Encroachment of shrubs into subalpine grasslands in the Pyrenees changes the plant-soil stoichiometry spectrum

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26   **ABSTRACT**

27   **Aims**

28   Shrub encroachment has been reported over a large proportion of the subalpine grasslands across Europe and

29   is expected to have an important impact on the biogeochemical cycle of these ecosystems. We investigated

30   the stoichiometric changes in the plant-soil system along the succession (e.g. increase in encroachment from

31   unencroached grassland to mature shrubland) at two contrasting sites in the Pyrenees.

32   **Methods**

33   We analyzed the chemical composition (C, N,^{15}N, P, K, Ca, Mg and Fe) in the soil and in the aboveground

34   plant compartments (leaves, leaf-litter and stems) of the main herbaceous species and shrubs at three

35   contrasting stages of the succession: unencroached grassland, young shrubland and mature shrubland.

36   **Results**

37   The plant-soil stoichiometry spectrum differed between the successional stages. Shrub encroachment

38   generally increased the concentration of C and Ca and the C:N ratio and often reduced to concentrations of N,

39   P and K in the leaves and leaf-litter, while several soil nutrient concentrations (N, P, K Ca and Mg) decreased.

40   The stocks of C, N, P, Ca, and Mg in the total aboveground biomass increased with encroachment.

41   **Conclusions**

42   Shrub encroachment favored the dominance of long-lived species with low concentrations of N and P in the

43   plant-soil compartments, high C:nutrient ratios in the aboveground biomass and increase the uptake of N

44   through ericoid or ectomycorrhizal fungi. We highlight the role of shrubs in the sequestration of C and

45   nutrients through the allocation to the aboveground biomass. The changes in plant-soil elemental composition

46   and stocks suggest a slowdown of the biogeochemical cycles in the subalpine mountain areas where shrub

47   encroachment occurred.

48

INTRODUCTION

The encroachment of shrub species into grasslands causes important changes in many grass-dominated ecosystems at landscape and regional scales from low to high latitudes (Van Auken 2009, Myers-Smith et al. 2011, Naito and Cairns 2011, Komac et al. 2013, Formica et al. 2014). The expansion of shrubs is mostly caused by changes in climatic conditions and land use (Eldridge et al. 2011). For example, the ongoing increase in mean annual temperature and the thinning of permafrost at high latitudes are promoting the expansion of shrubs across the Arctic and subarctic tundra (Tape et al. 2006; Hallinger et al. 2010; Myers-Smith et al. 2011). Shrub expansion has also been observed in most mountain ranges and massifs across Europe at lower latitudes, where humans have used the subalpine and alpine grasslands for traditional activities such as extensive livestock herding (Roura-Pascual et al. 2005; Anthelme et al. 2007; Hallinger et al. 2010; Targetti et al. 2010; Ferré et al. 2013) for many centuries or even millennia (Gassiot and Jiménez 2006; Pèlachs et al. 2007; Gassiot et al. 2016). The progressive abandonment of these practices in recent decades has favored the encroachment of shrubs into subalpine and alpine grasslands (MacDonald et al. 2000; Dullinger et al. 2003; Komac et al. 2013; Ameztegui et al. 2016). Climatic warming is also expected to promote the expansion of woody species in subalpine and alpine regions in the Pyrenees (Grau et al. 2013; Peñuelas et al. 2016; Angulo et al. 2019) and across Europe in the coming decades (Sanz-Elorza et al. 2003; Körner and Paulsen 2004; Wookey et al. 2009; IPCC 2013). The abandonment of subalpine and alpine grasslands is very apparent in the Pyrenees (northeastern Iberian Peninsula), where traditional extensive livestock grazing and the frequency of intentional fires to create or maintain pasture have substantially declined in the last century, mainly due to socio-economic changes in this area (Lasanta et al. 2000; MacDonald et al. 2000; Serrano et al. 2000; Roura-Pascual et al. 2005; Jiménez and Pujol 2010; Barrio et al. 2013).

Shrub expansion is expected to cause several changes in ecosystem functioning, but few studies have focused on the impacts of expansion on the functioning of subalpine or alpine grasslands in the Pyrenees (Vitousek 1984; Montané et al. 2007, 2010; Barrio et al. 2013; Catalan et al. 2017; Grau et al. 2019) and across Europe (MacDonald et al. 2000; Wookey et al. 2009). Some studies have reported that shrub encroachment has increased the carbon (C) and nitrogen (N) concentrations in the soil and reduced soil pH (Knapp et al. 2008; Eldridge et al. 2011), but such changes may strongly depend on shrub traits and

77 ecosystem features. Shrub and grass species have contrasting growth forms and differ in many functional

78 traits of their adaptive and reproductive capacities and their strategies of resource acquisition and allocation

79 (Chapin and Körner 1994). Different co-occurring shrub species may also have contrasting traits and

80 strategies (Illa et al. 2017), so the identity of shrubs is also crucial to our understanding of changes in

81 ecosystem functioning when encroachment occurs (Grau et al. 2019). Succession from grassland to shrubland

82 is thus expected to cause important changes in the distribution, cycling and stoichiometry of chemical

83 elements in the plant-soil system. Furthermore, the increase in woody biomass during the succession adds

84 complexity to the persistence and cycling of C and nutrients in the ecosystem. Wood is highly persistent and

85 stores C and nutrients such as N, phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) (Chave

86 et al. 2009; Sardans and Peñuelas 2015). The nutrient stocks stored in wood, however, depend mainly on

87 wood density and may vary greatly among species (Sardans and Peñuelas 2013; 2015).

88   Most of the research on the impacts of shrub encroachment into grasslands has focused on soil C and

89 N balances (Hibbard et al. 2001; Jackson et al. 2002; Hood et al. 2003; Throop and Archer 2008; Van-Auken

90 2009), including studies conducted in the Pyrenees (Garcia-Pausas et al. 2007; Montané et al. 2007; Garcia-

91 Pausas 2010). However, changes in the chemical composition of both plants and soils, including key elements

92 such as P, K, Ca, Mg and iron (Fe), along the succession from grassland to shrubland, have not yet been

93 investigated in detail. We studied two common and contrasting landscapes where shrub encroachment

94 typically occurs in the Pyrenees, one facing north (hereafter North face) and one facing south (hereafter South

95 face). Each study site represents an independent case study because the grassland and shrubland species differ

96 between the two sites. In each of the two sites, we established a replicated sampling design in three

97 contrasting stages along the succession: a) the unencroached grassland (initial stage of succession, where

98 shrubs are not yet present), b) the mixed, young shrubland (intermediate stage of succession, where grassland

99 has small patches of shrubs) and c) the mature shrubland (advanced stage of succession, where the dominant

100 shrub species form large, monospecific patches). The main aim was to analyze the changes in the chemical

101 composition (concentrations and stocks) of the aboveground-biomass compartments (leaves and leaf-litter in

102 herbaceous species; leaves, leaf-litter and stems in shrubs) and the soil along the succession from grassland to

103 shrubland in the Central Pyrenees.

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104   A previous study conducted in the same study sites in the Pyrenees highlighted that the

105   concentrations of some soil nutrients (e.g. N, P or K) generally declined along the succession from grassland

106   to shrubland (Grau et al. 2019), although there were differences between sites and shrub species. It was

107   hypothesized that shrubs may store nutrients in the biomass to take control of nutrients of the ecosystem to

108   avoid nutrient limitation and promote their further expansion along the succession, as hypothesized for

109   forested ecosystems with nutrient limitation in the soil (Grau et al. 2017). In this current study, we explore if

110   this hypothesis can be supported and investigate whether the decline in the concentrations of soil nutrients

111   along the succession was coupled with changes in concentrations and/or stocks of nutrients in the

112   aboveground biomass. Moreover, shrub expansion into grassland is expected to shape the plant-soil

113   stoichiometry spectrum and the biogeochemical cycle through changes in the abiotic and biotic conditions

114   along the succession from grassland to shrubland. We thus hypothesized that each successional stage would

115   show a contrasting plant-soil stoichiometric spectrum, resulting from changes in vegetation structure and

116   allocation strategies in each stage, and that C:nutrient ratios in the aboveground biomass and soil would

117   increase over the succession due to the lignification of plant compartments.

118   **MATERIAL AND METHODS**

119   *Study area, species and sampling design*

120   The two study sites were located in the Central Pyrenees (NE Iberian peninsula), one site on the North face

121   (Bargadèra, Val d'Aran, 42°39′53.9″N, 00°50′07.8″E, with Cambro-Ordovician schists, sandstones and

122   quartzites) and one on the South face (Fogueruix, Pallars Sobirà, 42°35′32.9″N, 01°04′28.8″E, with a

123   Devonian slaty limestone), both on the periphery of the 'Aigüestortes i Estany de Sant Maurici' National Park

124   (Figure SF1). The two sites are separated by only 22 km but are characterized by contrasting macro- and

125   microclimates; the site in Val d'Aran has a strong Atlantic influence, whereas the site in Pallars Sobirà is

126   more continental. The mean annual number of days with precipitation or fog and the relative air humidity are

127   therefore   higher   at   North   face   than   South   face   (Catalan   Meteorological   Service,

128   http://meteo.cat/wpweb/climatologia, accessed on March 2018). The North face is a smooth north-facing

129   slope at an altitude of 1800-1900 m a.s.l. Since 2015, annual precipitation at the nearest meteorological

130   station to North face (Bagergue, 1400 m a.s.l.) has ranged from 800 to 900 mm, mean annual temperature has

131   ranged from 8 to 9 °C and minimum and maximum absolute temperatures were -14 and 30 °C, respectively.

132   The South face site is a steep south-facing slope at an altitude of 2000-2100 m a.s.l. Since 2015, annual

133   precipitation at the nearest meteorological station to South face (Planes de Son, 1540 m a.s.l.) has ranged from

134   600 to 800 mm, mean annual temperature has ranged from 8 to 10 °C and minimum and maximum absolute

135   temperatures were -15 and 30 °C, respectively. Both meteorological stations are at altitudes (ca. 400 m) lower

136   than the study sites, so precipitation is expected to be higher and temperatures are expected to be lower

137   (average and range) than the reported values at both sites. Both sites are in areas that have been intensively

138   used for livestock grazing for many centuries, but socio-economic changes in the Pyrenees since the 1950s

139   reduced the density of livestock, and some summer pastures have been abandoned (MacDonald et al. 2000;

140   Jiménez and Pujol 2010), which have favored the expansion of shrubs into these former grasslands. The two

141   sites differ greatly in their abiotic conditions (macro and microclimate, bedrock lithology, topography) and in

142   the functional characteristics of their vegetation (biotic conditions); the dominant shrubs are:

143   *Calluna vulgaris* (L.) Hull, *Rhododendron ferrugineum* L. and *Vaccinium myrtillus* L. at North face and

144   *Arctostaphylos uva-ursi* (L.) Spreng, *Juniperus communis* L. and *Juniperus sabina* L. at South face.

