{CPC}$ = 1.35±0.25; mean $ratio_{yield}$ = 1.26±0.35; figure not shown), but that was not significant, due to a high variability between plots. For all species or mixtures (apart from red clover, garden vetch and Proteïs Alliance), there was a tendency for $ratio_{CPC}$ to be > 1 (all V = 10, P = 0.063), and white clover Aberdai and Herba'regarnir tended to have both the $ratio_{CPC}$ and $ratio_{yield}$ > 1 (V = 10, P = 0.063). In 2014, we found that no oversown species of legume or mixtures gave higher CPC (mean $ratio_{CPC}$ = 1.04±0.10) or yield (mean $ratio_{yield}$ = 1.02±0.36; figure not shown) compared to the control plots (all P > 0.05).

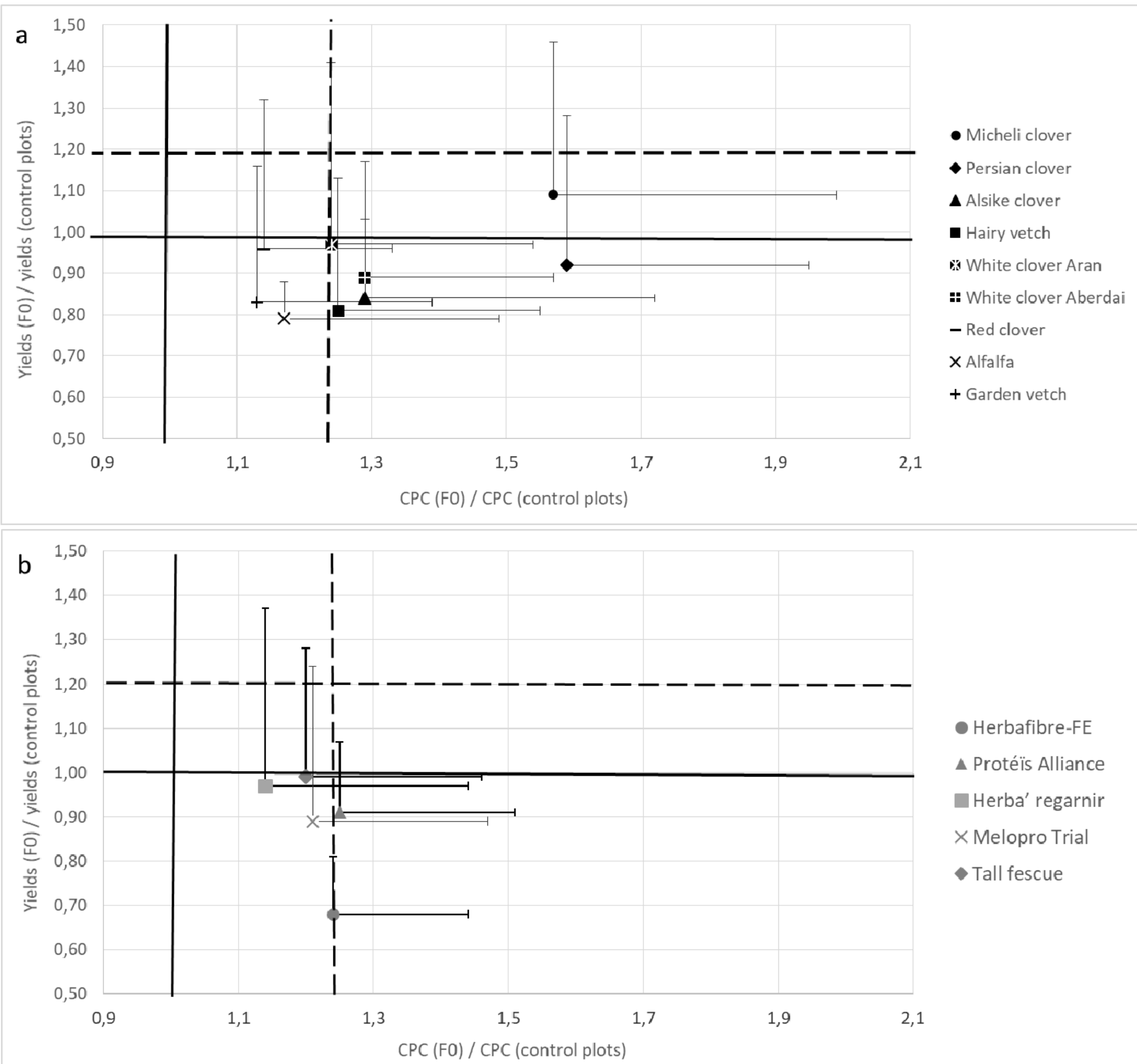

Figure 8. The $ratio_{CPC}$ and $ratio_{yield}$ in 2012, a) legumes and b) species mixtures. Each point represent the value of these two ratios±(positive) S.D.

The benefit of the fertilization was also investigated with the ratios [mean values in fertilized plots/mean values in F0]. In 2012, fertilization types generally had a positive effect on yield (all $P \geq 0.001$; mean $ratio_{yield}$ = 1.35±0.42); and 0-25-20, 20-16-0-21 and 33.5 even gave a yield gain of 20% and more (*i.e.*, their $ratio_{yield} \geq 1.2$; all $P \leq 0.01$; Figure 9). There was no significant effect on CPC (mean $ratio_{CPC}$ = 0.99±0.17). In 2013 and 2014, with rare exceptions, there was no positive effect of any fertilization types on both yield and CPC (P generally ≥ 0.05; figures not shown).

