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Abstract:

To improve sustainability in agricultural systems, winter cover crops are increasingly replacing fallow to stimulate soil functions that reduce nutrient losses and greenhouse gas production, reduce pests for the next cash crops, increase soil organic matter pools and reduce erosion. Several of these functions are highly dependent on soil microbes decomposing cover crop residues. Since cover crop species differ in their traits it is hypothesized that plant species residue mixtures with complementary characteristics perform better by stimulating soil microbial functional diversity. To test this, residues of cover crop monocultures and mixtures were mixed with agricultural soil in a microcosm experiment, and fungal and bacterial biomass, microbial metabolic potential, greenhouse gas emissions and soil nutrients were measured during 50 days. Fungal biomass increased for all treatments compared to the control (no additions). However, there were no significant differences between cover crop mixtures and monocultures. Biolog ECO plates were used as a proxy for the metabolic potential of the microbial community. The number of substrates used was significantly higher in soil amended with residues of cover crop mixtures indicating an increased number of substrate niches for microbes. C:N ratio of cover crop residues was shown to be an important variable in explaining dynamics of CO_2 and N_2O emissions. Mixtures of cover crops showed reduced N_2O and CO_2 emissions compared to monocultures at the start

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of the experiment, but did not reduce greenhouse gas emissions over the whole incubation period. Adding nitrogen to the cover crop treatment with the highest C:N ratio (oat) did increase N_2O emissions, but not CO_2 emissions suggesting that decomposition rate of oat residues is not limited by nitrogen availability. Overall, mixtures of cover crops stimulated microbial functional diversity in soil incubations. Although this may have positive implications for soil quality and functioning in agricultural fields, further studies are needed to verify if these results hold under field conditions.

Keywords: microbial functional diversity, decomposition, cover crops, greenhouse gas emissions, sustainable agriculture

1. INTRODUCTION

Sustainable management of soils is essential to ensure balanced soil functioning with maintenance or increase of soil organic matter and minimal losses of inorganic nutrients, an idea already raised a few decades ago (Holmberg *et al.*, 1991). Ecological processes such as decomposition are important for maintaining soil quality. These processes are modulated by soil biota, which are negatively affected by management in intensive agriculture (Tsiafouli *et al.*, 2015) such as predominant use of mineral fertilizers and monoculture cropping systems. This reduces the functional diversity in the soil (Tsiafouli *et al.*, 2015), which may affect plant performance. Creating more diverse cropping systems potentially leads to more sustainable agriculture as studies in (semi-)natural ecosystems have already shown that plant diversity improves soil microbial diversity leading to enhanced ecosystem functioning (Wardle *et al.*, 2004; Fierer *et al.*, 2009; Wagg *et al.*, 2014; Lange *et al.*, 2015). Additionally, it is known that high microbial diversity is important for maintaining multi-functionality in ecosystems (Eisenhauer *et al.*, 2012; Wagg *et al.*, 2014; Delgado-Baquerizo *et al.*, 2016). By stimulating the microbial diversity, the aim is to increase functional diversity belowground such as the ability to metabolize a wide spectrum of organic compounds (metabolic potential). Diversification of organic matter inputs into agricultural systems, e.g. use of cover crop mixtures, is expected to increase functional diversity belowground (Hattenschwiler *et al.*, 2005; Baumann *et al.*, 2009). This can have positive effects on nutrient availability (Olson *et al.*, 2010; Zhou *et al.*, 2012) and suppression of pathogens (Wang *et al.*, 2006).

Cover crops grow during autumn and winter when the fields are usually fallow. During fallow, fields are more vulnerable to leaching of nutrients to groundwater. Cover crops can be harvested or are incorporated into the soil as green manure before the cash crops are introduced. This coincides with changes in the abundance and composition of microbes (Baumann *et al.*, 2009; Shi and Marschner, 2014; Tao *et al.*, 2017), being the biological catalysers of degradation. The compositional changes are related to the different substrates added to the soil (Baumann *et al.*, 2009). However, the effects on functioning of the increased microbes are less clear.

Soil microbial functioning depends strongly on substrate quality and quantity (Mooshammer *et al.*, 2014; Zechmeister-Boltenstern *et al.*, 2015). Cover crops are usually fast growing species with a C:N ratio varying from 8 to 30, delivering residues with an initial stoichiometry that deviates from that of the microbial biomass as the C:N ratio of the soil microbial biomass is on average 7 (Xu *et al.*, 2013)). Mixtures with a wide range of plant species with deviating C:N ratios provide a range of substrates to the soil system, which may increase the niche breadth of the microbial community leading to an increased metabolic potential. For example, soil amended with residues composed of mixtures of plants with a wide range of chemical qualities resulted in additive effects for C and N mineralization (Redin *et al.*, 2014). Besides C and N, other nutrients like P, S and K can be provided by cover crop residues and these nutrients are important for microbial growth and activity (Tao *et al.*, 2017). Furthermore, potassium concentrations are positively correlated with soil microbial biomass (Nair and Ngouajio, 2012). Hence, well-selected plant species mixtures may produce residues with a broader nutrient and stoichiometric spectrum thereby creating conditions facilitating the establishment of a diverse community with increased metabolic potential and higher microbial biomass compared to the use of monocultures (Nicolardot *et al.*, 2007; Baumann *et al.*, 2009; Baumann *et al.*, 2011).