145   The grassland at North face is classified as a mesophilic, dense subalpine grassland '*Nardion*' (Galvánek and

146   Janák 2008), dominated mostly by grasses (*Festuca eskia* Ramond ex DC. and *Festuca nigrescens* Lam.) with

147   scarcer forbs (*Trifolium alpinum* L., and *Cerastium arvense* L.). The succession is mostly driven by the dwarf

148   shrubs *Calluna vulgaris*, *Rhododendron ferrugineum* and *Vaccinium myrtillus*. The grassland at South face is

149   classified as a xerophilic, open montane grassland '*Xerobromion*' (Carreras et al. 1983; EAE 2019),

150   dominated by grasses (*Festuca ovina* L. and *Festuca gautieri* (Hack.) K.Richt) that co-occur with small forbs

151   (such as *Hieracium pilosella* L., *Achillea millefolium* L. and *Potentilla neumanniana* Rchb.). The succession

152   from grassland to shrubland is mostly driven by the dwarf shrubs *Arctostaphylos uva-ursi*,

153   *Juniperus communis* and *Juniperus sabina* L. and to a much lesser extent by

154   *Helianthemum nummularium* (L.) Mill. or *Thymus pulegioides* L.

155   Soils at the North face site are built from Cambro-Ordovician schists, sandstone and quartzite, and, given the

156   moderate slope of the area, they are moderately deep, rich in organic matter, and acidic, corresponding in

157   general to Humudepts. Those at the South face site result mainly from Silurian pelite and also from Devonian

158   lime-rich slate. Being this site steeper, soils are shallower and irregular, moderately acidic and humic, broadly

159   corresponding to lithic Humudepts (Boixadera et al. 2014). Moreover, soils at the South face experience

160   summer drought episodes resulting into apparent drying off of the shallow soil level, which does not occur at

161   the North face. The soil pH in the North face tend be more acidic (pH around 4.7-5) than in South face (pH

162   5.7-6.3) (Grau et al. 2019). The percentage of soil organic matter measured in the same study sites were

163   higher at North face (herbaceous species: 21.2%, *Calluna vulgaris: 37.8%, Rhododendron ferrugineum:*

164   40.12% and *Vaccinium myrtillus* 40.6%*)* than South face (herbaceous species: 16.5%, *Arctostaphylos uva-*

165   *ursi*: 16.2%, *Juniperus communis*:13.2% and *Juniperus sabina:* 13.4%) (Grau et al. 2019).

166   We selected three stages at each site along the succession from grassland to shrubland: a) unencroached

167   grassland, which is still regularly grazed by domestic animals (Oriol Grau, pers. observation), is dominated by

168   herbaceous species and has no shrubs, b) young shrubland, composed of a mosaic of herbaceous species and

169   shrub patches of ca. 1-2 m^2 and c) mature shrubland, where the dominant shrub species form large

170   monospecific patches (at least 10×10 m). The mixed shrub patches in the young shrubland covered 25-30 %

171   of the ground, whereas the shrubs in the in the mature shrubland covered 90-100% of the ground.

172   We selected the most common shrubs at each site (*C. vulgaris*, *R. ferrugineum* and *V. myrtillus* at North face

173   and *A. uva-ursi*, *J. communis* and *J. sabina* at South face; see suplementary Table ST1a, b for an ecological

174   and functional characterization of the shrub species studied). The herbaceous species and each of the shrubs

175   occurring along the succession in each site will hereafter be referred to as 'vegetation types'.

176   The replication of the successional stages was done in each site separately; the two sites were not treated as

177   replicates in the analyses but as two independent study cases. Sampling plots were established to reproduce

178   the extant structure of the vegetation in each successional stage. We established four replicate 2×2 m plots

179   (separated by a minimum of 10 m) in the unencroached grassland, and in each of three mature shrublands at

180   each site (Fig. 1 and Grau et al. 2019 for further details). In the young shrubland, though, the plots for each

181   vegetation type (grass, shrub 1, shrub 2, shrub 3) were grouped because all vegetation types co-occurred; in

182   this intermediate stage, we established four groups of four plots. In total we sampled 32 plots per site along

183   the succession from unencroached grassland to mature shrubland (see Fig. 1). The distance between the

184   successional stages or among the mature shrublands of each shrub species was >100 m and the distance

185   between the four replicates inside each successional stage was 10 m. The plots in the unencroached grassland

186   were placed in areas that represented the mixture of grass species that co-occurred in this successional stage,

187   whereas the plots in the mature shrubland were placed in large patches dominated by each of the shrub

188   species.

189   We collected plant and soil samples towards the end of the growing season (September 2015) for the analysis

190   of their elemental compositions. The litter layer was removed prior to the soil sampling; root samples could

191   not be collected so this belowground compartment could not be accounted for this study. We collected a total

192   of 144 shrub samples: 2 sites $\times$ 2 successional stages where shrubs were present (young shrubland and mature

193   shrubland) $\times$ 3 shrub species $\times$ 4 replicates $\times$ 3 plant compartments (leaves, leaf-litter -dead leaves still

194   attached to the plant- and stems). We collected a total of 32 samples of a mixture of herbaceous species in the

195   unencroached grassland and the young shrubland: 2 sites $\times$ 2 successional stages where herbaceous species

196   were present (unencroached grassland and young shrubland) $\times$ 4 replicates $\times$ 2 plant compartments (leaves

197   and leaf-litter). We also collected soil samples to a depth of 10 cm next to each plant sample with a 5-cm

198   diameter soil corer, 48 samples for shrubs (2 sites $\times$ 2 successional stages where shrubs were present $\times$ 3

199   shrubs species $\times$ 4 replicates) and 16 samples for herbaceous species (2 sites $\times$ 2 successional stages where

200   herbaceous species were present $\times$ 4 replicates), making a total of 64 soil samples. The concentrations of C,

201   N, P, K, Mg, Ca and Fe (see details of the chemical analyses in the next section) and ^{15}N were measured in

202   leaves, leaf-litter, lignified stems and soils. The samples were transported to the laboratory in paper

203   envelopes, the soil samples were sieved (2 mm) and all samples were oven-dried at 60 °C for 48 h. They were

204   then ground with a ball mill (Retsch, model MM400. RestchGmbH. Haan, Germany), weighed with an

205   AB204 Mettler Toledo (Mettler Toledo, Barcelona, Spain) and analyzed in the chemistry laboratories at

206   Servei d'Anàlisi Química, Autonomous University of Barcelona (Barcelona, Spain), where the percentages of

207   P, K, Mg, Ca and Fe in dry weight were determined, and at the University of California Davis Isotope Facility

208   (Davis, USA), where the isotopic compositions (δ^{15}N) and percentages of C and N in dry weight were

209   determined.

210   We revisited the same sampling areas in September 2016 and collected more samples to characterize the

211   mean biomass of each plant compartment and the bulk density of the soil, which we used to estimate the

212   stocks of C and nutrients in the aboveground compartments (g m^{-2}) of the vegetation types and the soil. We

213   assumed that the aboveground biomass of the herbaceous species was similar between the two years, because

214   their aboveground structures are entirely or nearly newly produced each year and the climatic conditions

215   (mean annual temperature and precipitation) did not vary much (Catalan Meteorological Service,

216   http://meteo.cat/wpweb/climatologia, accessed on March 2018). The changes in biomass of shrubs are very

217   limited in this ecosystem (Ninot et al. 2010a), so we do not expect large significant variations between two

218   consecutive years with similar climatic conditions. We collected the aboveground vegetation within 25×25

219   cm quadrats in the young shrubland and 50×50 cm quadrats in the unencroached grassland and mature

220   shrubland. The biomass was then transported in plastic bags to the laboratory and sorted manually into leaves

221   and woody stems (only for shrubs). We collected a total of 48 foliar samples and 48 stem samples from

222   shrubs (2 sites $\times$ 2 successional stages where shrubs were present $\times$ 3 shrub species $\times$ 4 replicates) and 16

223   foliar samples from herbaceous species (2 sites $\times$ 2 successional stages where herbaceous species were

224   present $\times$ 4 replicates). The plant material was sorted and oven-dried at 60 °C to a constant weight, and the

225   dry weight was measured. We collected soil samples with a bulk-density corer (9 cm diameter to a depth of

226   10 cm) to estimate the bulk density for each vegetation type. The quantity of soil (g cm^{-2}) was calculated as

227   the product of the bulk density multiplied by the core depth. We also calculated the annual leaf-litter

228   production based on estimates of foliar persistence conducted in nearby locations (Ninot et al. unpublished

229   data). Leaf-litter productivity was used to estimate the annual input of nutrients from the leaf-litter for each

230   vegetation type.

231   *Chemical analyses*

232   For leaves, leaf-litter and stems, 4.5 mg of dry sample were weighed and encapsulated in tin capsules. For

233   soils, 8.6 mg of each sample were used. The samples were then analyzed for C and N with an Elementar

234   Cube system (Elementar Analyzen system GmbH, Hanau, Germany). ^{15}N isotope was analyzed by an

235   Elementar Vario EL Cube or Micro Cube elemental analyzer connected to a PDZ Europa 20-20 isotope-ratio

236   mass spectrometer (Sercon Ltd., Cheshire, UK). The plant samples were combusted at 1000°C in a reactor

237   packed with chromium oxide and silvered copper oxide and soils were combusted at 1080 °C in a reactor

238   packed with copper oxide and tungsten (VI) oxide. The oxides were then removed in a reduction reactor

239   (reduced copper at 650 °C). The samples were interspersed during the analysis with several replicates of at

240   least two laboratory standards. These standards, which were selected for their compositional similarity to the

241   samples, had been previously calibrated against National Institute of Standards and Technology (NIST)

242 Standard Reference Materials (IAEA-N1, IAEA-N2, IAEA-N3, USGS-40 and USGS-41). Preliminary

243 isotopic ratios were measured relative to the reference gases analyzed for each sample. These preliminary

244 ratios were refined by correcting for the entire batch based on the known ratios of the laboratory standards.