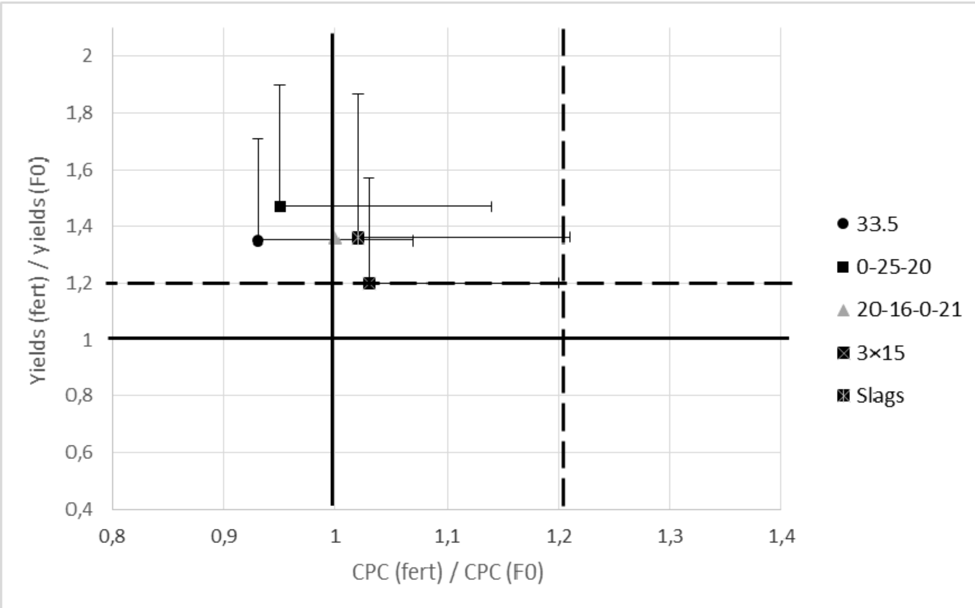

Figure 9. Relationships between the $ratio_{CPC}$ and $ratio_{yield}$ in 2012 for all fertilization treatments. Each point represent the value of these two ratios±(positive) S.D.

4. Discussion

4.1 Establishment of Oversown Species and Mixtures

The direct oversowing technique appeared to be successful in introducing plant species in permanent wet grasslands. It has been reported among efficient grassland restoration practices in previous studies (Jaurena et al., 2016; see also Golka et al., 2016 for a review). The early seedling stage is however a susceptible phase. Although we used no pre-oversowing treatments for disturbance of the existing sward (such as partial rotovation or low-dose application of herbicides), many seedlings survived through the spring of the first year. However, as soil surface moisture is important for the establishment of oversown plants (Awan et al., 1996), the lack of water in autumn 2011 probably resulted in lower numbers of seedlings than expected (the first rains came a month after oversowing). The freeze period in February 2012 certainly had an impact as well. Clearly, legumes that had a low establishment rate or low abundance in the sward were not those with smaller seeds. However, an apparently low establishment rate (about 10 seedlings m^{-2}) did not necessarily result in a failure in development. For instance, Micheli clover yields were significant despite a relatively poor seedling establishment.