Besides nutrients released from plant residues, turnover of microbial biomass formed during degradation of cover crop residues, may contribute to nutrient supply for the main crop following cover crop residue decomposition. However, sparse information is available considering nutrient release from microbial biomass in soils. It has been demonstrated that microbial products released after decomposition are the main precursors of SOM formation (Cotrufo *et al.*, 2013; Cotrufo *et al.*, 2015; Kallenbach *et al.*, 2016). Increases of SOM may be beneficial for plant growth by increasing the soil absorption complex and, therefore, nutrient retention (Lal, 2004; Lehmann and Kleber, 2015). SOM will only increase if the amount of carbon incorporation is higher than the carbon loss via respiration.

Both litter quality (e.g. C:N ratio) and agricultural practices (tillage) influence greenhouse gas emissions after cover crop incorporation (Petersen *et al.*, 2011; Abdalla *et al.*, 2014; McCourty *et al.*, 2018). Cover crop monocultures can have positive as well as negative effects on greenhouse gas emissions depending on the quality of the litter (Basche *et al.*, 2014). It is expected that the microbial community will be more efficient in decomposing residue mixtures compared to monocultures (Bardgett and Shine, 1999; Loreau, 2001). The effects of mixtures of cover crops, on GHG emissions have still to be investigated.

In this study, we executed a pot experiment in which residues of cover crop monocultures and - mixtures were added to a sandy, intensively managed agricultural soil with the aim to test the following hypotheses:

- Microbial biomass is positively affected by mixtures of cover crop residues due to increased substrate diversity.
- Mixtures of residues lead to a higher microbial functional diversity by increasing the niche breadth of the microbial community.
- Residue mixtures reduce greenhouse gas emissions due to increased nutrient use efficiency.

2. MATERIALS AND METHODS

2.1 Plant material and soil samples

Cover crop material was harvested in February 2017 from plots created and managed by Joordens Zaden company in an arable field in Neer, the Netherlands (51°18'24.7"N 6°05'08.8"E). Harvest was one week before the cover crops were ploughed in. Above- and belowground plant material of fifteen species was harvested from five different plant families (*Avena strigosa*, *Vicia sativa*, *Raphanus sativus*, *Guizotia abyssinica*, *Phacelia tanacetifolia*, *Sinapsis alba*, *Camelina sativa*, *Brassica carinata*, *Brassica napus*, *Lens culinaris*, *Medicago sativa*, *Pisum sativum*, *Trifolium alexantrinum*, *Secale cereale* and *Fagopyrum esculentum*). These fifteen species represent commonly used cover crops in the Netherlands. All cover crops were harvested from monoculture fields. Roots were collected simultaneously with the shoots by first making the soil loose with a shovel before pulling the complete plants out of the soil. The cover crops were dried at 40°C, cut in pieces of maximally 1 cm and stored at

room temperature. Both above- and belowground plant material was used in the experiment (in ratio present at harvest and mixed during processing of the samples). Elemental composition (carbon (C), nitrogen (N), phosphorus (P), potassium (K) and sodium (S)) was analyzed. C and N was analyzed by grinding 1 mg of plant material, transferred to tin cups and analyzed with an element analyzer (Flash EA 1112, Thermo Scientific). K, P and S were measured from 20 mg of grinded plant material with microwave-assisted digestion with nitric acid and hydrogen peroxide (Hansen *et al.*, 2009; Hansen *et al.*, 2013) and measured on an ICP-OES (iCAP 6500 DUO, Thermo Scientific).

Soil was collected in April 2017 from a conventionally managed agricultural field in Wageningen, the Netherlands (51°59'44.6"N 5°39'34.9"E). The soil type is loamy sand (83% sand, 12% silt and 2% clay). This is a highly disturbed arenic gleysol. Soil pH was 5.3 and soil organic matter content 3.1%. Prior to soil collection, the fields were from late summer after harvest of the cash crop (wheat). Fallow fields were used to ensure that the soil had no legacy of cover crops at the start of the experiment. The top 20cm of the soil was collected, as this is the plowing depth of the field. Prior to the experiment, the soil was sieved through a 4 mm sieve to remove stones. The sieved soil was stored at 4°C before the start of the experiment for maximally 1 month.

2.2 Experimental setup

250 gram of moist soil was mixed with 0.5 gram of plant material of different combinations, cut in pieces of 1 cm in length, and added in small plastic pots made from HDPE (6.5 cm in diameter and 14.5 cm height). The pots were loosely packed with a bulk density of 0.92 g/cm^3. The amount of added plant material is comparable with 5 ton per ha on an agricultural field scale, which is the common yield of cover crops in agriculture in the Netherlands. The treatments consisted of residues of monocultures of *Avena strigosa* (oat, A), *Vicia sativa* (vetch, V) and *Raphanus sativus* (radish, R), a three species mixture of vetch, radish and oat (VRA) and a 15 species mixture (15sp) containing all harvested cover crops. The plant material in the mixtures was equally divided among the species in the mixture (0.17 gram plant material per species in the 3 species mixture and 0.03 gram plant material per species in the 15 species mixture). The three monocultures and three species mixture were chosen as these three species are commonly used as cover crops, representing three different plant families. The 15 species mixture was used to create a highly diverse mixture of plant traits. The nutrient content of plant material in the different treatments is shown in Table 1. To identify if the microbial decomposing activity is nitrogen

limited, a treatment with added mineral nitrogen was used in the monoculture with the highest C:N ratio (oat) (A+N). In this treatment, 37 mg NH_4NO_3 was added to reduce the C:N ratio to approximately 10. As control (C), pots without added plant material were used.