245 The long-term standard deviation was 0.3‰ for $\delta^{15}N$.

246 The concentrations of P, Mg, K, Ca and Fe in the leaves, leaf-litter, stems and soil were determined by

247 inductively coupled plasma mass spectrometry (ICP-MS) after digestion. For leaves, leaf-litter and stems,

248 0.25g of dry material were diluted in 5 ml of concentrated HNO_3 and digested in a MARSXpress microwave

249 system (CEM GmbH, Kamp-Lintfort, Germany). The solution generated was analyzed by ICP-MS to

250 determine the elemental concentrations. The accuracy of the biomass digestions and analytical procedures

251 was assessed using certified biomass NIST 1573a (tomato leaf, NIST, Gaitherburg, USA) standards and

252 regularly analyzing blank solutions with no sample (5 mL of HNO_3 and 2 mL of H_2O_2 with no sample). Dry

253 soil subsamples of 0.1 g were dissolved in an acidic mixture of HNO_3, HCl and HF and digested as described

254 for the plant samples. The solutions were diluted with 1% HNO_3 (v/v) before injection into the spectrometer.

255 Blank solutions (5 mL of HNO_3 and 2 mL of H_2O_2 with no sample) were regularly analyzed. Total stocks of

256 the elements (g m^{-2}) for each plant compartment and the soil were calculated as the biomass (leaves, leaf-litter

257 or stems) and soil weights multiplied by the concentration of each element analyzed. The C:N, C:P and N:P

258 ratios were calculated on a mass basis.

259 *Statistical analyses*

260 We conducted principal component analyses (PCAs) of the elements to visualize the overall differences in the

261 chemical composition of the plant and soil samples collected for each vegetation type (shrub and herbaceous

262 species) and successional stage for each of the two sites. This allowed us to reduce the number of variables of

263 leaf, leaf-litter and soil chemistry and integrate the information in a more comprehensive way on a two-

264 dimensional plot. We standardized the variables and performed a PCA for the elements of all compartments

265 (leaves, leaf-litter and soil) for each site separately to assess the differences in the chemical compositions

266 among the successional stages and vegetation types within each site. If two variables were highly positively

267 correlated (Pearson's product moment correlation coefficient > 0.6), we excluded one of the two in the PCA

268 to avoid the overfitting of variables. We also conducted PCAs of each plant compartment (leaves, leaf-litter,

10

269   and stems) and soil using data from both study sites to identify differences between vegetation types and sites.

270   To identify links between the functional traits of the shrubs and the foliar elements concentrations (C, N, P, K,

271   Mg, Ca and Fe), we conducted PCAs of the foliar elemental concentrations and some key functional

272   measurements, as seed weight, specific leaf area (SLA), wood density, biomass per unit area, height and

273   annual leaf-litter production. All PCAs were conducted with the 'ggbiplot' (Wickham 2009) and

274   'FactoMineR' (Le et al. 2008) packages in R. The significance of the differences between vegetation types

275   and successional stages in each PCA were tested by permutational multivariate analyses of variance

276   (PERMANOVAs) with the R 'vegan' package (Oksanen et al. 2017). β dispersion homogeneity tests were

277   conducted prior to PERMANOVA analyses.

278   We analysed the differences in elemental concentrations (dry weight (dw) of nutrient/dw of sample $\times$ 100)

279   and stocks (g m^{-2}) in the plant and soil and between vegetation types (herbaceous species and shrub 1, shrub

280   2, and shrub 3) and successional stages at each site. Although in our sampling design we maximised the

281   independence of samples as much as possible, the extant structure of vegetation along the succession made it

282   not possible to find a completely independent, random distribution of combinations of successional stage $\times$

283   vegetation type in the field sites. We therefore firstly checked if there were any underlying patterns of spatial

284   aggregation in the elemental concentration and stocks data with autocorrelation semivariograms 'nlme'

285   package (Pinheiro et al. 2017) that are used for measuring the degree of spatial dependence between

286   observations as a function of distance. As in Grau et al. (2019) the initial visual interpretation of the

287   semivariograms indicated that in most cases the data were not autocorrelated despite the plots were not

288   completely randomized. Yet, we preferred to statistically check if autocorrelation should be accounted for in

289   the models. To do so we built two types of models: 1) Generalised least square (GLS) models that did not

290   account for spatial autocorrelation, 2) GLS models that included the 'corSpatial' R function to account for

291   potential spatial autocorrelation. For this second type of GLS models, the coordinates (longitude and latitude)

292   of each plot were specified and we fitted five different models using a different autocorrelation structure each

293   time (e.g. exponential, linear, gaussian, rational quadratic or spherical). After running all the models (one

294   without and five with autocorrelation structure) for the element concentrations and stocks in each soil or plant

295   compartment, we compared and selected the best model using scores of the Akaike Information Criterion

296   (AIC). We used maximised log-likelihood in all cases and checked the normal distribution of the data before

297   run the models. The unencroached grassland was the initial stage of the succession from grassland to

298   shrubland, and we considered it as the reference level in all models. The two study sites were analyzed as two

299   independent study cases, so 'site' was not included in the models as a factor. All analyses were performed

300   with R 3.2.4 (RCoreTeam 2017).

301   **RESULTS**

302   Significant differences in the elemental concentrations and ratios in the plant compartments (leaves and leaf-

303   litter and stems (only for shrubs) and soil for all the vegetation types along the encroachment succession in

304   both study sites are summarized in Table 1 and 2, the means ± sd values in Table ST2. Significant differences

305   for element stocks are summarized in ST3, ST4, ST5 and ST6.

306   *Patterns of plant-soil stoichiometry along the succession at North face*

307   The plant-soil stoichiometry spectrum at North face varied along the two axes of the PCA (Fig. 2a). The first

308   PC axis explained 32.6% of the variance mostly integrated the changes along the succession, whereas the

309   second axis explained 23% and mostly showed the variability within each successional stage, particularly for

310   shrublands. Such variation between successional stages and also vegetation types (PERMANOVA tests

311    $p<0.001$ in both cases). Because the β-dispersion homogeneity test showed significant differences between

312   groups (successional stages or vegetation types), the significance of these PERMANOVA tests may thus

313   result from differences in the position of the centroids as well as from differences in the dispersion of the data

314   around the centroids.

315   Leaves and leaf-litter in patches of herbaceous species in the young shrubland had a higher C:N ratio and

316   lower [N], [K] and [Fe] than the herbaceous species in the unencroached grassland; the soil [P] was lower in

317   patches with herbaceous species in the young shrubland than in the unencroached grassland (Table 1a).

318   Generally, shrub leaves in the young and mature shrubland had higher [C], C:N ratio and lower [N], [K], [Fe]

319   and N:P than herbaceous species in the unencroached grassland (Table 1a,b); leaf-litter [C] was higher in

320   shrubs than in the unencroached grassland. Foliar [Ca] and [Mg] in the mature shrubland were higher than in

321   the unencroached grassland. Regarding the soil, all vegetation types in young shrubland had higher soil C:N

322   ratio and lower [P] and [Mg] than in unencroached grassland. (Tables 1a). Soil N was also lower in the young

323   shrubland of *R. ferrugineum* and *V. myrtillus* and soil K, Ca and Fe were lower in the mature shrubland of *C.*

324   *vulgaris* compared to the unencroached grassland (Table 1b). Foliar $\delta^{15}N$ and [N] were lower for all the

325   vegetation types in the young and the mature shrubland than herbaceous species in unencroached grassland

326   (Fig. 3).

327   We found stoichiometric differences within shrub species between the young and mature shrublands (Table

328   2). In the mature shrubland, *C. vulgaris* had higher [K] and C:N ratio and lower [Mg] in the leaf-litter, higher

329   C:N ratio and lower [N] and [K] in the stems. *R. ferrugineum* had higher foliar N:P, higher [N], [K] and lower

330   C:N ratio in leaf-litter, higher stem [N] and N:P and lower C:N and higher soil [Mg], N:P and C:P ratio in the

331   mature than the young shrubland. *V. myrtillus* had higher foliar [C] and [Fe], lower leaf-litter N:P and C:P

332   ratios and higher soil [Mg] in the mature than the young shrubland (Table 2).

333   The stocks of nutrients were generally much larger (generally 100-fold in all successional stages) in the soil

334   than in the biomass. The stocks of N (9.4 g m^{-2}), P (0.71 g m^{-2}), K (8.5 g m^{-2}) and Fe (0.06 g m^{-2}) in leaves of

335   herbaceous species in the unencroached grassland were significantly larger than in the leaves of all vegetation

336   types in the young and mature shrublands (Fig. 4 and Table ST3 for significant differences). Nevertheless,

337   shrub stems generally stored larger amounts of C and nutrients compared to leaves, so that the stocks of C and

338   nutrients in the aboveground biomass in the young and mature shrubland were generally much larger than in

339   the grassland (except for K). *C. vulgaris* and *R. ferrugineum* in the mature shrubland had higher stocks of soil

340   C (8953.5 g m^{-2}, 7388.7 g m^{-2}, respectively) and soil N (507.5 g m^{-2}, only *C. vulgaris*) than the unencroached

341   grassland (C: 3645.7g m^{-2}, N: 294.7g m^{-2}). The stocks of soil P and K stocks in *C. vulgaris* in the mature

342   shrubland tend to increase but not significantly. Herbaceous species in unencroached grassland showed the

343   highest annual leaf-litter production (509.4 g m^{-2} year^{-1}) (Fig. SF2, see Table ST3 for significant differences);

344   also, the annual leaf-litter production in *V. myrtillus* was higher in the mature (99.8 g m^{-2} year^{-1}) than the

345   young shrubland (53.8 g m^{-2} year^{-1}) (Fig. SF2, see Table ST4 for significant differences), implying higher

346   inputs of C (49.5 g m^{-2} year^{-1}), N (1.1 g m^{-2} year^{-1}), P (0.12 g m^{-2}year^{-1}), K (0.68 g m^{-2} year^{-1}), Mg (0.2 g m^{-2}

347   year^{-1}), Ca (0.7 g m^{-2} year^{-1}) and Fe (0.007g m^{-2} year^{-1}) in the mature shrubland (Tables ST2 and ST4 for

348   significant differences). Leaf-litter production did not differ significantly for the other vegetation types.