4.2 Effect of Oversowing and Fertilization on DM yield

In spring 2012 (after the introduction of different species), there was on average 13% less herbage DM yield harvested from the oversown plots compared with the controls without oversowing. The oversowing certainly led to the death of some native plants, due either to disturbance to soil and plant roots by the seeder, or to competition between plant species. This may have reduced the beneficial effects of oversowing. Thus, if this trial were to be repeated, we would recommend a treatment by passing the oversowing seeder through control plots (without oversowing) to measure actual benefits from oversowing.

Considering the 9 legumes tested, we clearly identified 3 categories of species according to their ability to establish and persist. First, two clovers introduced successfully, *i.e.*, Micheli (despite poor seedling emergence) and Persian clovers were the most successful species in the vegetation cover. Although Persian clover decreased from 2012 to 2013, there was an increase in Micheli clover (up to 80-90%) because this species produced seeds before mowing in May 2012. In the second category, the two white clovers, alsike clover and hairy vetch had a proportion of 10-20% in 2012. They remained stable in 2013, and then disappeared in 2014. In the third category, 3 species (alfalfa, red clover and garden vetch) were not introduced successfully.

In 2014, there were no more oversown legumes in the plant cover. This seems to be mainly due to unfavorable conditions for legumes a few weeks before plant observations. It may be partly attributed to moderate drought conditions in the growing season of 2014, which would also explain the disappearance of native legumes in control plots. Oversowing of Micheli clover Paradana increased yield and nutritional quality but this big-leaf clover had clearly grown at the expense of other species. It is an invasive species as seeds can spread very easily if not controlled. Thus, it may be advisable to avoid oversowing it. Conversely, legumes such as Persian clover, both white clovers—Aran and Aberdai—alsike clover and hairy vetch could be better candidates for future studies. Both vetches are however annual, which means that they have to be re-sown every year, or else their seed dispersion must be facilitated by appropriate cutting management. For seed mixtures, the six-species mixture Protéïs Alliance retained a relatively good yield rank and presence of legumes over the first two years, although sown species disappeared in 2014, as did the legumes. Mélopro Trial can also be a candidate for oversowing as legumes were present in 2013. We found no benefit in sowing grass mixtures (Herba'Regarnir and Herbafibre-FE) or tall fescue, compared to mixtures with legumes.

It is well known that P is essential for legume establishment (Papanastasis, 1980; Osman et al., 1991), and annual P applications are required to maintain legume production and to ensure the persistence of oversown species. Surprisingly, we found that only some treatments with P applications positively affected the percentage of legumes. The effects of fertilization were however not clear-cut, due to a high variability within the 4 replicates, though slag tended to favor legumes, especially in the first year of establishment (2012). Judging by yields in 2012, 0-25-20 and 20-16-0-21 had an effect even if they were not sufficiently different from other fertilizers (apart from 3 × 15 and F0). In 2013, the higher productivity can partly be attributed to favorable weather conditions, but may also be due to the establishment of nitrogen-fixing and productive legumes, masking the effects of fertilizers (even though 0-25-20 tended here again to produce more than F0). Fertilization had no effect in 2014, surely because of the spring drought phase that could have prevented fertilizers from being effective. Another reason could be due to the application time of P fertilization, which is generally suggested in the autumn (Aydin and Uzun, 2005). In the present study, the application of P fertilizers was in early spring, which could have prevented the plant response in this drier spring than normal, although Koc (2013) found no difference between autumn and spring application.

4.3 Enhancement of Forage Quality

We posited that the introduction of legumes in the grass could increase the forage quality and production. We found that this introduction could partly increase the feeding value in terms of CPC of the herbage by some species. In 2012, oversowing had a slightly beneficial effect on forage quality, in particular the Micheli and Persian clovers. In 2013, this effect lasted but there was also the effect of earlier mowing (on May 13) to prevent the flowering and seeding of the invasive Micheli clover. At the time of harvesting, the majority of its plants were still in a vegetative stage, which had positive effects on the forage quality. This can also be seen from the slightly higher CPC in control plots in 2013 (85.6±11.2 g kg^{-1} DM) compared to 2012 (79.9±19.4 g kg^{-1}) or 2014 (67.2±4.2 g kg^{-1}). In this study, oversowing alone was thus able to improve forage quality.