The pots were incubated in the dark at 20°C for 50 days. The pots were watered twice a week to keep the water content of the soil at 60% of the water holding capacity (16.6 g water per 100 g of dry soil).

Soil samples were taken at 1, 3, 7, 12 and 50 days after the start of the experiment. At the start (T0), a soil sample was taken before mixing plant residues with the soil. The subsamples were taken destructively to prevent disturbance of the pots. Soil samples were taken after mixing the soil in pots to obtain a homogeneous sample.

2.3 Microbial biomass

Fungal biomass was measured in one gram of soil for all sampling time points using ergosterol as a proxy following the Bååth alkaline ergosterol extraction method (Bååth, 2001; De Ridder-Duine *et al.*, 2006). The ergosterol concentration was measured using LC-MSMS (6460 Triple Quad LC-MS, Agilent). Ergosterol was used to measure fungal biomass as this is a good method to estimate the saprotrophic fungi in the soil (Joergensen and Wichern, 2008). Bacterial biomass was measured using qPCR. DNA was extracted using DNeasy Powersoil Pro Kit (Qiagen, Venlo, The Netherlands) with 0.25 gram of soil according to the instructions provided by the supplier. The quantity and quality of the extracted DNA was analyzed with a spectrophotometer (Nanodrop 2000, Thermo Scientific). qPCR assays were used to target the 16S rRNA gene for bacteria. Each assay was performed with 2 $ng*\mu L^{-1}$ of DNA in duplicates for each DNA extract with the primer pair EUB338 and EUB518 (Fierer *et al.*, 2005). Each qPCR (total volume of 15 µL) consisted of 7.5 µL 29 SensiFAST SYBR (BIOLINE), 0.75 µL of forward and reverse primes each, 1.5 µL of bovine serum albumin (5 $mg*mL^{-1}$; Invitrogen), 1.5 µL DNase- and RNase-free water and 3 µL of the template DNA. Standard curves were obtained using 10-fold dilution series of a known amount of plasmid DNA from isolates of *Collimonas* (10^8 to 10^1 gene copies) containing the 16S rRNA fragment. The qPCR was performed with a C1000 Touch Thermal Cycler (CFX-real-Time system, Bio-Rad Laboratories, Inc.). As negative control, water was used instead of template DNA. Efficiencies for all assays were between 80.3 and 112.1 % with r^2 values between 0.951 and 0.989.

Bacterial biomass C was calculated by using the conversion factor 4.1 for 16s rRNA gene copy numbers for bacteria (Santelli *et al.*, 2008) and bacterial biomass estimates as described by Bloem *et al.* (1995).

178 The ergosterol data were converted to fungal biomass C using the conversion factor 5.4 mg
179 ergosterol*kg biomass C^{-1} (Klamer and Bååth, 2004). Both calculations were used to estimate the total
180 microbial biomass C (MBC) in the soil and the fungi:bacteria ratio.
181
182 2.4 Microbial functional diversity with Biolog ECO plates
183 Biolog ECO plates (Biolog Inc., Hayward, CA) were used to determine the ability of the microbial
184 community to grow on different substrates (metabolic potential). This is used as a proxy for microbial
185 functional diversity and niche breadth of the community (Mallon *et al.*, 2018). Biolog ECO plates contain
186 31 different C-substrates. To prepare Biolog ECO plates, microbial communities were extracted from 1
187 gram of soil with 10 ml TrisBis buffer as described by Rutgers *et al.* (2016). The soil samples were taken
188 at 12 and 50 days after the start of the experiment. Three dilutions (factor 8) from the microbial
189 suspension were prepared based on a test sample to identify the transition point between low and high
190 well color development to be able to distinguish the differences between the treatments. The ECO plates
191 were incubated at 20°C in the dark under humid conditions. The absorbance at 592 nm (purple) and at
192 750 nm (turbidity) was measured at 2h, 4h and 3, 5 and 7 days after plate preparation in a plate reader
193 (Spectramax 250, Molecular Devices Corp., Memlo Park, CA). If the microbial community was able to
194 grow on the substrate, absorbance 592 nm – 750 nm of the substrate was higher than the blank (water)
195 with a threshold of 0.1, this was scored as “1”. If there was no purple color development (no growth),
196 this was scored with “0”.
197
198 2.5 Nutrient analyses
199 At two time points (12 and 50 days), plant available soil nutrient concentrations (N, P, K and S) were
200 measured. 50 mL 1 M KCl was added to 10 g of soil to extract NO_3 and NH_4. Clear supernatant was
201 stored in the freezer before analysis on an AutoAnalyzer (SEAL QuAAtro SFA system, Beun- de Ronde
202 B.V. Abcoude, The Netherlands).
203 P, K and S were determined after extraction of 3 g soil added to 30.0 mL 0.01M calcium chloride solution.
204 This extraction procedure is used as it is considered as a proxy for plant available nutrients in the soil
205 solution (Menzies *et al.*, 2007). After 2 hours of shaking, the sample was centrifuged at 3000 rpm and
206 the supernatant was filtered through a Whatman Puradisc Aqua 30 filter with CA membrane. 130 µL