349   ***Patterns of plant-soil stoichiometry along the succession at South face***

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350   The plant-soil stoichiometry spectrum at South face varied greatly along the two axes of the PCA based on

351   the chemical composition of leaves, leaf-litter and soil (Fig. 2b). The first PC axis explained 30.7% of the

352   variance and mostly integrated the differences along the succession, whereas the second PC axis explained

353   20.1%, which highlights the variability within the successional stages and vegetation types. We found

354   significant differences between successional stages (PERMANOVA $p<0.001$) and vegetation types

355   (PERMANOVA $p<0.004$) (Fig. 2b). In general, herbaceous species in the young shrubland had lower [N] and

356   [K] in leaves, higher [C], [Fe], N:P and C:N ratio and lower [N] and [P] in the leaf-litter and higher [Fe] and

357   lower [N], [Ca] and [Mg] in soil than the unencroached grassland (Tables 1a). Shrub leaves in the young

358   shrubland generally had higher [C] and C:N ratios and lower [N], [K] and N:P ratio than herbaceous species

359   in unencroached grassland, and shrub leaf-litter generally had higher [C] and lower [N] than herbaceous

360   species in unencroached grassland. The soil in young shrubland had lower [N], [Ca], [Mg] and N:P ratio than

361   soil under herbaceous species in unencroached grassland. Leaves in the mature shrubland often had higher

362   [Ca] and [Mg] and lower [N], [K] and N:P ratio, and leaf-litter with higher [C] and lower [Fe] than the

363   unencroached grassland. Soil [N], [Ca], [Mg] were always lower in the young shrubland than the

364   unencroached grassland (Tables 1); soil [P] was also lower in the mature shrubland of *A. uva-ursi* and *J.*

365   *sabina*. Foliar δ^{15}N and [N] were also lower for all vegetation types in the young and the mature shrubland

366   than herbaceous species in unencroached grassland (Fig. 3), as observed at North face.

367   We also found some stoichiometric differences within shrub species between the young and mature stages

368   (Table 2). *A. uva-ursi* had lower foliar [P] and higher C:P ratio and higher leaf-litter N:P ratio in the mature

369   than the young shrubland, *J. communis* had lower [Ca] and C:N ratio in the leaf-litter and lower [Ca] in the

370   stems in the mature than the young shrubland and *J. sabina* had higher foliar [C], leaf-litter [C], [N] and [P]

371   and lower [Ca], C:N and C:P ratios and higher stem [Mg] and lower C:N ratio in the mature than the young

372   shrubland (Table 2). Soil stoichiometry did not differ significantly between the young and mature shrublands.

373   The stocks of nutrients were generally much larger in the soil (100-fold in unencroached grassland and young

374   shrubland and 10-fold for mature shrubland) than in the biomass. The stocks of C, N, P, Mg and Ca in the

375   leaves of *J. communis* and *J. sabina* in the young and mature shrubland were higher than in the leaves of the

376   herbaceous species in the unencroached grassland (C: 191.1 g m^{-2}, N: 8.2 g m^{-2}, P: 0.56 g m^{-2}, K: 6.7 g m^{-2},

377   Mg: 0.45g m^{-2}, Ca: 1.7g m^{-2}), and the leaves of *A. uva-ursi* had lower K stocks (Fig. 5 and Table ST5 for

378 significant differences). As observed at North face, shrub stems generally stored larger amounts of C and

379 nutrients compared to leaves, so that the overall stocks of C and nutrients in the aboveground biomass were

380 generally much larger than in the grassland. The soil for most shrubs had smaller stocks of Mg and Ca in the

381 young and mature shrublands than in the unencroached grassland (Mg 77.1 g m^{-2} and Ca: 431.1 g m^{-2}). The

382 soil for *A. uva-ursi* (348.19 g m^{-2}) and *J. communis* (315.76 g m^{-2}) in the mature shrubland had lower N stocks

383 than in young shrubland. Soil P stocks tended to decrease along the succession, but not significantly (Fig. 5

384 and Table ST6 for significant differences). Annual leaf-litter production by *J. sabina* was higher in the mature

385 (938.9 g m^{-2} yr^{-1}) than the young shrubland (566.2 g m^{-2} year^{-1}) (Fig. SF2 and Table ST6 for significant

386 differences). Leaf-litter production did not differ significantly for the other vegetation types.

387 *General patterns in the plant-soil stoichiometry spectrum at North and South face*

388 The PCA of the functional traits and foliar elemental composition of the shrub species from both study sites

389 (Fig. SF3) showed that the shrub species at North face differed from those at South face (PERMANOVA

390 $p<0.01$) along the PC2 (31.2% of variance explained). Moreover, differences between shrub species within

391 each site were also discriminated (PERMANOVA $p<0.01$) along the PC1 (39.1% of variance explained).

392 The PCA based on the foliar stoichiometry including both sites showed significant differences between shrubs

393 and herbaceous species (PERMANOVA $p<0.5$), mostly along the first axis (39,4% of variance explained,

394 SF4a). The chemical composition of leaf-litter showed a similar pattern (PERMANOVA $p<0.001$) but

395 varying mostly along the second axis (24% of variance explained, Fig. SF4b). The chemical composition of

396 the soil, however, differed more between the two sites (PERMANOVA $p<0.001$) (41.4% of variance

397 explained along the first axis, SF4c) than between herbaceous species and shrubs. In this case, though,

398 because the β-dispersion homogeneity test in Fig. SF4c was significant, the significance of the

399 PERMANOVA test may result not only from differences in the position of the centroids but also from

400 differences in the dispersion of the data around the centroids. Finally, the chemical composition of the stems

401 differed mostly between sites (PERMANOVA $p<0.001$) (39.3% of the variance explained along the first axis,

402 Fig. SF4d).

403

404 **DISCUSSION**

15

405   The plant-soil stoichiometry spectrum differed greatly along the succession from unencroached

406   grassland to mature shrubland (Fig. 2), as initially hypothesized. The shift in plant-soil stoichiometry

407   spectrum was more apparent and consistent between the unencroached grassland and the young shrubland

408   than between the young and mature shrubland (Tables 1 and 2), suggesting that changes in growth forms

409   (from herbaceous-dominated to shrub-dominated) play a greater role in shaping the plant-soil stoichiometry

410   spectrum than differences in successional stage as such. In fact, the plant-soil stoichiometry spectrum is

411   expected to be primarily shaped by changes in abiotic and/or biotic factors, such as changes in vegetation

412   along the succession. This is evidenced by the shift between herbaceous species and shrubs, from high foliar

413   [N] and [K] for herbaceous species to high foliar [C], [Ca], [Mg], C:N and C:P ratios and low [N], [P] or [K]

414   for shrubs (Table 1, Table ST2); or from low to high [C], C:N and C:P ratios in litter, or from high to low [N],

415   [P], [Mg] and [Ca] in soil. Such changes in stoichiometry may have major consequences on the functioning of

416   ecosystems (Eldridge et al. 2011). For example, the high [C] and C:N and C:P ratios in the shrub leaves and

417   leaf-litter promote the formation of recalcitrant organic matter accumulation with slow decomposition rates in

418   the top soil of shrubland compared to the grassland (Ninot et al. 2010b; Garcia-Pausas et al. 2017).

419   The changes in foliar chemical spectrum enabled us to detect shifts in the biogeochemical niche

420   (Urbina et al. 2017; Peñuelas et al. 2019) (Fig. SF4). We found that shrubs and herbaceous species differed

421   significantly in their biogeochemical niche, as expected from these two contrasting growth forms. For

422   example, herbaceous species in the unencroached grassland had higher foliar nutrient concentrations (N, K),

423   characteristic of plants with fast growth rates; whereas shrubs had higher C concentration and C:N and C:P

424   ratios in leaves, characteristic of plants with slow growth rates (Ågren 2004; Sardans et al. 2012;

425   Zechmeister-Boltenstern et al. 2015).

426   The changes in stoichiometry in the plant aboveground compartments along the succession were

427   coupled with a decrease of the soil concentrations of some essential nutrients needed for plant development.

428   Grau et al. (2019) suggested that this decrease in nutrient concentrations in the soil was possibly due to the

429   decline in nutrient inputs from excrements of domestic herbivores and to the transfer of nutrients from the soil

430   to the biomass of the shrubs (Horton et al. 2009). The allocation of nutrients to the biomass of shrubs could be

431   a mechanism of nutrient accumulation by which shrubs control nutrients in an ecosystem through a positive

432   feed-back (Chapin et al. 1997) to outcompete herbaceous species and expand into the grassland (Grau et al.

16

433 2019). In our study we found that the concentrations of N, P and K often decreased in the aboveground

434 biomass along the succession in both study sites (Table 1); at the same time, the concentrations of soil N, P or

435 K often decreased in the mature shrubland of several shrubs. The stocks of N and P in the aboveground

436 biomass (leaves and stems), though, increased for several shrubs along the succession, particularly in *R.*

437 *ferrugineum*, *J. communis* and *J. sabina* (Figs. 3 and 4). This indicates that the dilution of these nutrients in

438 the aboveground biomass of shrubs was outweighed by their increase in aboveground biomass. This

439 evidences that shrubs may act as net reservoirs of essential nutrients in the biomass even if the concentration

440 in the biomass and soil decrease along the succession. The amount of C and nutrient stocks in aboveground

441 biomass differed notably amongst shrub species, which implied differences in the final stocks in the mature

442 shrubland within each site. Shrub identity was therefore crucial to understand the variability in concentrations

443 and stocks of nutrients in the vegetation and in the soil within each site. However, we also found differences

444 between the North and South face. Shrubs were generally smaller and less sclerophyllous at North face (Fig.

445 SF3), where solar radiation and evapotranspiration are lower, the slope is smoother, and the soil is deeper than

446 at South face (Ninot et al. 2010a). The increases in the C and nutrient stocks (g m^{-2}) in the aboveground plant

447 compartments along the succession from grassland to shrubland were less evident at North face than at South

448 face. In contrast, shrubs developed more woody tissue, were taller and had higher wood density and more

449 sclerophyllous leaves at South face. This is probably because shrubs are adapted to harsher conditions, with

450 more solar radiation, higher evapotranspiration, less precipitation, a steeper slope and shallower soil with

451 more rocky outcrops than at North face. The differences in shrub traits therefore, seem to also play a key role

452 in the C and nutrient cycles with the advance of the encroachment, as previously described by other authors

453 (Eldridge et al. 2011).

454   We also found that there was a marginally significant decrease (p-value 0.08) of P stocks in the

455 young shrubland at North face, but not at South face, when vegetation and topsoil stocks were summed

456 together. This specific pattern could be related to differences in the way P is mobilized across the plant-soil

457 compartment in each site after the abandonment of the grassland, leading to putative losses of P from the

458 ecosystem. Moreover, *A. uva-ursi* at the South face site showed lower storage of N, P and K in the plant-soil

459 compartments in the mature shrubland stage, which also suggests a possible loss of nutrients from the system

17

460 with the advance of the encroachment dominated by this species. These results though should be explored in

461 more detail to fully understand the underlying mechanism of this loss of nutrients from the ecosystem.

