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## Ley grassland under temperate climate had a legacy effect on soil organic matter quantity, biogeochemical signature and microbial activities

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1 **Ley grassland under temperate climate had a legacy effect on soil organic matter**  
2 **quantity, biogeochemical signature and microbial activities**

3

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15



## 16 Abstract

17 Temporary (ley) grassland introduced into cropping cycles has been advocated as  
 18 being beneficial for the delivery of ecosystem services by agricultural soils. The management  
 19 of these temporary grasslands has unknown effects on soil organic matter (SOM)  
 20 concentrations and biogeochemical properties of the cropland soils following the grassland  
 21 phase. Here, we investigated the legacy effect of differently managed temporary grasslands,  
 22 i.e. change of soil properties lasting beyond three years of crop. We assessed soil organic  
 23 carbon (SOC) quantity and SOM biogeochemical signature (composition of soil neutral  
 24 carbohydrates and lignin), as well as microbial activities (potential C and N mineralization  
 25 and denitrification). We used a long-term field experiment on Cambisol with temperate  
 26 climate in western France, where temporary grassland management practices differed in terms  
 27 of duration (3 or 6 years) and presence or absence of N fertilization. Topsoil (10 cm) samples  
 28 were collected after a 3-yr crop rotation (maize, wheat, barley).

29 Our results showed that N fertilization during the grassland phase was necessary to  
 30 maintain soil C and N concentrations beyond three years of crop. Temporary grassland  
 31 management may affect microbial activities as indicated by contrasting polysaccharide and  
 32 lignin composition. It had however, no effect on potential CO<sub>2</sub> and N<sub>2</sub>O emissions during  
 33 laboratory incubations. The biogeochemical signature of SOM was close to continuous  
 34 grassland only in treatments with 6 yrs of fertilized temporary grassland. We thus, conclude  
 35 that the legacy effects of a grassland phase on SOC quantity and properties of SOM depend  
 36 on its management.

37  
38

## 39 Graphical abstract

	Control		Temporary grassland		
	3 years	Cropland	Grassland	Cropland	Grassland
6 years	Cropland	Grassland	Grassland	Grassland	Grassland N-
9 years	Cropland	Grassland	Cropland	Cropland	Cropland
	9 yrs crop rotation maize-wheat-barley	9 yrs fertilized grassland	 3yrs                      6yrs		 N+                      N-
	Cropland SOM ≠ Grassland SOM		Unexpected non-progressive age dependent SOM effect		Positive legacy effect on SOM quantity and chemical signature
	6yrs fertilized grassland introduction in crop rotation have positive legacy effects on SOM content and biochemical signature				

40  
41  
42

43 **Keywords**

44 Temporary grassland management, microbial activities, potential N<sub>2</sub>O emission, soil organic  
45 matter biogeochemistry, long-term field experiment.

46

47 **Abbreviations**

48 SOM, Soil organic matter; SOC, Soil organic carbon; TG, temporary grasslands; TG3N+,  
49 temporary grassland with 3 years of fertilized ley; TG6N+, temporary grassland with 6 year  
50 of fertilized ley; TG6N-, temporary grassland with 6 years ley without fertilization; CC,  
51 permanent cropland; GG, permanent grassland; POM, particulate organic matter; IN,  
52 inorganic nitrogen; ON organic nitrogen; IP inorganic phosphorus; OP, organic phosphorus;  
53 V, vanillyl; S, syringyl; Co, *p*-coumaryl; Ac, acid; Al, aldehyde; C5, pentoses; C6, hexoses;  
54 MB-C, microbial carbon; MB-N, microbial nitrogen; CLPP, community-level physiological  
55 profiles; AWCD, average well color development; Cmin, carbon potential mineralization;  
56 Nmin, nitrogen potential mineralization; *k*, carbon mineralization constant rate; PD, potential  
57 denitrification; PCA, principal component analysis.

58

## 59 **1. Introduction**

60 Agricultural soils of the temperate regions have lost much of their organic carbon  
61 since the 1960s due to the intensification of crop production (Lal, 2004). Nowadays,  
62 rebuilding soil organic matter (SOM) stocks has become a priority because SOM greatly  
63 improves soil properties and functions. Moreover, SOM storage may be able to counteract  
64 increasing atmospheric greenhouse gas concentrations (Chabbi et al., 2017; Smith, 2016). In  
65 this context, grassland soils with high belowground C stocks can be carbon sinks (Conant et  
66 al., 2001). Therefore, the introduction of temporary or ley grassland in cropland cycles may  
67 be a suitable negative emission technology (Smith, 2016), if it increases soil C sequestration  
68 as a legacy effect (i.e. soil C lasts beyond the following cropland phase).

69  
70 Temporary grasslands were shown to have several beneficial effects on soil quality related to  
71 SOM increase (Studdert et al., 1997; Franzluebbers et al., 2014). Moreover, they reduce  
72 nitrate leaching as compared to continuous cropping systems (Kunrath et al., 2015). Recent  
73 work also showed that introduction of temporary mowed grassland in the cropping cycle led  
74 to higher contribution of SOM in macro-aggregates as compared to permanent arable land  
75 (Panettieri et al., 2017). This effect was observed as a legacy effect after 3 yrs of continuous  
76 cropping, and explained by higher amounts of root litter input during the grassland phase  
77 (Armas-Herrera et al., 2016; Attard et al., 2016). However, in order to secure the C sink  
78 function of grasslands, judicious management is necessary (Smith et al., 2015). Indeed,  
79 management of temporary grasslands in terms of cutting or grazing, fertilisation and duration,  
80 influences the decomposition and stabilisation processes of SOM through its impact on C, N  
81 and P coupling and decoupling (Acharya et al., 2012; Attard et al., 2016; Conant et al., 2001;  
82 Crème et al., 2016; Klumpp et al., 2009; Lemaire et al., 2015; Rumpel et al., 2015). Beneficial  
83 effects of temporary grasslands may depend on the interplay between environmental  
84 constraints, soil microorganisms and SOM characteristics. To date the effect of temporary  
85 grassland management on these variables is still unknown.

86 In this study, we investigated the legacy effect (i.e. changes observable after 3 years of  
87 crop) of differently managed temporary grasslands on SOM and microbial properties. The  
88 aim of the study was to better apprehend the impact of temporary grassland management on  
89 SOM biogeochemical composition, nutrient forms as well as potential microbial activities.  
90 We analysed SOM molecular signatures of soil neutral carbohydrates and lignin, susceptible  
91 to be affected by grassland management (Crème et al., 2017). These two macromolecules  
92 were chosen because their nature and fate in soil are contrasting (Kögel-Knabner and Rumpel,  
93 2018). As biomarkers, their quantity and composition may give valuable information on

94 degradation and stabilisation processes of SOM. Non-cellulosic carbohydrates have a low  
95 degree of polymerization and may be plant or microbial derived (Murayama, 1984; Kögel-  
96 Knabner, 2017, 2002; Kuzyakov et al., 2000; Rumpel and Dignac, 2006). In contrast,  
97 comparatively more recalcitrant compounds such as aromatic lignins are exclusively plant-  
98 derived. They are mineralizable only by a restricted number of decomposers (white-rot fungi)  
99 (Frei, 2013; Kögel-Knabner, 2017, 2002). In addition, we determined SOM C:N:P  
100 stoichiometry, which may change in response to grassland management (Crème et al., 2016).  
101 We also quantified the amount of particulate organic matter and potential microbial activities  
102 involved in CO<sub>2</sub> and N<sub>2</sub>O emissions and nutrient release. We hypothesised (i) that temporary  
103 grassland has legacy effects on SOM quantity, molecular composition and microbial  
104 activities, observable after a 3 year crop rotation following the end of the grassland phase; (ii)  
105 that the magnitude of the legacy effect would depend on the length of the grassland phase (6  
106 versus 3 years); and (iii) that the magnitude of the legacy effect would be sensitive to nitrogen  
107 fertilization applied during the grassland phase.