207 HNO_3 was added to 12.87 ml filtrate and stored for maximal one day at 4°C before analysis with an ICP-
208 OES (iCAP 6500 DUO, Thermo Scientific).
209
210 2.6 Greenhouse gas measurements
211 Greenhouse gas (GHG) fluxes were measured daily up to 9 days. From day 9 on, measurement
212 frequency was decreased to once a week until the end of the experiment. Thus, measurements were
213 done at days: 1-9, 11, 12, 14, 16, 18, 21, 24, 28, 35, 42 and 50. CO_2 and N_2O fluxes were measured
214 over an hour by closing the pots and taking an air sample at 0 and 60 min after closing. 20 mL of air
215 was taken from the pots of which 14 ml was flushed in a 6 mL exetainer (Labco, UK) and the last 6 mL
216 was used to create an overpressure of 1 bar. The exetainers were analyzed using an autosampler
217 (TriPlus RSH, Thermo Fisher Scientific, Bleiswijk, The Netherlands) connected to a gas chromatograph
218 (GC1300, Thermo Fisher Scientific) equipped with a Methanizer and a Flame Ionization Detector (FID)
219 to detect CO_2 and CH_4 an electron capture detector (ECD) for detection of N_2O. The gas chromatograph
220 contained two sets of a pair Rt-Q-Bond capillary columns (L; 15m and 30m, ID; 0.53mm, Restek,
221 Interscience, Breda, The Netherlands). Chromeleon™ Chromatography Data System 7.1 (CDS, Thermo
222 Fisher Scientific) software was used to analyze the obtained gas chromatograms from the GC. Fluxes
223 of CO_2 and N_2O were calculated on basis of the accumulation during 1 hour. Gas concentrations were
224 calculated in ppm values by comparing with calibration curves, which were generated by dilution of a
225 certified gas mixture (1 ppm N_2O, 2 ppm CH_4 and 2000 ppm CO_2; Linde Gas, The Netherlands). The
226 concentrations (ppm) were converted into absolute amounts (mmol) with the gas law: $pV = nRT$ in which
227 p is the pressure in the pot, V is the volume, n is the amount gas in mol, R is the gas constant (8.31
228 $J \cdot K^{-1} mol^{-1}$) and T is the temperature. Cumulative fluxes of the GHG emissions were calculated by
229 assuming that the time between the samples was similar to the average of both samples:

230 $$\text{Cumulative flux } (\Phi) = \Phi_{T_1} + T_{j-i} * \left(0.5 * \Phi_{T_i} + 0.5 * \Phi_{T_j}\right) + \cdots$$

231 where Φ is the flux at time point i and T is the time between the different time points (i and j).
232
233 2.7 Data analysis
234 The data were analyzed with R (version 3.5.1) with attached packages: agricolae, car and plyr for
235 statistical analysis; ggplot2, grid and gridExtra for making figures; dplyr, survival, Formula and Hmisc for
236 making correlation tables; and klaR and MASS for RDA analysis. First, the data were tested for normality

and for homogeneity of variances. If this was not the case, Tukey's ladder of powers transformation in the package Rcompanion was used to meet the criteria for parametric statistical testing as log transformation was not sufficient to make the data normally distributed. The data were analyzed for significant treatment effects using a linear model and Anova. A post hoc Tukey test was used to identify the significant differences between the treatments. The greenhouse gas emissions data over time were not normally distributed and were analyzed using Kruskal-Wallis rank sum test combined with Scheffe test to determine treatment and time effects. Correlations were calculated between the GHG and nutrient content in the plant residues and in the soil.

The Biolog data were analyzed in R and Canoco (version 5). RDA analysis in Canoco was used to find the dissimilarity between the treatments. To determine if microbial biomass was driving differences in well color development, these parameters were added in the RDA analysis as environmental variables. All environmental variables were included in the analysis for visualization of the effecs. 500 permutations were used. Correlations were tested between the environmental variables.

Comparison of the effect of mixing of cover crops was done for the three species mixture. First, the expected values for contribution by the 3 species were determined by calculating the sum of all individual monocultures for a certain trait divided by the amount of species in the three species mixture. Next, it was tested if the values for the traits in the three species mixture were significantly different from the expected values. Results were statistical significant when the p-value was smaller than 0.05.

3. RESULTS

3.1 Microbial biomass

Addition of plant material stimulated growth of fungi as indicated by increased soil ergosterol contents (Figure 1). The fungal biomass was not significantly different between the different residue treatments, but was higher than the control treatment (C) without added plant residues ($p < 0.001$). There was no significant difference between the different time points (Table S1, $p = 0.42$). Residue mixtures (VRA and 15sp) did not have an additive effect over monocultures, but mixtures showed a more gradual increase of ergosterol in the soil compared to the monocultures. Monocultures led to a fungal biomass peak at different time points, for example: vetch (V) at 3 days and oat (A) at 12 days.

Bacterial biomass (based on 16S rRNA gene copy numbers) was significantly different between cover crops additions three days after cover crop incorporation (Figure 2, $p < 0.001$). At this time point, radish (R), vetch alone and the three species mixture (VRA) led to an increase of the bacterial biomass compared to the control. Overall vetch, radish and both mixtures led to an increase of bacteria during the experiment compared to the other treatments, which disappeared after 50 days of incubation.

Dynamics of bacterial and fungal biomass development differed between treatments. Vetch led to an early increase of both fungal and bacterial biomass (at three days) while the oat residue led to an increase at a later time point (12 days). Mixtures had a more gradual increase and decrease and not a clear peak at one of the measured time points.