462   Finally, herbaceous species in young shrubland and shrubs in young and mature shrubland showed

463 lower foliar δ^{15}N and [N] than herbaceous species in unencroached grassland (Fig. 3). This was often coupled

464 with a decrease in soil [N] in the young and/or mature shrubland for several shrub species (Table 1). Ruiz-

465 Navarro et al. (2016) argued that low soil N and low foliar δ^{15}N were good indicators of a decline in soil

466 fertility in Mediterranean ecosystems, with major implications on the biogeochemical cycling rates and

467 nutrient availabilities. In the same way, our results indicate that N may become limiting in these subalpine

468 ecosystems, particularly once the shrub cover is high (Angulo et al. 2019). Moreover, lower δ^{15}N generally

469 indicates more N uptake by ectomycorrhizal or ericoid mycorrhizal fungi compared to arbuscular mycorrhizal

470 plants such as herbaceous species (Michelsen et al. 1998), with more recycled N leading to lower N losses

471 from the ecosystem (Garten 1993; Robinson 2001; Craine et al. 2009; Anadon-Rosell et al. 2016). All shrubs

472 at North face are ericaceous and have ericoid mycorrhizae; in the South face, *A. uva-ursi* hosts both ericoid

473 and ectomycorrhizal fungi, whereas *J. communis* and *J. sabina* host arbuscular mycorrhizal fungi. The lower

474 δ^{15}N in young and mature shrubland possibly indicates that more N had been taken up by ectomycorrhizal or

475 ericoid fungi (Angulo et al. 2019). The N uptake by ectomycorrhizal or ericoid mycorrhizal fungi possibly

476 promotes the uptake of organic N (e.g. from litter) by shrubs (Akhmetzhanova et al. 2012) as inorganic N

477 becomes more limiting in the soil. The fact that the arbuscular mycorrhizal species occurring in the young or

478 mature shrubland (herbaceous species at North and South face and *J. communis* and *J. sabina* at South face)

479 also show low δ^{15}N values compared to the unencroached grassland possibly indicates that these species may

480 have taken up part of their N in organic form derived from litter of surrounding ericoid or ectomycorrhizal

481 shrubs (e.g. from patches of *A. uva-ursi*), but this should be further investigated. These results suggest that the

482 advance of the succession possibly changed the mechanisms by which plants uptake N to overcome N

483 limitation.

484 **CONCLUSIONS**

485 Shrub expansion has a major influence on the plant-soil stoichiometry spectrum along the succession from

486 grassland to shrubland in the Pyrenees. Grassland ecosystem are dominated by species with faster turnover of

487 nutrients between the plant and soil compartments, high concentrations of N, P and K in the plant
488 aboveground biomass and topsoil, but limited capacity to store biomass. The expansion of shrubs, though,
489 favored the dominance of long-lived species, with a more conservative strategy, high C: nutrient ratios and
490 low concentrations of N, P and K in the aboveground biomass and low nutrient concentrations in soils. The
491 total stocks of C and nutrients in the aboveground biomass of the shrubland were nevertheless high because
492 the biomass of the mature shrubland was very high compared to the grassland. We thus highlight the role of
493 shrubs in the sequestration of C and nutrients, through the allocation to the aboveground biomass. Moreover,
494 shrub encroachment altered the strategy with which N was acquired, possibly through an increased uptake of
495 N through ectomycorrhizal or ericoid fungi. Our results suggested that shrubs play an important role in the C
496 and nutrient sequestration in the aboveground biomass (through the allocation into the plant compartments)
497 along the succession, where the woody tissues play a main role as storage compartment. The changes in plant-
498 soil elemental composition and stocks suggest a slowdown of the biogeochemical cycles in the subalpine
499 mountain areas where shrub encroachment occurred, but experimental manipulations are still needed to better
500 understand the mechanisms involved.

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Figure captions

Fig. 1 **Experimental design**. The dotted lines represent the sampling area in each successional stage: (a) Unencroached grassland, (b) young shrubland and (c) mature shrubland. The squares inside each successional stage represent the replicate plots (approximately 2×2 m). In the young shrubland we established four groups of four plots; the herbaceous species, shrub 1, shrub 2 and shrub 3 were grouped because all vegetation types co-occurred. The distance between the successional stages or among the mature shrublands of each shrub species was >100 m and the distance between the four replicates inside each successional stage was 10 m.

Fig. 2 **Principal component analysis (PCA) based on the concentrations of chemical elements in** leaves (lf), leaf-litter (ltt) and soil (0-10 cm) for the successional stages and vegetation types at (a) North face and (b) South face. Only variables that were poorly correlated with each other (Pearson's coefficients <0.6) were included in the analyses. Different colors indicate the successional stages and different shapes indicate the vegetation types. The ellipses represent the dispersion around the centroid for each successional stage with a normal probability of 0.85.

Fig. 3 **Polynomial regression between foliar N and δ^{15}N for herbaceous species** and shrubs along the succession from unencroached grassland to mature shrubland in both study sites. Different shapes show the

535 different vegetation types (herbaceous species or shrub) and colors show the successional stage

536 (unencroached grassland, young shrubland or mature shrubland) in each study site (NF: North face, SF: South

537 face).

538 Fig. 4 **Stocks of chemical elements (C, N, P, K, Ca, Mg and Fe) in leaves, stems and soil (0-10 cm) (g m^{-2})**

539 **for each successional stage and vegetation type at the North face**. The left column in each figure

540 represents the unencroached grassland, the columns in the middle represent the vegetation types in the young

541 shrubland, and the columns on the right represent the vegetation types in the mature shrubland. Significant

542 differences are summarized in Table ST3. Leaf-litter data are not included because the data available for leaf-

543 litter could only be used to estimate the productivity (g m^{-2} yr^{-1}), not the stocks. See Fig. SF2 and Table ST2

544 for the leaf-litter productivity values.

545 Fig. 5 **Stocks of chemical elements (C, N, P, K, Ca, Mg and Fe) in leaves, stems and soil (0-10 cm) (g m^{-2})**

546 **for each successional stage and vegetation type at the South face**. The left column in each figure

547 represents the unencroached grassland, the columns in the middle represent the vegetation types in the young

548 shrubland, and the columns on the right represent the vegetation types in the mature shrubland. Significant

549 differences are summarized in Table ST5. Leaf-litter data are not included because the data available for leaf-

550 litter could only be used to estimate the productivity (g m^{-2} y^{-1}), not the stocks. See Fig. SF2 and Table ST2

551 for the leaf-litter productivity values.

552

553   Fig. 1 **Experimental design**. The dotted lines represent the sampling area in each successional stage: (a) Unencroached grassland, (b) young shrubland and (c)

554   mature shrubland. The squares inside each successional stage represent the replicate plots (approximately 2×2 m). In the young shrubland we established four

555   groups of four plots; the herbaceous species, shrub 1, shrub 2 and shrub 3 were grouped because all vegetation types co-occurred. The distance between the

556   successional stages or among the mature shrublands of each shrub species was >100 m and the distance between the four replicates inside each successional stage

557   was 10 m.
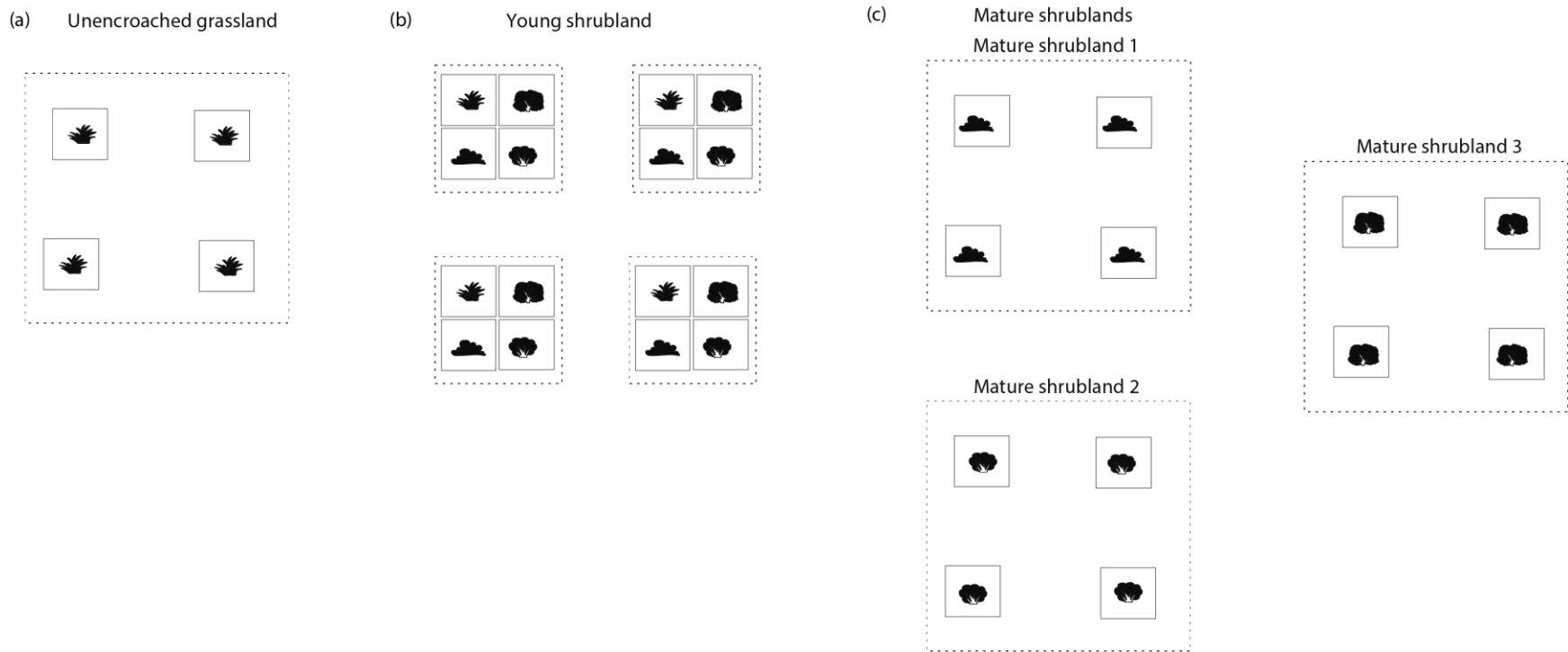
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Fig. 2 **Principal component analysis (PCA) based on the concentrations of chemical elements in** leaves (lf), leaf-litter (ltt) and soil (0-10 cm) for the

561   successional stages and vegetation types at (a) North face and (b) South face. Only variables that were poorly correlated with each other (Pearson's coefficients

562   <0.6) are included in the analyses. Different colors indicate the successional stages and different shapes indicate the vegetation types. The ellipses represent the

563   dispersion around the centroids for each successional stage with a normal probability of 0.85.