The tested fertilizers had no positive effect on the forage quality. Favoring the development and maturity of the general cover (grasses), fertilization such as 33.5% would even tend to have a depressing effect on CPC (ratio < 1), because it tended to favor the development of grasses at the expense of legumes. For multi-species mixtures (*i.e.*, more than 2 species), their advantage is nutritional quality which is fairly constant (unlike some legumes that have very low or very high CPC values). Finally, in terms of forage quality, we found no benefit in oversowing grass mixtures.

Our results showed that, in the conditions of this study, legumes were difficult to maintain in an existing sward. This is consistent with previous studies, as the lack of persistence of legumes is a recognized constraint to their use (Muir et al., 2011; Laberge et al., 2005). As we know, the establishment of legumes is affected by management practices. Some limitations may be attributed to the hay-cutting management applied in this study, which may have impeded the development of some species that do not tolerate competition for resources such as light. During the establishment phase, slight defoliation (by cutting) may have provided light for slower-growing young legumes. On the other hand, early cutting (in mid-May 2012 and 2013 for two years after sowing) may have prevented the flowering of legumes and seed setting to occur. We however believe that the loss of all sown legume species was due to a 'weather effect': dry in early 2014 (when even local legumes did not persist in the cover). Our results, consistent with those of Del Pino et al. (2016), have thus shown that when there are some benefits of oversowing (and fertilizer applications), they are short-lived. This raises the issue of the frequency of oversowing to maintain its positive effects over time. The frequency of oversowing in meadows (generally every 4-5 years) may therefore need to be more frequent in permanent wet grasslands.

4.4 Implications of This Study

This study showed that oversowing is feasible in permanent wet grasslands such as those typical of the dry marshes of the French Atlantic coast (Durant et al., 2014). However, as these grasslands being of conservation value (Michelet et al., 2016), this raises the question of the relevance of oversowing for a biodiversity-oriented management of these meadows. Although it may improve the quantity and the protein content of the diet for animals, and thus increase livestock productivity on small farms (MacLeod & Cook, 2004; Ferreira et al., 2011), the question is whether oversowing should be encouraged under the current agroecological prescriptions for natural grasslands.

During the experiment, there was concern that oversowing would have negative consequences on local plant communities, especially in the case of Micheli clover which turned out to be an invasive species. The objective was to enhance the forage quality and quantity, rather than to replace local flora with legumes. Our findings suggest that a trade-off between the provision of high-quality hay and the risk of a reduced diversity of the plant community may arise, as Jaurena et al. (2016) had already pointed out. More importantly, the short-lived (limited) benefits of oversowing and fertilizer applications do not really advocate for the use of this technique.

We therefore believe that practices supporting the enhancement of hay meadows diversity in marshes warrant further investigation. For instance, if the use of commercial pasture mixtures (generally made for temporary grasslands) is considered incompatible with the overall environmental objectives, more attention may be paid to native herbaceous legumes as potential species to oversow. Trials could consist in oversowing local legumes, *i.e.*, seeds collected from species such as *Trifolium michelianum* naturally present in wet grasslands. Other successful techniques on historically N fertilized grasslands could also be tested, such as stopping nitrogen inputs and combining water maintenance, particularly in autumn/winter (water control in duration of flooding, through hydraulic engineering) and appropriate grass management to see if legumes develop or reappear naturally. There is thus a need for research to continue to invest in agroecological management of wet grasslands.

In conclusion, we tested the introduction of legumes (and mixtures) in a hay meadow. In the conditions of this experiment, introduction was feasible and successful for some species. It can partially increase forage quality, particularly in the first year after establishment. We found however that the benefits were limited and short-lived, as the maximum persistence of legumes was limited to two years after oversowing. As conservation targets of wet grasslands are of concern, we recommend further research on practices allowing the natural return of legumes to these wet grasslands.

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