Microbial biomass C (MBC) was calculated to estimate the total amount of microbial biomass in the soil. The MBC ranged between 0.45 to 28 mg C per kg soil (Table S3). The highest values for radish monoculture and the three species mixture were mainly driven by high bacterial abundance in the samples compared to the other treatments. The F:B ratio increased during decomposition, after 50 days, this increase disappeared (Table S3).

3.2 Microbial functional diversity

Microbial metabolic potential, as proxy for functional diversity, was measured with Biolog-ECO plates. Microbes in soil suspensions obtained from mixed residue additions showed a significant increase in substrate use compared to those obtained from monocultures at both time points (Figure 3 and Figure S1, $p = 0.0016$), except for vetch residue. For radish and oat (with and without added N), there was no significant difference compared to the control. Recalcitrant substrates like D-cellobiose (G1) and glycogen (F1) could be used at low concentrations by microbes derived from the mixtures of residues, while microbes from monocultures only degraded these substrates at the highest inoculum density. These differences between substrate use are shown in a RDA analysis (Figure 4). Radish, oat and oat with N grouped together, while 15sp and VRA are significantly different from vetch (V). Both mixtures could use several substrates including polymers and carboxylic acids while microbes from vetch-amended soil used more amino acids. Microbial biomass had no significant effect on substrate use in the Biolog plate ($p = 0.44$). Only C, 15sp and V explained a significant part of the variation observed in the redundancy analysis using forward selection (Table S8, adjusted P-values are lower than 0.05).

296 **3.3 Greenhouse gas fluxes**

297 Cover crop addition to the soil showed an immediate response of greenhouse gas emissions. For all
298 treatments, the first measurement at day 1 showed the highest GHG emissions. Vetch-amended soil
299 showed the highest peak for both CO_2 and N_2O at the start followed by reduction that was faster than
300 for the other residue treatments (Figure 5).

301 Cumulative fluxes showed that after 50 days, vetch decomposition resulted in the highest N_2O emissions
302 while oat (with the highest C:N ratio) had the lowest (Figure S2B). Based on the average greenhouse
303 gas emissions of the three monoculture treatments, expected values for the three species mixture were
304 calculated. The N_2O emissions of the three species mixture was not significantly different from the
305 expected N_2O emissions. Cumulative N_2O fluxes were negatively correlated with the C:N ratio of the
306 initial plant material (Figure S4, $r = -0.57$, $p = 0.0014$), plant and soil N content correlated positively with
307 N_2O emissions ($r = -0.67$, $p < 0.001$ and $r = -0.61$, $p < 0.001$ respectively).

308 CO_2 emissions showed a similar trend as N_2O (Figure 5A). For vetch- and radish amendments there
309 was a decrease during prolonged incubation, while CO_2 fluxes for oat and oat with N were nearly
310 constant over the experimental period. Cumulative CO_2 emissions did not correlate with C:N ratio of the
311 residues (r -0.16, $p = 0.40$), but did correlate negatively with C content ($r = -0.37$, $p = 0.049$), C:P ($r = -$
312 0.66, $p < 0.001$) and N:P ratio ($r = -0.57$, $p = 0.0016$). Interestingly, CO_2 emissions did correlate positively
313 with initial plant P ($r = 0.73$, $p < 0.001$), K ($r = 0.71$, $p < 0.001$) and S ($r = 0.70$, $p < 0.001$) content and
314 soil K ($r = 0.71$, $p < 0.001$) and S ($r = 0.65$, $p < 0.001$) content after 50 days (Figure S4).

315 The total global warming potential (GWP) was calculated based on the global warming potential for each
316 separate gas from the cumulative fluxes over 50 days of incubation ($CO_2 = 1$ and $N_2O = 265$). Overall,
317 cumulative greenhouse gas fluxes showed that amendment with vetch and radish only resulted in the
318 highest greenhouse gas emissions after 50 days (Figure 6).

319

320 **3.4 Soil nutrients**

321 Plant-available soil nutrient content was differently affected by cover crop residue additions. For N, only
322 NO_3^- content significantly accumulated in the soil during decomposition (Figure 7A). The materials with
323 the lowest C:N ratio (vetch) showed the largest mineral N accumulation at both time points. Oat alone
324 immobilized N from the soil. Both mixtures show intermediate accumulation of mineral nitrogen in the

soil. Mineral N accumulation for the three species mixture was similar to the expected value based on the three monocultures.

Both vetch and radish have high S contents (Table 1). This is reflected by the extractable S content in the soil after incubation at both 12 and 51 days after incubation (Figure S3C). Similar to S, vetch led to the highest extractable K and P content in soil as well (Figure S3A and B). Oat with added N had a significantly higher P increase over time than oat alone. The plant species mixtures had significantly higher concentrations of all three nutrients compared to oat, but not compared to the other two monocultures. Most of the K in the plant material was already available in the soil after 12 days. Approximately 90% of the K in the plant material was released for vetch, radish and the three species mixtures (Figure 8A), while only 5% of the P present in the total amount of residue (Figure 9B) and 50% of the added residue S was released in vetch and radish (Figure 8C), as measured with $CaCl_2$ extraction. Decomposing oat residues showed the lowest increase of mineral nutrients in the soil.