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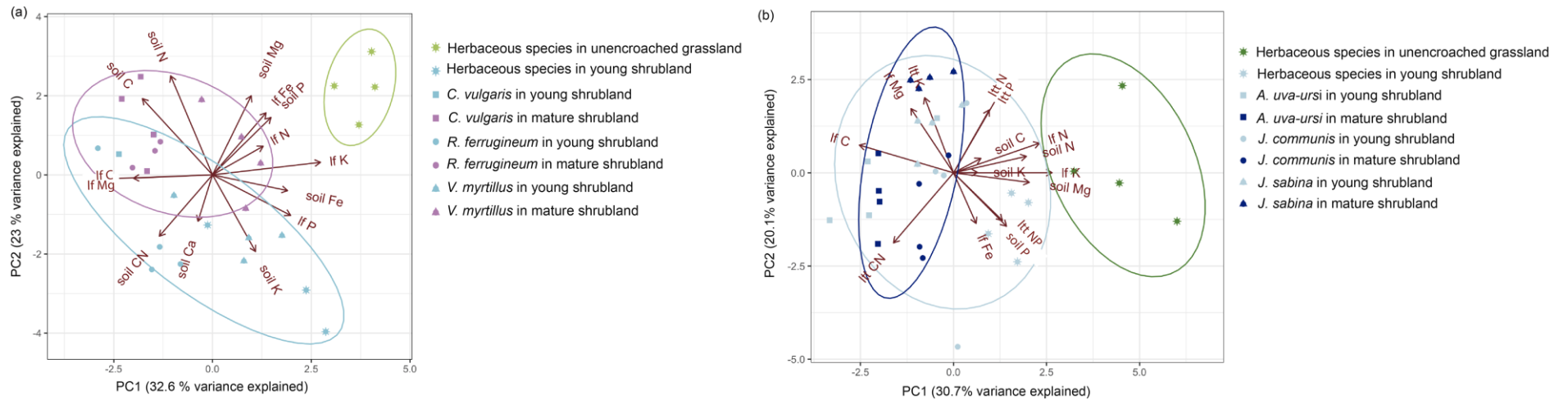
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567 Fig. 3 **Polynomial regression between foliar N (%) and foliar delta ^{15}N (δ^{15}N) for herbaceous species and**

568 **shrubs along the succession from unencroached grassland to mature shrubland in both study sites**.

569 Different shapes show the different vegetation types (herbaceous species or shrub) and colors show the

570 successional stage (unencroached grassland, young shrubland or mature shrubland) in each study site (NF:

571 North face, SF: South face)
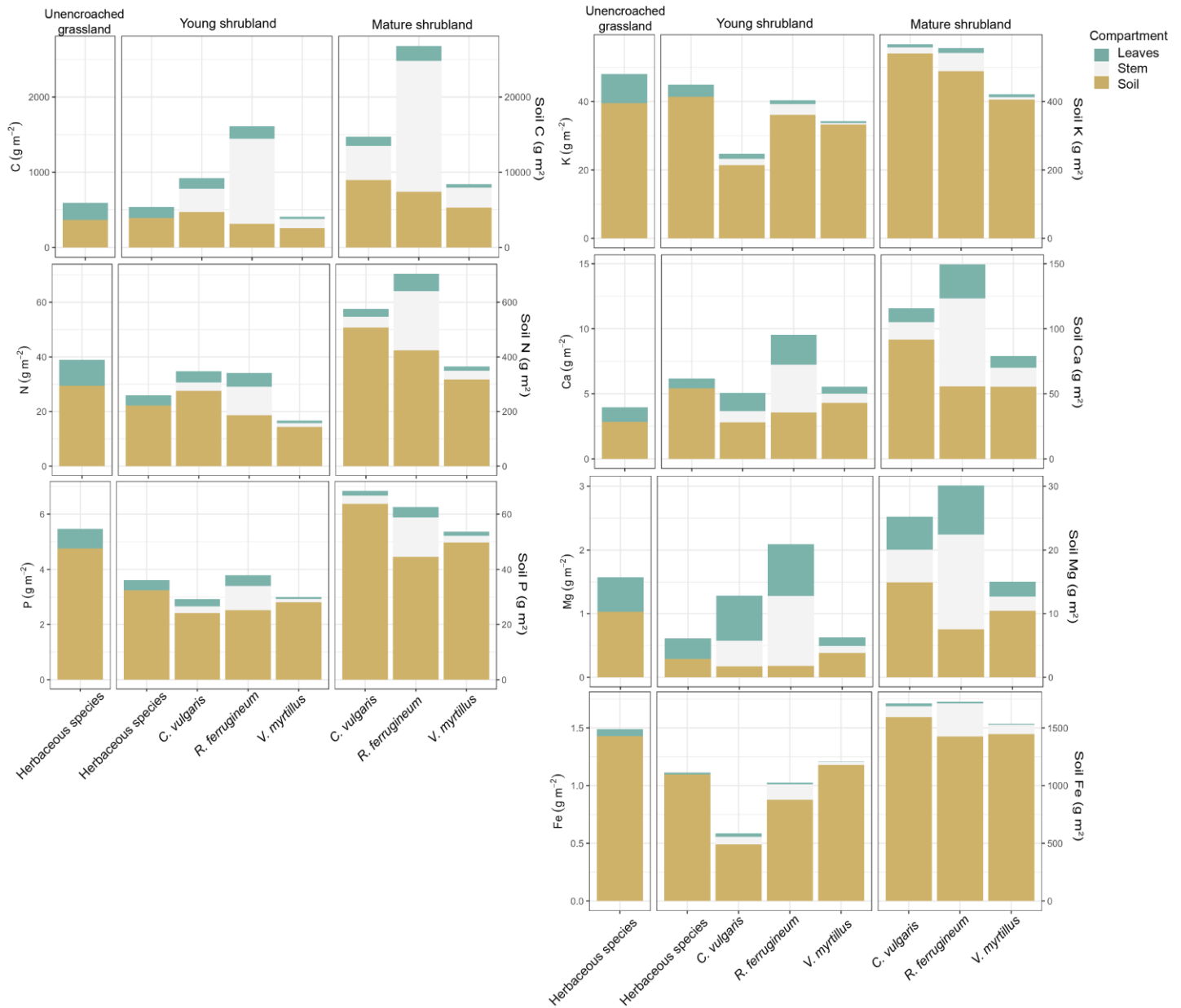
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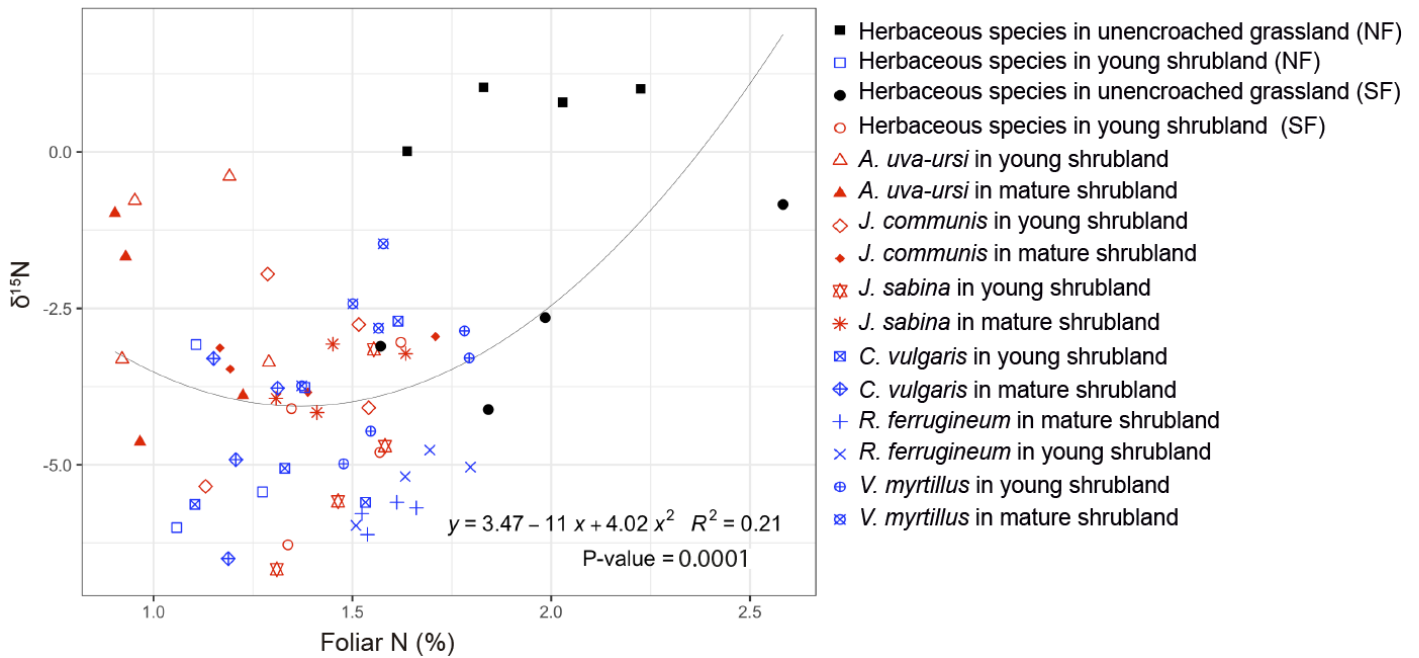
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581 Fig. 4 **Stocks of chemical elements** (C, N, P, K, Ca, Mg and Fe) in leaves, stems and soil (0-10 cm) (g m^{-2})

582 **for each successional stage and vegetation type at the North face**. The left column in each figure

583 represents the unencroached grassland, the columns in the middle represent the vegetation types in the young

584 shrubland, and the columns on the right represent the vegetation types in the mature shrubland. Significances

585 are summarized in table ST3. Leaf-litter data are not included because the data available for leaf-litter could

586 only be used to estimate the productivity (g m^{-2} y^{-1}), not the stocks. See Fig. SF2 and Table ST2 for the leaf-

587 litter productivity values.