108

## 109 **2. Materials and methods**

110

### 111 *2.1. Site description*

112 The experimental site is the long term observatory on environmental research called  
113 “agrosystems biogeochemical cycles and biodiversity” (SOERE ACBB) situated at the INRA  
114 research station in Lusignan (46°25'12.91" N; 0°07'29.35" E), western France.

115 The mean annual temperature is 12°C and the mean annual precipitation is around 750  
116 mm. The experiment was established in 2005 on a Cambisol with a silty-loam texture and a  
117 bulk density of 1.43 kg m<sup>-3</sup> (Chabbi et al., 2009; Moni et al., 2010). The profile can be divided  
118 into two main domains: three red-brown upper horizons characterised by a loamy texture  
119 overlying three lower clayey rubefied horizons rich in kaolinite and iron oxides (Ducloux and  
120 Chesseron, 1988). The lower rubefied horizons were classified as Paleo-Ferralsol from the  
121 late Mesozoic period, while the upper horizons were classified as Cambisol formed from  
122 Ferralsol horizons under temperate climate (Ducloux and Chesseron, 1988).

123 At this site, in 2005, a long-term field experiment was established consisting of ley  
124 grassland introduction in the cropping cycle with 5 different treatments in terms of  
125 fertilization and duration of the grassland phase (Table 1): TG3N+ corresponds to 3 yrs of  
126 fertilized ley, TG6N+ to 6 yrs of fertilized ley and TG6N- is a 6 yrs ley without fertilization.  
127 In addition, two control treatments with permanent cropland (CC) consisting of maize-wheat-  
128 barley rotation and with permanent grassland (GG) were established. Treatments were

129 replicated 4 times in 4 separated blocks. Before the beginning of the experiment, the whole  
130 surface had been homogenized by 3 years of cropping (barley, barley and wheat).

131 The species sown for CC in April 2005, was *Zea mays* L. (maize) with a density of 8.5  
132 seeds m<sup>-2</sup>. In October 2005, *Triticum aestivum* L. (wheat) was sown with density of 150 seeds  
133 m<sup>-2</sup>. In October 2006, *Hordeum vulgare* L. (barley) was sown with density of 165 seeds m<sup>-2</sup>.  
134 This crop rotation of 3 yrs was repeated when the soil was under permanent cropland (CC).  
135 For ley grassland, this crop rotation was alternated with a grassland phase (TG). To establish  
136 grassland, a species mixture containing *Dactylis glomerata* L. (cocksfoot), *Festuca*  
137 *arundinacea* Schreb (tall fescue) and *Lolium perenne* L. (rye-grass) was sown.

138 The grasslands were harvested 3-4 times a year depending on their productivity. The  
139 biomass was removed. Nitrogen was the only element added through mineral fertilizer  
140 application. The N-fertilizer amount needed to account for biomass removal was determined  
141 each year by soil analyses. In consequence, fertilization varied with years and ranged between  
142 36 and 160 kg N ha<sup>-1</sup> year<sup>-1</sup> for cropland, and between 170 and 380 kg N ha<sup>-1</sup> year<sup>-1</sup> for  
143 fertilized grassland phases according to the residual soil N amount determined at the end of  
144 the previous growing season. It is important to note that in unfertilised temporary grassland  
145 (TG6N-) differed from the other treatments only with regards to fertilisation during the  
146 grassland phase.

147

## 148 2.2. Soil sampling

149 The top 10 cm soil was sampled in March 2014 for each plot (i.e. 5 treatments × 4  
150 blocks), nine years after implantation of the experiment. All soils except GG soils had been  
151 under cropland rotation for the last three years (year 7 to 9, Table 1). Two soil cores (8 cm  
152 diameter) were randomly taken from each plot, and mixed to yield one composite sample per  
153 plot. Fresh sub-samples were used for biological analysis. The remaining soil was air-dried,  
154 and aliquots were ground for chemical analysis.

155

## 156 2.3. Soil elemental composition

157 Total carbon (C) and total nitrogen (N) concentrations were determined using an  
158 elemental analyzer (CHN NA 150, Carlo Erba). No carbonate was present in the soil,  
159 therefore soil C is considered to be exclusively organic.

160 Nitrate-N (NO<sub>3</sub><sup>-</sup>) and ammonium-N (NH<sub>4</sub><sup>+</sup>) were determined colorimetrically after  
161 extraction of soil with a 0.5 M KCl solution (1:5 weight/volume) (Page *et al.*, 1982).

162 Total soil phosphorus (P) was determined with the ignition method (Walker and  
163 Adams, 1958) using the modifications introduced by Ivanoff *et al.*, (1998). Briefly 0.5 g of

164 dry soil was ignited for 1 h at 550°C before extraction during 24 h by shaking with 1 M  
165 sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) for total P. A second (unignited) replicate of 0.5 g dry soil was  
166 extracted with 30 mL of 1 M H<sub>2</sub>SO<sub>4</sub> for 24 h for inorganic phosphorus (IP) determination.  
167 The suspensions were centrifuged at 17000 g for 10 min and the supernatants analyzed. The  
168 orthophosphate content in each extract was measured as acid molybdenum complex  
169 colorimetrically with detection at 880 nm (Murphy and Riley, 1962). The organic phosphorus  
170 (OP) content was determined by difference.

171

#### 172 2.4. Particulate organic matter

173 Particulate organic matter (POM) was separated from soil by physical fractionation  
174 (Zimmermann, 2007 modified by Poeplau et al. 2013). Briefly, 30 g of soil were dispersed in  
175 150 mL of deionized water using an ultrasonic probe with a calibrated energy output of 22 J  
176 mL<sup>-1</sup> to break down macro-aggregates. The soil suspension was sieved over a 63 µm sieve  
177 with a minimum of 2 L of deionized water. The fraction > 63 µm was freeze-dried and  
178 separated by density fractionation into POM and sand and stable aggregate. Thirty mL of  
179 sodium polytungstate solution with a density of 1.8 kg m<sup>-3</sup> was added and stirred 1 h followed  
180 by centrifugation for 15 minutes at 10000 RPM. The floating POM fraction and the  
181 submerged sand and stable aggregate fraction were separated and recovered, after vacuum  
182 filtration using glass fiber filters (Whatman GF/C 0.45 µm). They were rinsed with deionized  
183 water, freeze-dried, ground and analyzed for their C and N concentrations (see above).

184

#### 185 2.5. Lignin

186 Soil lignin content was determined after alkaline cupric oxide oxidation (Hedges and  
187 Ertel, 1982; Koegel and Bochter, 1985) and quantification of the released monomers by gas  
188 chromatography. Briefly, oxidation was carried out with CuO at high temperature (172°C)  
189 under alkaline conditions (2 M NaOH) using 500 mg of soil, 250 mg of CuO, 50 mg of  
190 ammonium ferrous hexahydrate and 50 mg of glucose. After cooling the samples, 0.5 mL of  
191 the recovery standard ethylvanillin (60 mg L<sup>-1</sup>) was added to assess losses during the  
192 purification procedure. Thereafter, samples were acidified with 5 M HCl before removal of  
193 humic acids by centrifugation (10 minutes at 17000 RPM) and solid-phase extraction of the  
194 phenolic oxidation products using C18 reversed phase columns. The phenols were derivatised  
195 with BSTFA and quantified as trimethylsilyl derivatives by gas chromatography with an HP  
196 gas chromatograph (HP GC 6890) equipped with a flame ionization detector and a SGE BPX-  
197 5 column (50 m length, 0.25 mm inner diameter, 0.32 µm coating). Samples were injected in  
198 split mode (1:10). The GC oven temperature was programmed at 100°C during 2 min, then



199 from 100°C to 172°C with a heating rate of 8°C min<sup>-1</sup>, from 172 to 184°C at 4°C min<sup>-1</sup>, and  
200 from 184 to 300°C at a rate of 10°C min<sup>-1</sup>.