The amount of nutrients in the plant material added to the soil correlates positively with the nutrient content in the soil at the end of the experiment (Figure S4). The amount of P, K and S in the plant strongly correlates with each other not only in the plant residue ($r > 0.9$, $p < 0.001$), but also in the soil after 50 days ($r > 0.48$, $p < 0.01$). Furthermore, the C:N, C:P and N:P ratio of the added cover crop residues correlates negatively with the amount of K, P and S in the plant residue and in the soil.

4. DISCUSSION

We investigated the effect of soil amendment with cover crop residues of mixtures and of monocultures on respiration, mineralization, greenhouse gas emission and microbial functional diversity. As previously shown, above- and belowground biodiversity is important to maintain ecosystem functioning (Wagg *et al.*, 2014). The current study aimed to determine if application of mixtures of cover crop residues has a positive effect on the functioning of soil microbes, compared to the residue of a monoculture cover crop. In this study, soil without a cover crop legacy was used to prevent effects of cover crops growing in the field (e.g. presence of root fragments). The cover crop residue amount used in this experiment is comparable to biomass production of winter cover crops. Although different cover crops in mixed cultures in the field do not produce the same amount of biomass, we used equal amounts of each species in the added mixtures to be able to identify if diversity effects could occur.

355

356 **4.1 Microbial biomass dynamics**

357 Fungal and bacterial biomass did increase when cover crop residues were added to the soil (Figure 1
358 & 2). The dynamics of fungal and bacterial biomass differed between residues added, which appeared
359 to be mainly caused by C:N ratio of the plant residues. These results partially support the first hypothesis:
360 different cover crop species can indeed lead to different microbial biomass dynamics. Mixtures of cover
361 crop residues, however, did not result in increased biomass as compared to those of single species.
362 Similar results have been published by Shi and Marschner (2014) who found only increased microbial
363 activity, but no increased microbial growth in residue mixtures of root and shoot of a diverse set of plants
364 as compared to either root or shoot residues alone. Furthermore, litter mixture effects in forests are
365 mainly caused by the plant species identity (Hattenschwiler and Gasser, 2005) and do not lead to
366 increased biomass of the decomposer community (Wardle *et al.*, 2006). These studies show that
367 increase in microbial biomass also depends on the residue mixture, amount of residue and the
368 environment.

369

370 **4.2 Microbial functional diversity**

371 Mixing plant material might lead to an increased amount of substrate niches that can lead to increased
372 microbial functional diversity in the soil. In this experiment, the metabolic response of the microbial
373 community as assessed by Biolog Eco plates was influenced by cover crop residue additions. As
374 hypothesized, mixtures led to increased functional diversity as compared to monocultures leading to
375 increased metabolic potential of the community (Figure 4). It should be considered that Biolog ECO
376 plates, in potential, do reflect the substrate usage of the aerobic carbon utilizing microbial community.
377 Dormant and microbes with another physiology are not captured. However, the substrate range of the
378 ECO plates is so broad that a wide range of microbes will be detected, wide enough to assess effects
379 of changes in environmental conditions in microbial functioning in comparative experimental designs.
380 Hence, Biolog can be used as indicator of microbial potential for substrate usage and potential changes
381 therein as the results of the changes in physical chemistry (i.e. niches) of the soil.
382 For natural systems, it was already shown that litter mixtures increase soil microbial diversity (Wardle *et*
383 *al.*, 2006; Chapman and Newman, 2010; Chapman *et al.*, 2013; Byrnes *et al.*, 2014; Delgado-Baquerizo
384 *et al.*, 2016). Several studies indicate that microbial functional diversity is enhancing soil ecosystem

functions such as disease suppression (Van Elsas *et al.*, 2002; Garbeva *et al.*, 2006; Postma *et al.*, 2008; Mallon *et al.*, 2015b), decomposition and nutrient cycling (Wagg *et al.*, 2014; Delgado-Baquerizo *et al.*, 2016; Delgado-Baquerizo *et al.*, 2017). Van Elsas *et al.* (2012) showed that a higher microbial diversity could lead to reduced ability for pathogens to invade soil environments. This may be a result of the higher competition for resources in diverse microbial communities with a more diverse metabolic potential leading to reduced niche space for the invader (Mallon *et al.*, 2015a; Mallon *et al.*, 2018). As with disease suppression, decomposition may need a functional diverse community to be able to decompose residues faster and use the nutrients more efficiently (Loreau, 2001; Hattenschwiler *et al.*, 2005). This does not necessary mean that the microbial biomass is higher as well, as this experiment showed increased functional diversity, but not increased microbial biomass in mixtures compared to monocultures.

4.3 Greenhouse gas emissions

We hypothesized that residue mixtures lead to reduced GHG emissions compared to monocultures as we expected that the microbial community would be more efficient in using nutrients in residue mixtures compared to monocultures as a result of increased carbon use efficiency (Hattenschwiler *et al.*, 2005). In this experiment, mixtures did not reduce cumulative GHG emissions compared to residues from monocultures (Figure 6). Vetch had the highest cumulative emissions. The nitrogen concentration in the vetch residue was nearly double compared to the other residues (Table 1). This may have increased the decomposability of the vetch compared to the other residue treatments. Management practices, including cover crops and tillage, play an important role in mitigating GHG emissions (Kallenbach *et al.*, 2010; Abdalla *et al.*, 2012; Abdalla *et al.*, 2014). Furthermore, as shown by Basche *et al.* (2014) in a meta-analysis, decomposition of cover crops led to increased N_2O emissions, mainly caused by the amount of N added with cover crops. This shows that nutrient content of the plant material is a main driver of soil GHG fluxes and microbial activity in the soil (Basche *et al.*, 2014; Marschner *et al.*, 2015; Nguyen *et al.*, 2016).