Fig. 5 **Stocks of chemical elements** (C, N, P, K, Ca, Mg and Fe) in leaves, stems and soil (0-10 cm) (g m^{-2})

589 **for each successional stage and vegetation type at the South face**. The left column in each figure

590 represents the unencroached grassland, the columns in the middle represent the vegetation types in the young

591 shrubland, and the columns on the right represent the vegetation types in the mature shrubland. Significances

592 are summarized in table ST5. Leaf-litter data are not included because the data available for leaf-litter could

593 only be used to estimate the productivity (g m^{-2} y^{-1}), not the stocks. See Fig. SF2 and Table ST2 for the leaf-

594 litter productivity values.
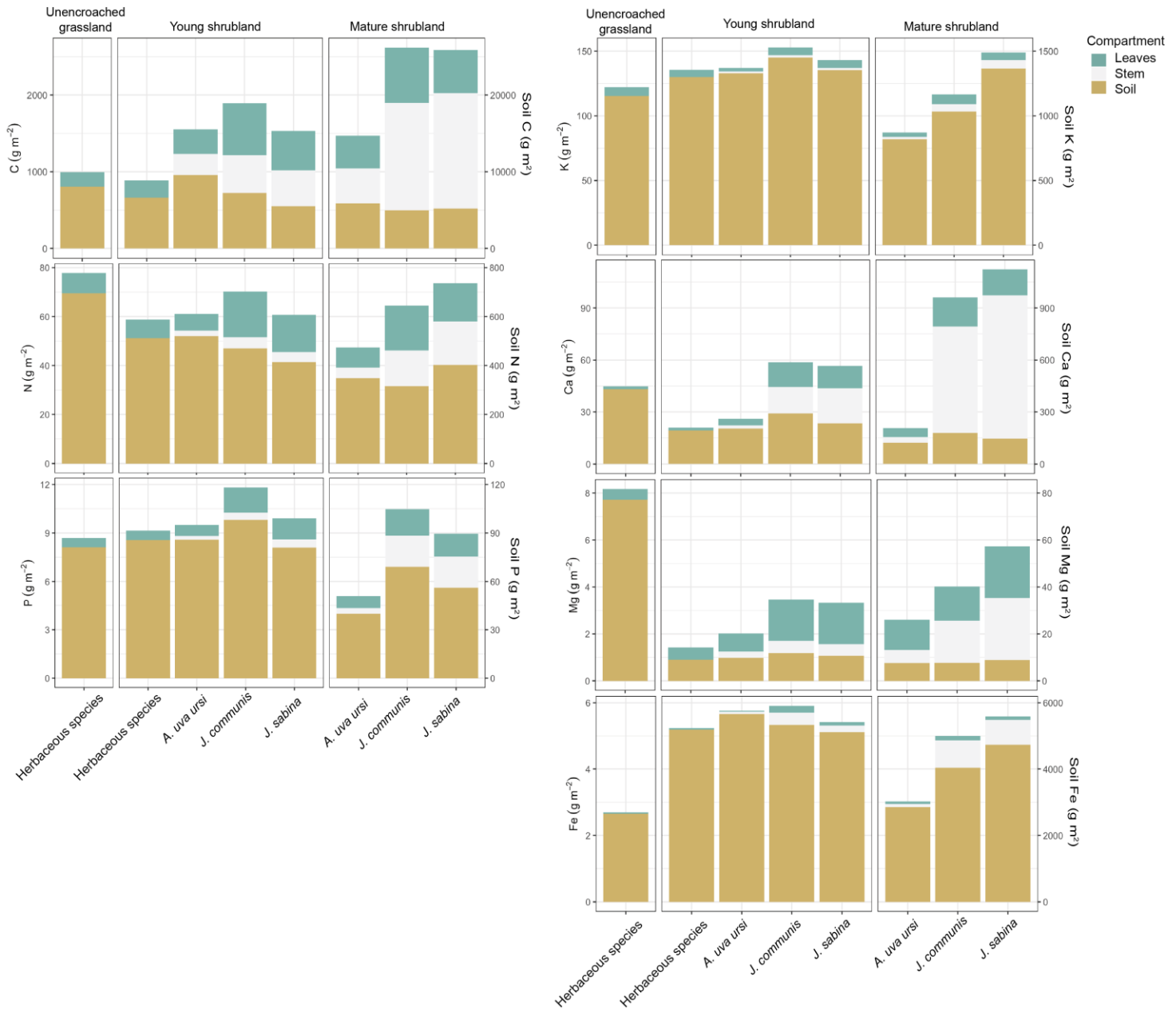
Table 1. **Significant changes in the element concentrations for the different vegetation types in (a) the young shrubland and (b) mature shrubland compared to the herbaceous species in the unencroached grassland.** Changes (increases (↑) or decreases (↓)) in the elemental concentrations (C, N, P, K, Ca, Mg and Fe) (dw/dw) and in the C:N, C:P and N:P ratios were identified by generalized least square models. The n.s abbreviation indicates that the results were not statistically significant ($P > 0.05$). Means ± sd are summarized in Table ST2 and significance values are shown in Tables

(a)		Increase or decrease in the elemental concentration and ratios between the **young** shrubland and **unencroached grassland** stage					
		Leaves		Leaf-litter		Soil (0-10 cm)	
	Chemical changes (dw/dw)	↑	↓	↑	↓	↑	↓
North Face	herbaceous species	C:N	C, N, K, Fe	C:N	N, K, Mg, Fe	Ca C:N	P
	Calluna vulgaris	C, Ca, Mg N:P, C:N, C:P,	N, P, K	C, N, Ca, Mg	K, Fe	Ca N:P, C:N, C:P	P, Mg
	Rhododendron ferrugineum	C, Ca, Mg C:N	N, K, Fe	C, Ca, Mg C:N, C:P	N, K, Fe	Ca, K C:N	N, P, Mg
	Vaccinium myrtillus	C, Ca, Mg	N, K, Fe	C, Ca, K, Mg	Fe	K, Ca C:N	N, P, Mg
South Face	herbaceous species	n.s	N, K	C, Fe N:P, C:N	N, P	Fe	N, Ca, Mg
	Arctostaphylos uva-ursi	C C:N, C:P	N, K N:P	C, K, Mg C:N	N, Fe N:P	Fe C:N	N, K, Ca, Mg N:P
	Juniperus communis	C, Ca C:N, C:P	N, K N:P	C, Ca C:P	N, P, Fe	n.s	N, Ca, Mg, Fe N:P
	Juniperus sabina	C, Ca, Mg C:N	N, K N:P	C, K, Ca, Mg	n.s	Fe	N, Ca, Mg N:P
(b)		Increase or decrease in the elemental concentration and ratios between the **mature** shrubland and **unencroached grassland** stage					
North Face	*Calluna vulgaris*	C, Ca, Mg N:P, C:N, C:P	N, P, K, Fe	C, Ca, Mg	n.s	C:N, C:P	K, Ca, Fe
	Rhododendron ferrugineum	C, Ca, Mg C:N, C:P	N, K, Fe	C, Ca, Mg C:N, C:P	N, P, K, Fe	C, Ca N:P, C:N, C:P	Mg
	Vaccinium myrtillus	C, Ca, Mg C:N	N, K, Fe N:P	C, P, K, Ca, Mg	Fe	Ca C:N	n.s
South Face	*Arctostaphylos uva-ursi*	C, Ca, Mg	N, P, K N:P, C:N, C:P	C, Mg C:N, C:P	N, P, Fe N:P	C:N	N, P, K, Ca, Mg
	Juniperus communis	C, Ca C:N	N, K N:P	C, Ca C:N, C:P	N, P, Fe	n.s	N, Ca, Mg N:P
	Juniperus sabina	C, Ca, Mg C:N	N, K N:P	C, P, K, Mg, Ca	Fe N:P	n.s	N, P, Ca, Mg

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614   ST3 and ST5.

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626   Table 2. **Significant changes in the element concentrations for the vegetation types in the mature shrubland compare to young shrubland**. Changes

627   (increases or decreases) in the elemental concentrations (C, N, P, K, Ca, Mg and Fe) (dw/dw) and in the C:N, C:P and N:P ratios were identified by generalized

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	Chemical changes	Changes in the elemental composition between the **mature** and **young** shrubland stage							
		Leaves		Leaf-litter		Stems		Soil (0-10 cm)	
		↑	↓	↑	↓	↑	↓	↑	↓
North face	*Calluna vulgaris*	n.s	n.s	K C:N	Mg	C:N	N, K	n.s	n.s
	Rhododendron ferrugineum	N:P	n.s	N, K	C:N	N N:P	C:N	Mg N:P, C:P	n.s
	Vaccinium myrtillus	C, Fe	n.s	n.s	N:P, C:P	n.s	n.s	Mg	n.s
South Face	*Arctostaphylos uva-ursi*	C:P	P	N:P	n.s	n.s	n.s	n.s	n.s
	Juniperus communis	n.s	n.s		Ca C:N	n.s	Ca	n.s	n.s
	Juniperus sabina	C	n.s	C, N, P	Ca C:N, C:P	Mg	C:N	n.s	n.s

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641   **Reference**

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819   Supplementary material

820   Figure SF1. **Map of the study sites**. Location of (a) the study area in the Central Pyrenees (red square) in southwestern Europe and (b) the two study sites (red

821   dots).

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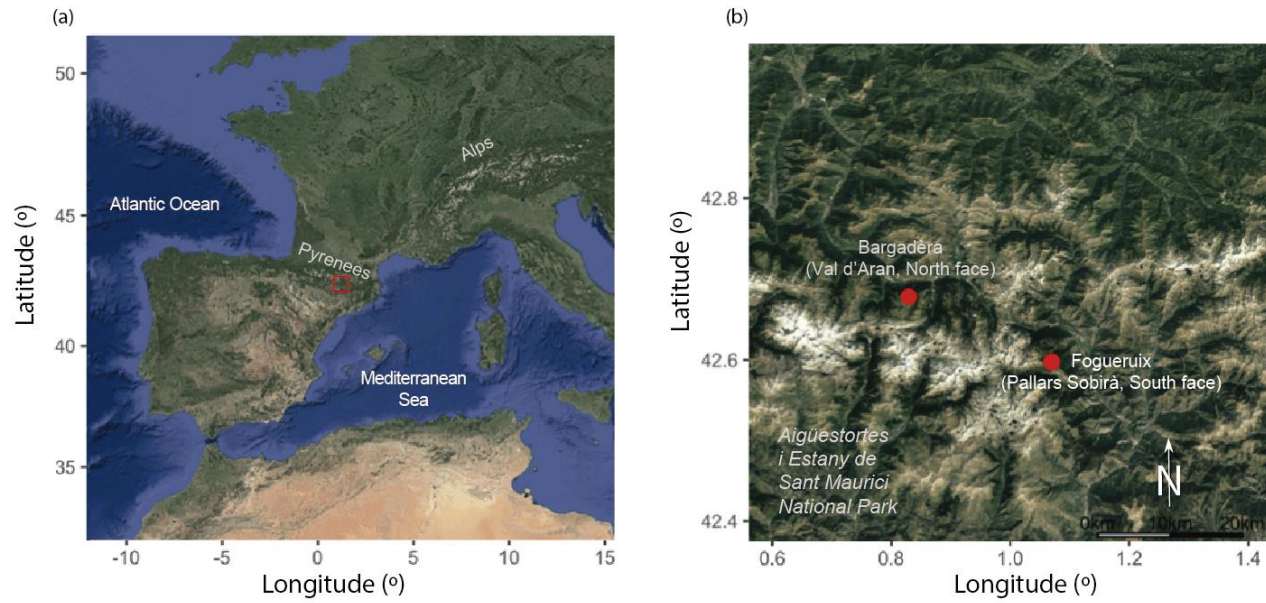
831 Figure SF2. **Leaf-litter productivity (g m^2 y^{-1}) for each vegetation type in the two study sites:** (a) North face and (b) South face in the unencroached grassland

832 (left panel in each figure), young shrubland (middle panel in each figure) and mature shrubland (right panel in each figure). Leaf-litter productivity was calculated

833 using the foliar persistence analysis from Ninot et al. (unpublished data). The upper and lower line of the boxes indicates the second and third quartile,

834 respectively; the black line inside de box indicates the median; the bars represent the first and fourth quartiles and dots represent the outliers. Note the different

835 vertical scales for North face and South face.