201 The content and state of decomposition of lignin were characterized by this method.  
202 The total lignin concentration of the sample is represented by the sum of phenolic oxidation  
203 products vanillyl (V), syringyl (S) and p-coumaryl (Co) with their acid (Ac), aldehyde (Al)  
204 and ketone side chains. Thus, the concentration of V phenols corresponded to the sum of the  
205 concentrations of vanillin, acetovanillone and vanillic acid. S phenol concentrations were  
206 calculated as the sum of syringaldehyde, acetosyringone and syringic acid. The Co phenol  
207 concentrations were estimated as the sum of ferulic and p-coumaric acid. The ratios between  
208 the acid and aldehyde forms of the phenol monomers V and S can be used to infer the state of  
209 lignin degradation. Usually (Ac/Al)<sub>V</sub> and (Ac/Al)<sub>S</sub> are increasing with increasing lignin  
210 degradation (Koegel and Bochter, 1985). Likewise, the ratios Co/V and S/V are decreasing  
211 with increasing lignin degradation because of the preferential degradation of S and Co as  
212 compared to V units (Bahri et al., 2006).

213

## 214 2.6. Sugar

215 The concentrations of neutral non-cellulosic carbohydrates were determined after  
216 trifluoroacetic acid (TFA) hydrolysis and reduction-acetylation of the monomers released by  
217 gas chromatography (Rumpel and Dignac 2006; modified by Eder et al 2010). The analysis  
218 was performed using 500 mg of air-dried and ground soil samples. Hydrolysis was carried out  
219 at 105°C for 4 h with 5 mL of 4 M TFA. After cooling the samples, 0.5 mL of the recovery  
220 standard methylglucose (3 g L<sup>-1</sup>) was added and the soil was removed by filtration through  
221 glass fiber filters (Whatman GF/C 0.45 µm). After evaporation of TFA using a vacuum  
222 speedvac (Thermo scientific, USA), the sugars were re-dissolved in 0.9 µL Na<sub>2</sub>-EDTA and  
223 the internal standard myo-inositol (1.75 g L<sup>-1</sup>) was added. Reduction of aldoses to alditols was  
224 performed at 40°C for 1 h 30 after addition of 1 mL of sodium borohydride (NaBH<sub>4</sub>) in  
225 dimethylsulfoxide (20 g L<sup>-1</sup>). After cooling, acetylation was carried out by addition of 0.2 mL  
226 acetic acid. Thereafter, 2 ml of acetic anhydride were added and methylimidazole (0.2 mL)  
227 was used as a catalyst of the reaction. After 10 min, the reaction was stopped by addition of  
228 10 mL ice-cold distilled water. The derivatised monosaccharide units were extracted by  
229 liquid—liquid extraction using a vortex mixer following addition of 1 mL dichloromethane.  
230 The gas chromatographic measurements of the monosaccharides in dichloromethane were  
231 performed with an HP GC 6890 gas chromatograph equipped with a flame ionization detector  
232 operating in split mode (1:10). Separation of the monosaccharide units was achieved with a  
233 60 m fused silica capillary column (SGE BPX 70, 0.32 mm internal diameter, 0.25 mm film

234 thickness) and the following temperature program: from 200 to 250°C at 8 °C min<sup>-1</sup> and 15  
235 min isothermal at 250°C. Carrier gas was helium with a flow rate of 1.0 mL min<sup>-1</sup>. The  
236 injector was kept at 250°C and the detector at 260°C. The concentrations of individual sugars  
237 were calculated based on the total ion current of the recovery standard methylglucose and the  
238 internal standard myo-inositol. The non-cellulosic sugar content of the samples was  
239 determined as sum of monosaccharides released: pentoses (xylose, ribose and arabinose),  
240 hexoses (glucose, galactose and mannose), and desoxyhexoses (fucose and rhamnose)  
241 (Koegel-Knabner, 2002). To determine the plant and microbial origin of soil carbohydrates,  
242 the ratios C6/C5 and desoxyC6/C5 were calculated. The decay degree of plant-derived sugars  
243 was determined by the ratio of xylose/mannose (Oades, 1984).

244

### 245 2.7. Microbial biomass

246 Microbial biomass was determined on fresh soil samples by the chloroform  
247 fumigation-extraction method (Vance *et al.*, 1987). Briefly, the fumigation-extraction  
248 technique is based on fumigation of soil with chloroform (CHCl<sub>3</sub>) for 16 hours, which allows  
249 lysis of microbial cells. For each plot, dissolved C and N were extracted from a fumigated soil  
250 sample and an unfumigated control with a 0.03 M K<sub>2</sub>SO<sub>4</sub> solution (1:4 weight/volume). The  
251 extracts were filtered at 0.7 µm and freeze-dried. C and N of the dry extracts were determined  
252 with an elemental analyzer (CHN NA 1500, Carlo Erba). Microbial C (MB-C) and N (MB-N)  
253 were calculated as differences between paired fumigated and non fumigated soils, using a  
254 multiplication factor of 2.22 for C and N (Jenkinsen *et al.*, 2004).

255

### 256 2.8. Potential mineralization of C and N

257 The potential C and N mineralization (C<sub>min</sub> and N<sub>min</sub>) of soils was determined under  
258 controlled conditions. The fresh soil sieved at 4 mm was incubated at field moisture in three  
259 replicates of 30 g and one of 50 g for the last step (around 140 g in total) in jars of 1 liter at  
260 20°C for 56 days. All samples were pre-incubated for 2 days under the same condition and the  
261 atmosphere was flush with CO<sub>2</sub>-free atmosphere at the beginning of the experiment. To avoid  
262 anaerobic conditions, the atmosphere was flushed regularly with CO<sub>2</sub>-free synthetic air.

263 The accumulated CO<sub>2</sub> concentration was measured at day 0, 1, 3, 7, 10, 14, 21, 28, 42  
264 and 56 with a micro gas chromatograph (Agilent 590). C mineralization was modeled with R  
265 (Studio Version 0.98.1062) using a first order exponential model with one C pool. This model  
266 gave the best fit after model evaluation. We used the following equation:

267

$$C(t) = C_0(1 - e^{(-kt)})$$

268 Where  $C(t)$  is the cumulative amount of C mineralized after time  $t$  (%  $C_0$ ),  $C_0$  is the  
269 initial amount of organic carbon,  $t$  is the incubation time (day) and  $k$  is the C mineralization  
270 rate constant.

271 The soil was collected at day 7, 14, 28 and 56 to follow the N mineralization. The  
272 concentration of inorganic N of each sample was determined on fresh soil as described above  
273 (1.3).

274

### 275 *2.9. Potential denitrification*

276 Potential gross rate of  $N_2O$  production, i.e. potential denitrification (PD), was  
277 measured as the  $N_2O$  production rate from soil samples amended with  $NO_3^-$  and labile C,  
278 headspace atmosphere being replaced by a He: $C_2H_2$  mixture (90:10) to ensure anaerobic  
279 conditions and inhibition of  $N_2O$  reductase (Patra et al., 2005). Soil samples were incubated at  
280  $28^\circ C$  for 8h.  $N_2O$  concentration was measured after 2h, 4h, 6h and 8h with a gas  
281 chromatograph. A linear rate of  $N_2O$  production was always observed during the  
282 denitrification assays.