In the current study, the total amount of plant material was the same for all treatments, leading to different amounts of C and other nutrients added to the soil (Table 1) leading to correlations between the amount of C and N and greenhouse gas emissions. The correlation between the amount of C and CO_2 emissions was weaker compared to the amount of N with N_2O emissions, probably because the

amount of C is not only important but also the recalcitrance of the material. Furthermore, adding N in the treatment with oat residue did increase mainly N_2O emissions but not CO_2 emissions. This lack of response in respiration may be explained by the recalcitrance of the material to degradation or shortage of other nutrients.

4.4 Nutrient release

The impact of decomposing cover crop residues on nutrient availability depended on the plant species, as shown in the results (Figure 7 & 8). K availability in the soil increased up to 90% of the amount added with the plant material while P availability only increased up to 10%. The available P released from the cover crops residues is low compared to a field study by Cobo *et al.* (2008) where only 30% of N and P remained in the plant material after 50 days of decomposition of two different leguminous plants. Rapid release of K from decomposing plant material has been shown earlier (Cobo *et al.*, 2002). Decomposition of oat was not stimulated by the addition of NH_4NO_3, as the CO_2 emissions (respiration rate) are similar to oat without added N. The question arises: by what factor are these microbes limited other than N? Are other nutrients, e.g. P, K or S, missing? As shown before, adding nitrogen to decomposing plant residues can increase, decrease or have no effect on decomposition rates (Liu *et al.*, 2006; Hobbie, 2008; Norris *et al.*, 2013). In this experiment, the treatment with highest N content, vetch, led to an increase of the fungal biomass quickly after start of the experiment and higher CO_2 emissions, but vetch also had higher P, K and S amounts in the plant residues. Microbes may lack these nutrients in the treatment where only N was added to oat. Interestingly, P availability in the soil is increased when NH_4NO_3 is added, indicating that other nutrients are limiting. N addition to decomposing litter in other systems led to increased P availability as well (Liu *et al.*, 2006; Zhong *et al.*, 2017). These results show that nutrient content in residues at the start of the experiment is a major driver for decomposition and nutrient release (Sakala *et al.*, 2000; Partey *et al.*, 2014; Marschner *et al.*, 2015).

Microbial activity might be important for the incorporation of plant derived carbon and nutrients in soil organic matter (SOM). In this experiment, the microbial biomass ranged between 0.45 to 28 mg C per kg soil. Microbes can mobilize nutrients during decomposition of plant residues, but also as a result of turnover of the microbial biomass. In this experiment, the nutrient concentration in the soil mainly increased during the first two weeks. As shown by Achat *et al.* (2010), microbial P is important in the P cycling and availability of P for plants. The available P is mainly present in the microbial community and

this P has a short turnover time of only a few days (Achat *et al.*, 2010). Thus, even though the microbial biomass represents a small portion of the soil, it can have large influence on nutrient availability.

5. Conclusion

In this study, decomposition of mixtures of cover crops and monocultures resulted in differences in dynamics of microbial biomass and functional diversity. The microbial biomass development in mixtures was more gradual as compared to monocultures. Furthermore, the microbial community became more functionally diverse in mixtures as shown by a higher ability to degrade substrates in Biolog ECO plates. Mixtures of residues did not increase GHG emissions and nutrient availability compared to the average of the monocultures. Overall, these results show that adding residue mixtures can lead to more balanced soil functioning. Further studies should test whether the microbial community will be more diverse in the field when using cover crop mixtures instead of monocultures to test if these microcosm results can be extrapolated to field conditions. This will help to determine how long this microbial community will be positively changed due to residues of cover crop mixtures.

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643 **Figure legends**

644

645 **Figure 1**: Fungal biomass (mg ergosterol *kg soil^{-1}) over time (mean ± SE; n = 4). **A:** control,
646 monocultures V, R, A and A+N; **B:** control, mixtures VRA and 15sp. expVRA is the average of the
647 three monocultures (V, R and A). The results of the statistical test are shown in Table S1.

648

649 **Figure 2**: Bacterial biomass (based on copy numbers of the 16S rRNA gene with qPCR) over time
650 (mean ± SE; n = 4). **A:** control, monocultures V, R, A and A+N; **B:** control, mixtures VRA and 15sp.
651 expVRA is the average of the three monocultures (V, R and A). The results of the statistical test are
652 shown in Table S2.

653

654 **Figure 3**: Microbial functional diversity: number of wells that showed a positive response in Biolog
655 ECO plates after incubation of 7 days (mean ± SE; n = 4). As the treatments are not significantly
656 different over time (p = 0.59), the average of both time points (T12 and T50) is shown here (complete
657 graph is Figure S1). The different letters indicate significant differences between treatments. The
658 results of the statistical test are shown in Table S6.

659

660 **Figure 4**: RDA (Redundancy Analyses) ordination plot based on positive/negative (1/0) results of the
661 data from the Biolog ECO 96 well plates after 7 days of incubation. The letters indicate the different
662 substrates and are listed in Table S9. Time was significantly related to the results of the Biolog ECO
663 plates (P = 0.004), microbial biomass was not significantly correlated with the results. The results of
664 the RDA are shown in Table S7 and Table S8. Correlations between the environmental variables are
665 shown in Table S10.