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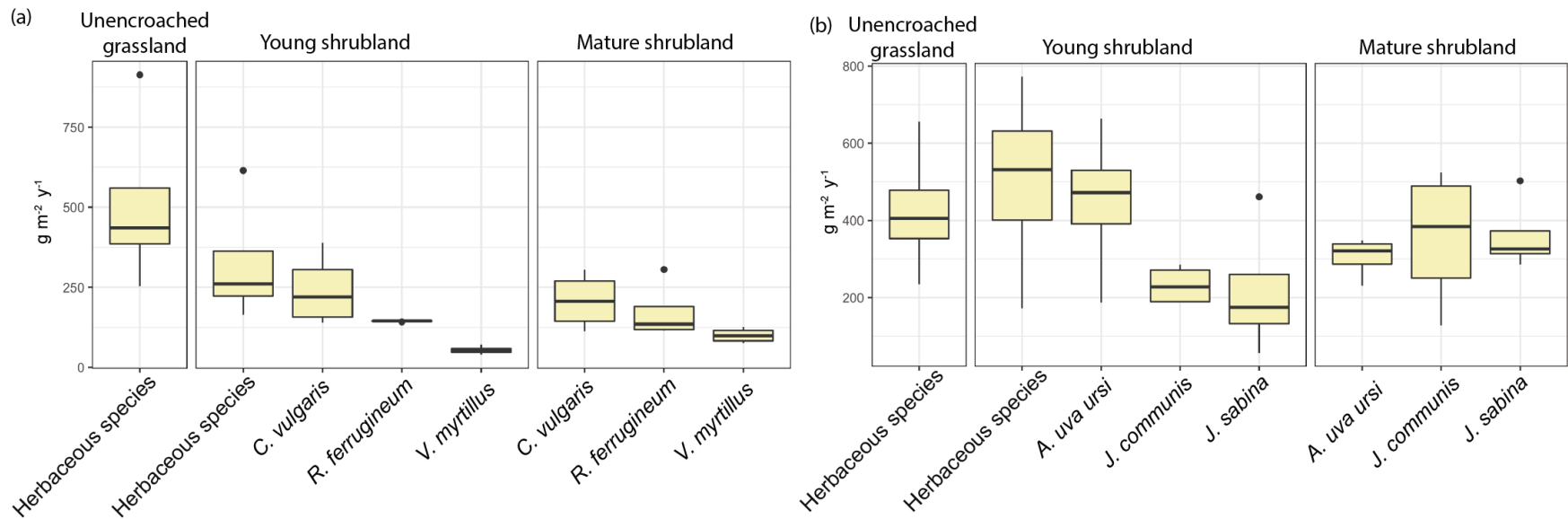
Figure SF3. **Principal component analysis of the functional traits and foliar elemental composition of the shrub species in the mature shrubland including both study sites**. The ellipses represent the dispersion around the centroids for each shrub species with a normal probability of 0.85.

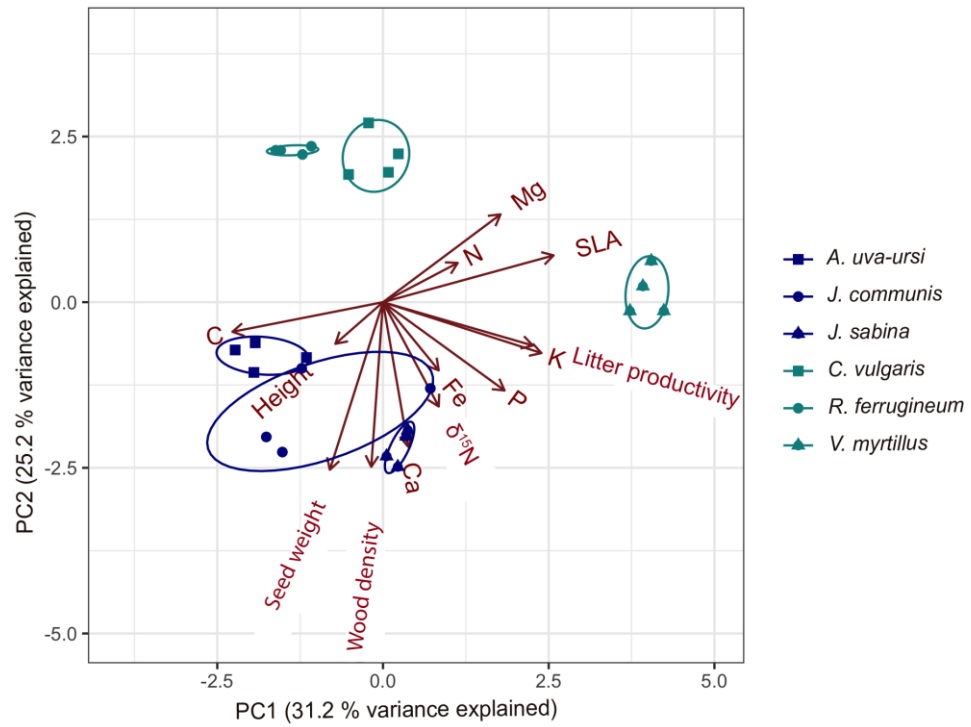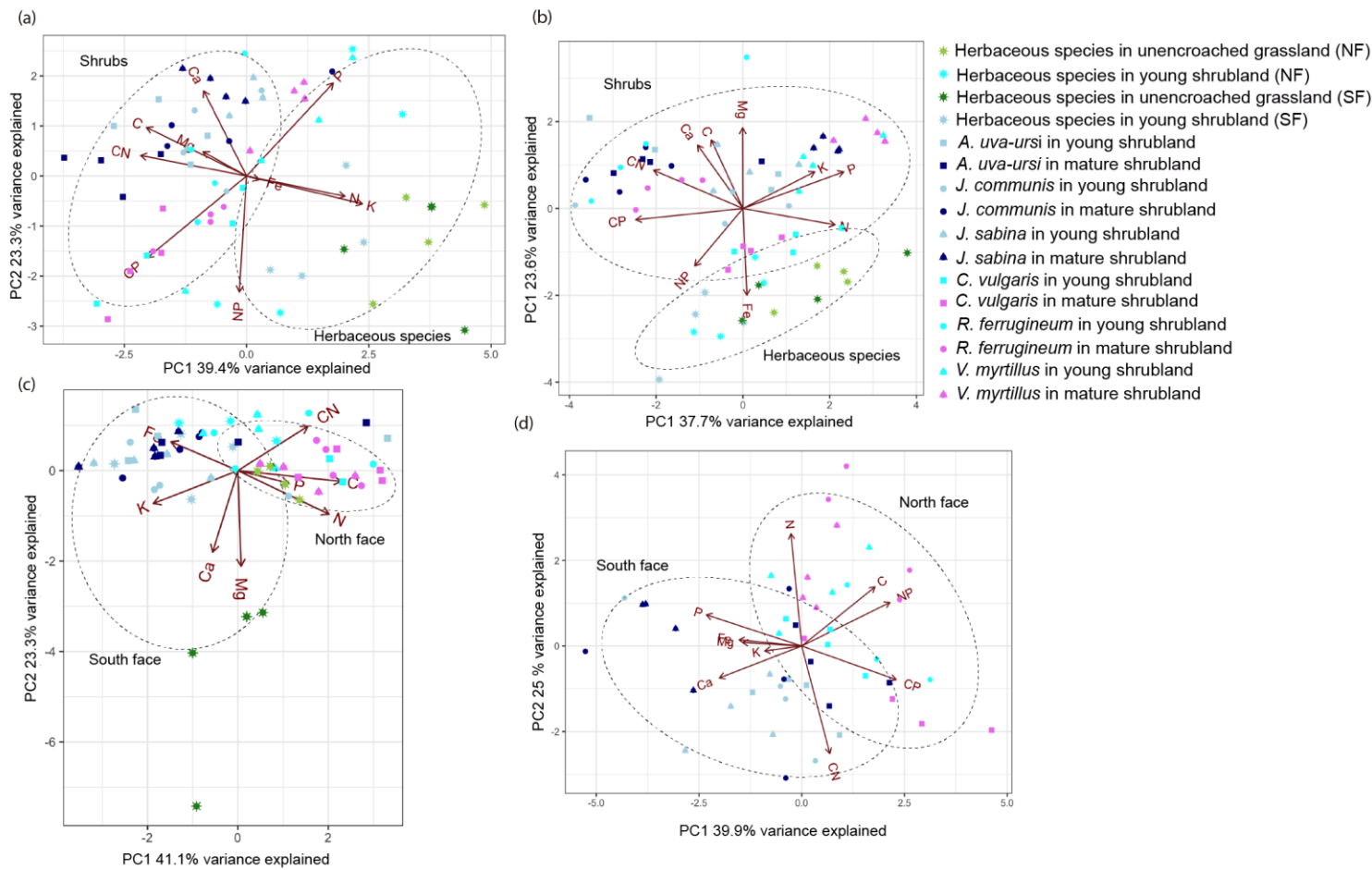849 Figure SF4. **Principal component analysis (PCA) based on the elemental concentrations for (a) leaves,**

850 **(b) leaf-litter, (c) soil and (d) stems for all vegetation types and successional stages**. Only variables that

851 were poorly correlated with each other (Pearson's coefficients <0.6) were included in the analyses. Different

852 colors indicate the successional stages and different shapes indicate the vegetation types. The ellipses in (a)

853 and (b) represent the dispersion around the centroids for herbaceous species and shrubs, and in (c) and (d) for

854 the different sites, with a normal probability of 0.85.

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