283

### 284 *2.10. Statistical analysis*

285 A two-way ANOVA was used followed by Tukey HSD post hoc test. For sugar and  
286 lignin data, the first factor accounted for different treatments, while the second factor was the  
287 analytical series, which impacted significantly the results. For all other data, the first factor  
288 was the treatments and the second factors the field plot.

289 Principal component analysis (PCA) was carried out for soil parameters with package  
290 FactoMineR. The PCA function of the FactoMineR package automatically transforms data to  
291 a standardized normal distribution.

292

## 293 **3. Results**

294

### 295 *3.1. Soil C, N and P concentrations and forms*

296 The C concentrations ranged between 9.1 and 16.8  $mg\ g^{-1}$ , and those of N between  
297 1.01 and 1.65  $mg\ g^{-1}$  (Table 2). Lowest concentrations of both elements were observed for  
298 unfertilised temporary grassland soil (TG6N-), and highest for continuous grassland (GG).  
299 Both elements followed a similar pattern and C/N ratios were thus unaffected by treatments.  
300 Temporary grassland duration had no linear effect on soil C and N as demonstrated by the  
301 significant increase of C and N concentrations for TG3N+ as compared to continuous  
302 cropping (CC), while no such an effect was observed for TG6N+.

303 Total soil phosphorus (P) concentrations did not vary significantly among treatments  
304 (Table 2). Significant differences were noted for the C/P ratio, which increased following  
305 fertilisation and was highest in GG (Table 2). Continuous grassland also showed the highest  
306 proportion of C in the POM fraction and the highest  $\text{NH}_4^+/\text{NO}_3^-$  ratio (Table 2).

307

### 308 *3.2. Soil carbohydrate signature*

309 Total soil carbohydrate concentrations ranged between 3.9 and 5.9  $\text{mg g}^{-1}$  soil, and  
310 decreased in the order  $\text{GG} > \text{TG6N}^+ > \text{TG3N}^+ > \text{CC} > \text{TG6N}^-$  (Table 3). The carbohydrate  
311 content expressed on a total C basis was with 43% highest for  $\text{TG6N}^-$  and similar for all other  
312 treatments (Table 3). A  $\text{C}_6/\text{C}_5 > 2$  was recorded for the soils of the treatments CC and TG  
313 (Table 3). This indicated a primarily microbial origin of the carbohydrates. Both ratios  
314 indicating microbial sugar contribution ( $\text{C}_6/\text{C}_5$  and  $\text{desoxyC}_6/\text{C}_5$ ) were lowest for GG and  
315 highest for CC, with temporary grasslands being in an intermediate position (Table 3).

316

### 317 *3.3. Lignin signature*

318 Total soil lignin concentrations varied between 0.20 and 0.35  $\text{mg g}^{-1}$  soil, and were not  
319 different for GG and CC, in contrast to TG6 and TG3, which showed significantly lower  
320 values (Table 4). Lignin contents were comprised between 1.9 and 2.4 % of total soil C  
321 without any differences among treatments (Table 4). The  $\text{Ac}/\text{Al}$  ratios of vanillyl and syringyl  
322 indicated similar lignin degradation for GG, CC and TG, except for the ratio  $(\text{Ac}/\text{Al})_s$ , which  
323 differentiated  $\text{TG3}^+$  and  $\text{TG6}^+$  (Table 4). The ratio  $\text{Co}/\text{V}$  was significantly higher for CC than  
324 GG, indicating a lower degree of soil lignin degradation.  $\text{TG6N}^+$  and  $\text{TG6N}^-$  showed  
325 intermediate values, while the highest  $\text{Co}/\text{V}$  and  $\text{S}/\text{V}$  ratios were recorded for  $\text{TG3N}^+$  (Table  
326 4).

327

### 328 *3.4. Microbial biomass*

329 The C and N concentrations of soil microbial biomass (MB-C and MB-N) are  
330 presented in Table 5. MB-N did not vary significantly among treatments. In contrast, MB-C  
331 with decreased in the order  $\text{GG} > \text{TG6N}^+ > \text{TG3N}^+ > \text{CC} > \text{TG6N}^-$  (Table 5) and the MB-  
332 C/MB-N ratio consequently showed highest values for GG (Table 5).

333

### 334 *3.5. Potential microbial activities*

335 Treatment effects on C mineralisation during 56 days of incubation are presented in  
336 Fig. 1. At the end of incubation, between 3.8 and 10.5 % of initial C were mineralised, highest  
337 mineralisation rate constant  $k$  being recorded for soils of the treatment GG (Fig. 1).

338 N mineralisation dynamics during the incubation (for N-NH<sub>4</sub><sup>+</sup> and N-NO<sub>3</sub><sup>-</sup>) are shown  
339 in Fig. 2. During the first 15 days of incubation, a decrease of soil ammonium concentration  
340 was observed (Fig. 2A). Thereafter, the N-NH<sub>4</sub><sup>+</sup> concentrations increased again to reach  
341 highest values for GG and TG6N+. The other three treatments showed similar NH<sub>4</sub><sup>+</sup>  
342 concentrations at the end of the incubation, with TG3N+ and TG6N- presenting significantly  
343 lower values than GG. Nitrate (NO<sub>3</sub><sup>-</sup>) concentrations increased during the incubation in all  
344 treatments (Fig. 2B). GG showed significantly higher nitrate accumulation than CC.  
345 Temporary grasslands were in an intermediate position (Fig. 2B).

346 Potential denitrification ranged between 0.72 and 1.13 μgN-N<sub>2</sub>O g<sup>-1</sup> soil h<sup>-1</sup>, and did  
347 not reveal any differences among treatments (Table 5).

348

### 349 3.6. Principal component analyses

350 To investigate the parameters most affected by land use legacy, we carried out a PCA  
351 (Fig. 3). Based on Pearson correlation coefficients among variables of the dataset, it appeared  
352 that C, N, MB-C, lignin, sugar concentration and *k* were positively correlated (all  $r > 0.57$  and  
353  $p < 0.001$ ) and all were negatively correlated to C6/C5 sugar ratio ( $p < 0.01$ ). Soil P was  
354 positively correlated only with Co/V ( $r = 0.61$ ) and S/V ( $r = 0.51$ ) lignin ratios; S/V itself  
355 correlated negatively with sugar ( $r = -0.53$ ) and POM ( $r = -0.64$ ). PD was positively  
356 correlated with the S/V lignin ratio ( $r = 0.54$ ;  $p = 0.05$ ).

357 The first two dimensions of the PCA explained 73,17 % of the variability. The first  
358 dimension, representing 52.15% of the variability, was strongly and positively ( $r > 0.78$ )  
359 correlated with sugar and lignin concentrations, soil C and N concentrations, and the C  
360 mineralization rate constant *k*, and negatively with the C6/C5 sugar ratio (Fig. 3B). The  
361 second dimension, which represented 21.02 % of the variability, was strongly and positively  
362 ( $r > 0.8$ ) correlated to the concentration of soil P and Co/V and S/V lignin ratios and more  
363 weakly with PD ( $r = 0.51$ ). It was negatively correlated to POM ( $r = -0.45$ ) (Fig. 3B).

364 On the PCA plan (Fig. 3A), GG was separated in the first dimension from all other  
365 treatments that had been under crop rotation for the last three years. However, grassland  
366 management before the cropland phase affected the location of the treatments within the PCA  
367 plan. While six-year fertilised grassland TG6N+ was in an intermediate position between CC  
368 and GG, TG6N- is located next to CC in the opposite direction (Fig. 3 A). The treatment  
369 TG3N+ overlaps with CC.

370

## 371 4. Discussion

372

373 *4.1. Grassland soil and arable soil – two different systems with contrasting SOM*  
374 *characteristics and microbial activities*

375

376 Nine years of continuous grassland (GG) led to higher soil C and N concentrations  
377 than 9 yrs of continuous crop (CC), in accordance with literature results (Guggenberger et al.,  
378 1994; INRA, 2002; Lal, 2004; Soussana et al., 2004). The PCA clearly showed differentiation  
379 of GG and CC along the first dimension, which correlated to C, N, MB-C, lignin, sugar, *k* and  
380 the C6/C5 sugar ratio. Soils under both land use systems can thus be distinguished not only  
381 according to their C and N concentrations but also according to the biochemical nature of  
382 their SOM (Rumpel et al., 2009; Rumpel and Chabbi, 2010) and potential microbial activities  
383 (Attard et al., 2016).

384

385 Highest concentrations of particulate organic matter, indicating the presence of fresh  
386 litter, were found for GG. This may be explained by (1) lower mineralisation of SOM in soil  
387 permanently covered by vegetation and absence of tillage (Soussana et al., 2004) and/or (2)  
388 higher constant inputs of fresh plant material originating from grass roots (Acharya et al.,  
389 2012; Post and Kwon, 2000). High inputs of litter and rhizodeposition under grassland may  
390 also sustain a more active microbial community (Attard et al., 2016; Carter et al., 1998;  
391 Murphy et al., 2007). This is in agreement with the higher potential C mineralisation and  
392 microbial biomass (MB-C) in GG as compared to CC.

393

394 Lignin concentrations and contents were similar for CC and GG. These results are in  
395 contrast with those recorded by other authors (Guggenberger et al., 1994; Lobe et al., 2002;  
396 Rumpel and Chabbi, 2010). In the present study, differences in the lignin signature between  
397 the two systems concerned the Co/V ratio. The lower Co/V ratio for CC indicates a more  
398 degraded lignin signature as compared to GG, in accordance with observations made by other  
399 authors (Lobe et al., 2002). This is supported by a higher contribution of microbial sugars in  
400 CC as compared to GG, suggesting more processed SOM in soil under continuous cropping.  
401 Moreover, the C/P ratio of 11.6 for CC as compared to 18.4 for GG could indicate a more  
402 stabilized SOM under continuous crop (Kirkby et al., 2013).

403

404 Overall, nine years after the establishment of mowed and fertilised grassland, soils  
405 showed clear differences as compared to cropland concerning the nature of their SOM,  
406 nutrient concentrations as well as amount and activity of microbial biomass. According to our  
407 data, it seems that while long-term grasslands are beneficial for increasing SOM concentrations,

408 at the same time they contain less processed SOM, which may increase the potential for CO<sub>2</sub>  
409 emissions but on the other hand is beneficial in terms of nutrient retention, as illustrated for  
410 example by the higher NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> ratio.

411

#### 412 *4.2. Legacy effect of N fertilisation during 6 yrs of temporary grassland*

413

414 Our data showed that the unfertilised 6 yrs temporary grassland (TG6N-) presented the  
415 lowest soil C and N concentrations of all treatments. This may be explained by biomass  
416 export following mowing leading to nutrient removal and thus loss of productivity during the  
417 grassland phase. Although soils were fertilised during the following three years of cropland, C  
418 and N concentrations were significantly lower for TG6N- than TG6N+. Moreover, absence of  
419 fertilisation during 6 yrs of temporary grassland induced a legacy effect concerning SOM  
420 stoichiometric ratios, with the lowest C/N and C/P ratios of all treatments. These data suggest  
421 low amounts of plant derived matter, and a high proportion of soil C being composed of  
422 microbial derived material in TG6N- (Kirkby et al., 2013). This is supported by the high  
423 proportions of microbial sugars present in soil of TG6N-. In contrast, the lignin  
424 concentrations on a soil or C basis were not impacted by fertilisation during the grassland  
425 phase, even if lignin degradation is generally affected by N availability (Kiem and Kögel-  
426 Knabner, 2003; Knicker, 2011).

427

428 The strong legacy effect of absence of fertilisation during the grassland phase on soil  
429 parameters is illustrated by PCA analyses, as the soils of TG6N- were distinguished from all  
430 treatments along the first dimension. This shows that N fertilisation of mowed grasslands is  
431 necessary to allow grassland soil to acquire a specific grassland SOM signature and maintain  
432 important soil functions (Conant et al., 2001; Lal, 2004; Rumpel et al., 2015). Indeed, N  
433 fertilisation is a limiting factor for biological productivity, plant biomass formation and SOM  
434 input (Knicker, 2011). Thus, beneficial effects of temporary grasslands related to soil C are  
435 dependent on N fertilization.

436

#### 437 *4.3. Legacy effects of grassland duration*

438

439 To investigate legacy effects of temporary grassland duration, we compared the effects  
440 of 3 and 6 yrs fertilised mowed temporary grassland (TG3N+ and TG6N+). Our data show  
441 that 3 yrs of temporary grassland increased concentrations of major elements as compared to  
442 continuous crop and even as compared to TG6N+. The biogeochemical signature of SOM is

443 distinct for TG3N+ as illustrated by lower lignin degradation state for TG3N+ than TG6N+.  
444 Parameters related to the sugar signature are intermediate between CC and TG6N+,  
445 suggesting an age dependent legacy effect. It is interesting to note that comparatively labile  
446 compounds like carbohydrates, which control the initial phase of plant litter degradation,  
447 showed an age dependent (non-significant) legacy effect of duration, while for lignin, a  
448 recalcitrant compound, the legacy effect was not age dependent and significant.

449  
450 In the PCA plan, TG6N+ was placed along the first dimension between CC and GG,  
451 whereas TG3N+ was not different from CC and differentiated from TG6N+ along the second  
452 dimension characterised by P, Co/V and S/V lignin ratios, PD and POM. It was interesting to  
453 see an unexpected non-linear response of these parameters to TG age. Non age-dependent  
454 effects for grasslands of short duration have already been observed by other authors for root  
455 biomass (Acharya et al., 2012) and for C and N fluxes (Attard et al., 2016). These data  
456 indicate that after 3 yrs of grassland, the nature of SOM was still in a transitional state and did  
457 not yet acquire grassland characteristics, for example a typical biogeochemical grassland soil  
458 signature with high lignin concentration. In our system, grasslands of longer duration were  
459 needed to be able to differentiate temperate grassland from continuously cropped soils beyond  
460 the three-year cropland phase.

461  
462 **5. Conclusion**

463 In this study, we investigated the biogeochemical nature of SOM in 0-10 cm of  
464 cropland and grassland soils as well as the legacy effect (i.e. changes in parameters lasting  
465 beyond three years of crop) of differently managed temporary grasslands inserted into crop  
466 rotations. Our results show that even if no effect on potential microbial activity was detected,  
467 C concentrations of arable soil may be increased through the introduction of temporary  
468 grasslands with specific management in terms of fertilisation and duration Strong legacy  
469 effects were related to N-fertilisation, which was necessary to preserve a typical  
470 grassland biogeochemical signature and SOM concentrations beyond three years of crop.  
471 Grassland duration showed no age-dependent legacy effects concerning C concentrations and  
472 other soil properties. However, overall it seems that only after temporary grassland duration  
473 of 6 years, soils acquired biogeochemical characteristics close to continuous grassland. In  
474 contrast, soils, which supported 3 years of temporary grassland were similar to those  
475 supporting continuous crop after the end of the cropland phase.

476  
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483

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- 663
- 664

**Table 1.** Experimental design of the study site (SOERE ACBB)\* with the 5 treatments\*\*. The vertical bold line indicates the date of sampling (March 2014)

		2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
<b>CC</b>	Continuous cropping	Maize	Wheat	Barley	Maize	Wheat	Barley	Maize	Wheat	Barley	Maize
<b>TG3N+</b>	TG fertilized, 3 yrs	Maize	Wheat	Barley	Grassland	N +	Maize	Wheat	Barley		
<b>TG6N+</b>	TG fertilized, 6 yrs				Grassland	N +	Maize	Wheat	Barley		
<b>TG6N-</b>	TG unfertilized, 6 yrs				Grassland	N -	Maize	Wheat	Barley		
<b>GG</b>	Continuous grassland				Grassland	N +					

665 \* Long-term observatory on environmental research called “agrosystems biogeochemical  
666 cycles and biodiversity”

667 \*\* permanent cropland (CC), 3 yrs fertilised temporary grassland (TG3N+), 6 yrs fertilized  
668 temporary grassland (TG6N+), 6 yrs unfertilised temporary grassland (TG6N-) and 9 yrs  
669 permanent grassland (GG).

**Table 2.** Soil characteristics\* under the 5 treatments\*\*. Data are presented as mean of 4 replicates. For each column, different letters indicate significant differences ( $p < 0.05$ ).

	<b>C</b>	<b>N</b>	<b>P</b>	<b>C/N</b>	<b>C/P</b>	<b>POM</b>	<b>NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup></b>	<b>IP</b>	<b>OP</b>
	mg g <sup>-1</sup>	mg g <sup>-1</sup>	μg g <sup>-1</sup>			% C	μg g <sup>-1</sup>	μg g <sup>-1</sup>	μg g <sup>-1</sup>
<b>CC</b>	11.33 <sup>c</sup>	1.22 <sup>c</sup>	979.9 <sup>ab</sup>	9.3 <sup>a</sup>	11.6 <sup>bc</sup>	11.5 <sup>b</sup>	0.22 <sup>b</sup>	539.7 <sup>ab</sup>	440.2 <sup>a</sup>
<b>TG3N+</b>	12.97 <sup>b</sup>	1.39 <sup>b</sup>	1135.8 <sup>a</sup>	9.4 <sup>a</sup>	11.7 <sup>bc</sup>	9.7 <sup>b</sup>	0.13 <sup>ab</sup>	605.7 <sup>a</sup>	530.1 <sup>a</sup>
<b>TG6N+</b>	12.25 <sup>bc</sup>	1.27 <sup>c</sup>	915.6 <sup>ab</sup>	9.8 <sup>a</sup>	13.5 <sup>b</sup>	14.3 <sup>b</sup>	0.16 <sup>b</sup>	430.4 <sup>ab</sup>	485.3 <sup>a</sup>
<b>TG6N-</b>	9.14 <sup>d</sup>	1.01 <sup>d</sup>	827.3 <sup>b</sup>	9.0 <sup>a</sup>	11.1 <sup>c</sup>	14.1 <sup>b</sup>	0.16 <sup>ab</sup>	472.3 <sup>ab</sup>	354.9 <sup>a</sup>
<b>GG</b>	16.83 <sup>a</sup>	1.65 <sup>a</sup>	926.1 <sup>ab</sup>	10.2 <sup>a</sup>	18.4 <sup>a</sup>	19.9 <sup>a</sup>	0.74 <sup>a</sup>	392.8 <sup>b</sup>	533.3 <sup>a</sup>
<i>F-value</i>	187.6	91.4	4.2	3.3	41.5	45.5	10.3	3.7	0.9

671 \*POM, particulate organic matter; IN, inorganic nitrogen; IP, inorganic phosphorus; OP,

672 organic phosphorus.

673 \*\* permanent cropland (CC), 3 yrs fertilised temporary grassland (TG3N+), 6 yrs fertilized

674 temporary grassland (TG6N+), 6 yrs unfertilised temporary grassland (TG6N-) and 9 yrs

675 permanent grassland (GG).

**Table 3.** Sugar signature\* of soil under the five treatments\*\*. Data are presented as mean of 4 replicates. For each column, different letters indicate significant differences ( $p < 0.05$ ).

	<b>Content</b>	<b>Concentration</b>	<b>C6/C5</b>	<b>DesoxyC6/C5</b>	<b>Xylose</b>
	% C	mg g <sup>-1</sup> soil			/mannose
<b>CC</b>	38.3 <sup>b</sup>	4.29 <sup>bc</sup>	2.92 <sup>a</sup>	0.37 <sup>a</sup>	0.23 <sup>c</sup>
<b>TG3N+</b>	34.8 <sup>c</sup>	4.50 <sup>b</sup>	2.61 <sup>ab</sup>	0.30 <sup>b</sup>	0.26 <sup>bc</sup>
<b>TG6N+</b>	37.6 <sup>bc</sup>	4.61 <sup>b</sup>	2.37 <sup>b</sup>	0.33 <sup>b</sup>	0.31 <sup>b</sup>
<b>TG6N-</b>	43.1 <sup>a</sup>	3.94 <sup>c</sup>	2.90 <sup>a</sup>	0.29 <sup>b</sup>	0.23 <sup>c</sup>
<b>GG</b>	35.5 <sup>bc</sup>	5.98 <sup>a</sup>	1.51 <sup>c</sup>	0.22 <sup>c</sup>	0.60 <sup>a</sup>
<i>F-value</i>	21.7	85.6	60.6	33.8	131.8

676 \*C6/C5, hexose / pentose ratio; desoxyC6/C5, desoxyhexose / pentose ratio.

677 \*\* permanent cropland (CC), 3 yrs fertilized temporary grassland (TG3N+), 6 yrs fertilized

678 temporary grassland (TG6N+), 6 yrs unfertilized temporary grassland (TG6N-) and

679 permanent grassland (GG).

**Table 4.** Lignin signature\* of soil under the five treatments. Data are presented as mean of 4 replicates. For each column, different letters indicate significant differences ( $p < 0.05$ ).

	<b>Content</b>	<b>Concentration</b>	<b>(Ac/Al)<sub>v</sub></b>	<b>(Ac/Al)<sub>s</sub></b>	<b>Co/V</b>	<b>S/V</b>
	% C	mg g <sup>-1</sup> soil				
<b>CC</b>	2.4 <sup>a</sup>	0.27 <sup>ab</sup>	0.45 <sup>a</sup>	0.54 <sup>ab</sup>	0.46 <sup>b</sup>	1.40 <sup>b</sup>
<b>TG3N+</b>	1.9 <sup>a</sup>	0.25 <sup>b</sup>	0.39 <sup>a</sup>	0.51 <sup>b</sup>	0.66 <sup>a</sup>	1.60 <sup>a</sup>
<b>TG6N+</b>	2.0 <sup>a</sup>	0.25 <sup>b</sup>	0.48 <sup>a</sup>	0.56 <sup>a</sup>	0.52 <sup>ab</sup>	1.40 <sup>b</sup>
<b>TG6N-</b>	2.2 <sup>a</sup>	0.20 <sup>b</sup>	0.46 <sup>a</sup>	0.52 <sup>ab</sup>	0.50 <sup>ab</sup>	1.43 <sup>ab</sup>
<b>GG</b>	2.1 <sup>a</sup>	0.35 <sup>a</sup>	0.47 <sup>a</sup>	0.52 <sup>ab</sup>	0.66 <sup>a</sup>	1.29 <sup>b</sup>
<i>F-value</i>	2.7	6.9	0.9	5.2	7.0	9.0

680 \*(Ac/Al)V, (acid/aldehyde)vanillyl ratio ;(Ac/Al)S, (acid/aldehyde)syringyl ratio ;Co/V, p-  
681 coumaryl/vanillyl ratio ;S/V, syringyl/vanillyl ratio.

682 \*\* permanent cropland (Cr), 3 yrs fertilized temporary grassland (TG3N+), 6 yrs fertilized  
683 temporary grassland (TG6N+), 6 yrs unfertilized temporary grassland (TG6N-) and  
684 permanent grassland (GG).



**Table 5.** Microbial parameters\* for soil under the five treatments\*\*. Data are presented as mean of 4 replicates. For each column, different letters indicate significant differences ( $p < 0.05$ ).

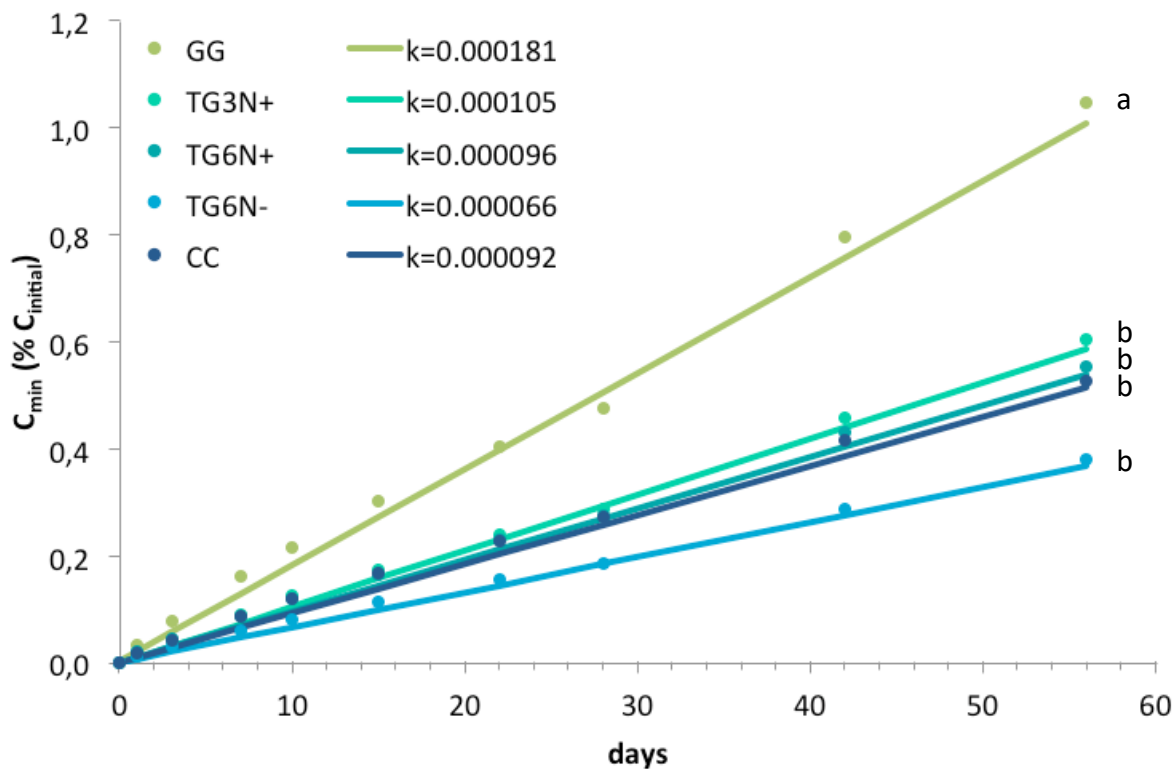
	<b>MB-C</b>	<b>MB-N</b>	<b>MB-C/MB-N</b>	<b>Potential denitrification</b>
	$\mu\text{g g}^{-1}$	$\mu\text{g g}^{-1}$		$\mu\text{gN-N}_2\text{O g}^{-1} \text{h}^{-1}$
<b>CC</b>	143.6 <sup>b</sup>	24.1 <sup>a</sup>	5.9 <sup>b</sup>	1.09 <sup>a</sup>
<b>TG3N+</b>	180.9 <sup>ab</sup>	30.8 <sup>a</sup>	5.8 <sup>b</sup>	1.13 <sup>a</sup>
<b>TG6N+</b>	204.4 <sup>ab</sup>	26.7 <sup>a</sup>	7.6 <sup>a</sup>	1.05 <sup>a</sup>
<b>TG6N-</b>	135.2 <sup>b</sup>	25.2 <sup>a</sup>	5.3 <sup>b</sup>	0.93 <sup>a</sup>
<b>GG</b>	231.3 <sup>a</sup>	33.8 <sup>a</sup>	6.8 <sup>ab</sup>	0.72 <sup>a</sup>
<i>F-value</i>	6.3	2.4	7.3	1.5

685 \* MB-C, microbial carbon; MB-N, microbial nitrogen; AWCD, average well color  
686 development.

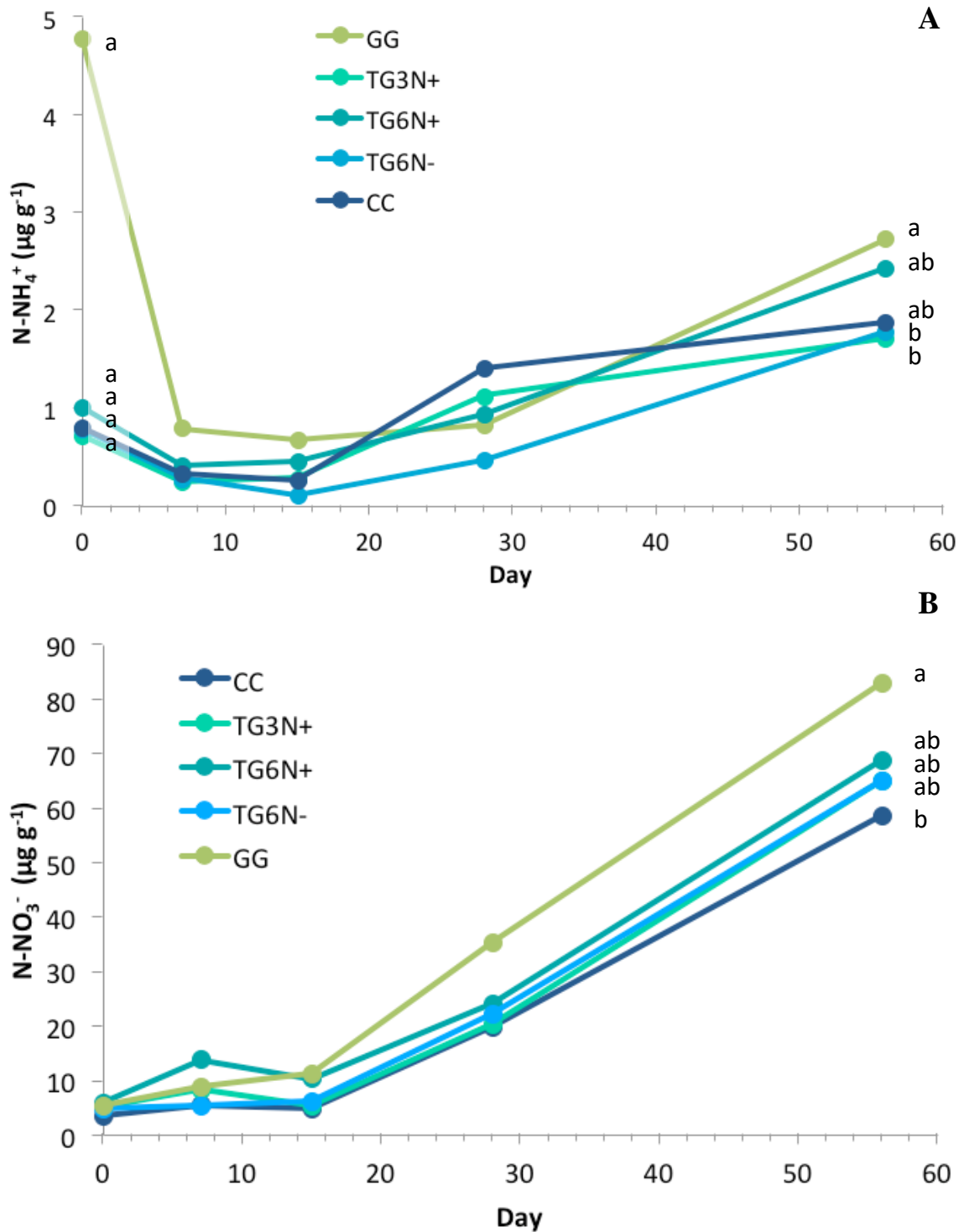
687 \*\* permanent cropland (CC), 3 yrs fertilized temporary grassland (TG3N+), 6 yrs fertilized  
688 temporary grassland (TG6N+), 6 yrs unfertilized temporary grassland (TG6N-) and  
689 permanent grassland (GG).

**Table 6.** Pearson's regression correlation of variables and treatments with dimension 1 and 2 of PCA for analysis for soil under permanent cropland (CC), 3 yrs fertilized temporary grassland (TG3N+), 6 yrs fertilized temporary grassland (TG6N+), 6 yrs unfertilized temporary grassland (TG6N-) and permanent grassland (GG).

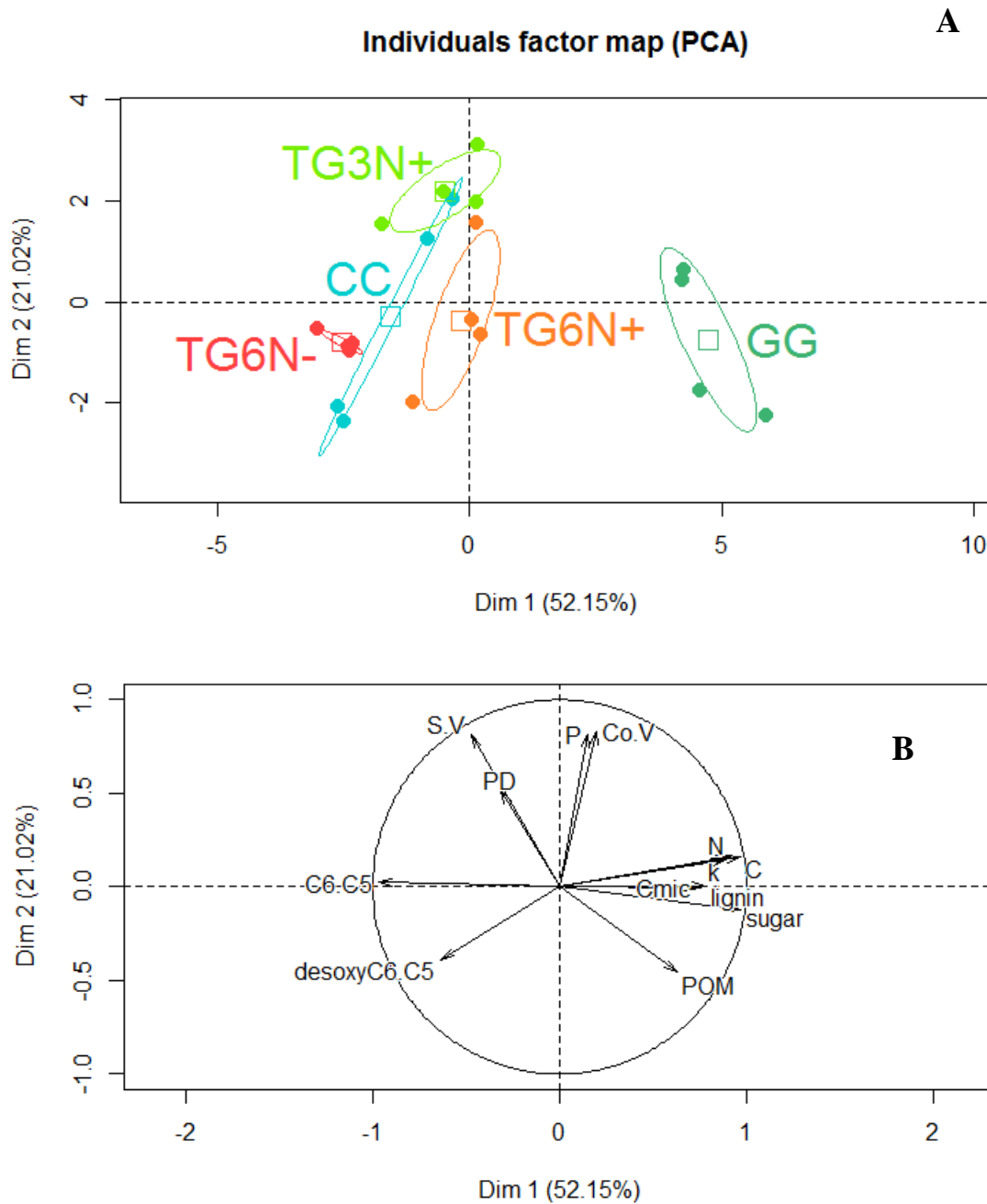
<b>Dimension 1 (52.15%)</b>		<b>Dimension 2 (21.02%)</b>	
<b>treatment</b>	<b>estimate (<i>p</i>-value)</b>	<b>treatment</b>	<b>estimate (<i>p</i>-value)</b>
GG	5.11 (2.32e <sup>-08</sup> )	TG3N+	2.22 (0.00129)
TG6N-	-2.56 (4.25e <sup>-07</sup> )		



**Fig. 1.** Potential mineralization of soil carbon with observed (point) and simulated data (line) of soil under permanent cropland (CC), 3 yrs fertilized temporary grassland (TG3N+), 6 yrs fertilized temporary grassland (TG6N+), 6 yrs unfertilized temporary grassland (TG6N-) and permanent grassland (GG).  $k$  is the C mineralization constant rate used for simulation ( $C(t) = C_0(1 - e^{-kt})$ ). Different letters indicate significant differences ( $p < 0.5$ ) between treatments. Data are presented as mean of 4 replicates.



**Fig. 2.** Potential mineralization and organization of soil inorganic nitrogen for (A) ammonium and (B) nitrate of soil under permanent cropland (CC), 3 years fertilized temporary grassland (TG3N+), 6 years fertilized temporary grassland (TG6N+), 6 years unfertilized temporary grassland (TG6N-) and permanent grassland (GG). \* indicates significant differences ( $p < 0.5$ ) for Anova 2 ways and Tukey HSD post hoc test results between treatments per day. Data are presented as mean of 4 replicates.



**Fig. 3.** Principal component analysis of soil under permanent cropland (CC), 3 years fertilized temporary grassland (TG3N+), 6 years fertilized temporary grassland (TG6N+), 6 years unfertilized temporary grassland (TG6N-) and permanent grassland (GG). (A) Variable factor map and (B) Individual factor map.

693

694