666

667 **Figure 5**: Greenhouse gas fluxes over time (days) (mean ± SE; n = 4). **A:** mmol CO_2 per hour per m^2;
668 **B:** µmol N_2O per hour per m^2; **C:** close-up of N_2O graph. The results of the statistical test are shown in
669 Table S11 and Table S12.

670

671 **Figure 6**: Cumulative greenhouse gas fluxes of both CO_2 and N_2O calculated as CO_2 equivalent
672 values (mean ± SE; n = 4). The letters indicate significant differences between the treatments. The

results of the statistical test are shown in Table S13. The separate cumulative greenhouse gas fluxes of both CO_2 and N_2O are shown in Figure S4.

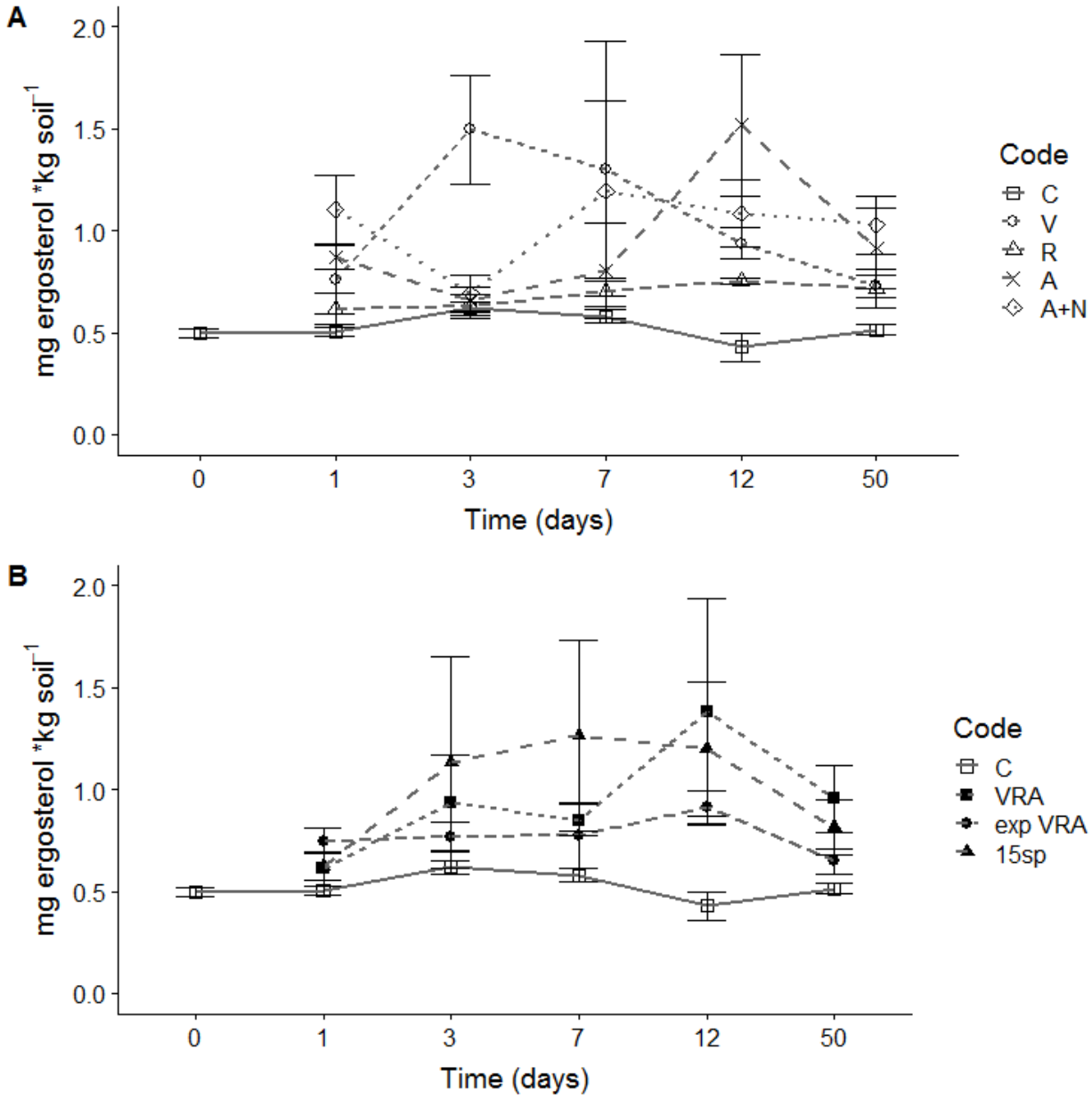
Figure 7: N content (mg per kg of dry soil) in the soil at the different time points (in days) (mean ± SE; n = 4). **A:** NO_3; Letters indicate significant differences between the treatments. The treatments show similar increase over time. **B:** NH_4. There are no significant differences between the treatments in B (only A+N was significantly different at T12), the asterisk indicate the significant difference between T12 and T50 of A+N. The results of the statistical test are shown in Table S14 and Table S15.

Figure 8: Percentage of nutrients that is released from the added plant material in the soil at the different time points (in days) (mean ± SE; n = 4). **A:** K; **B:** P; **C:** S.

Tables

Table 1: Nutrient content of the added plant material

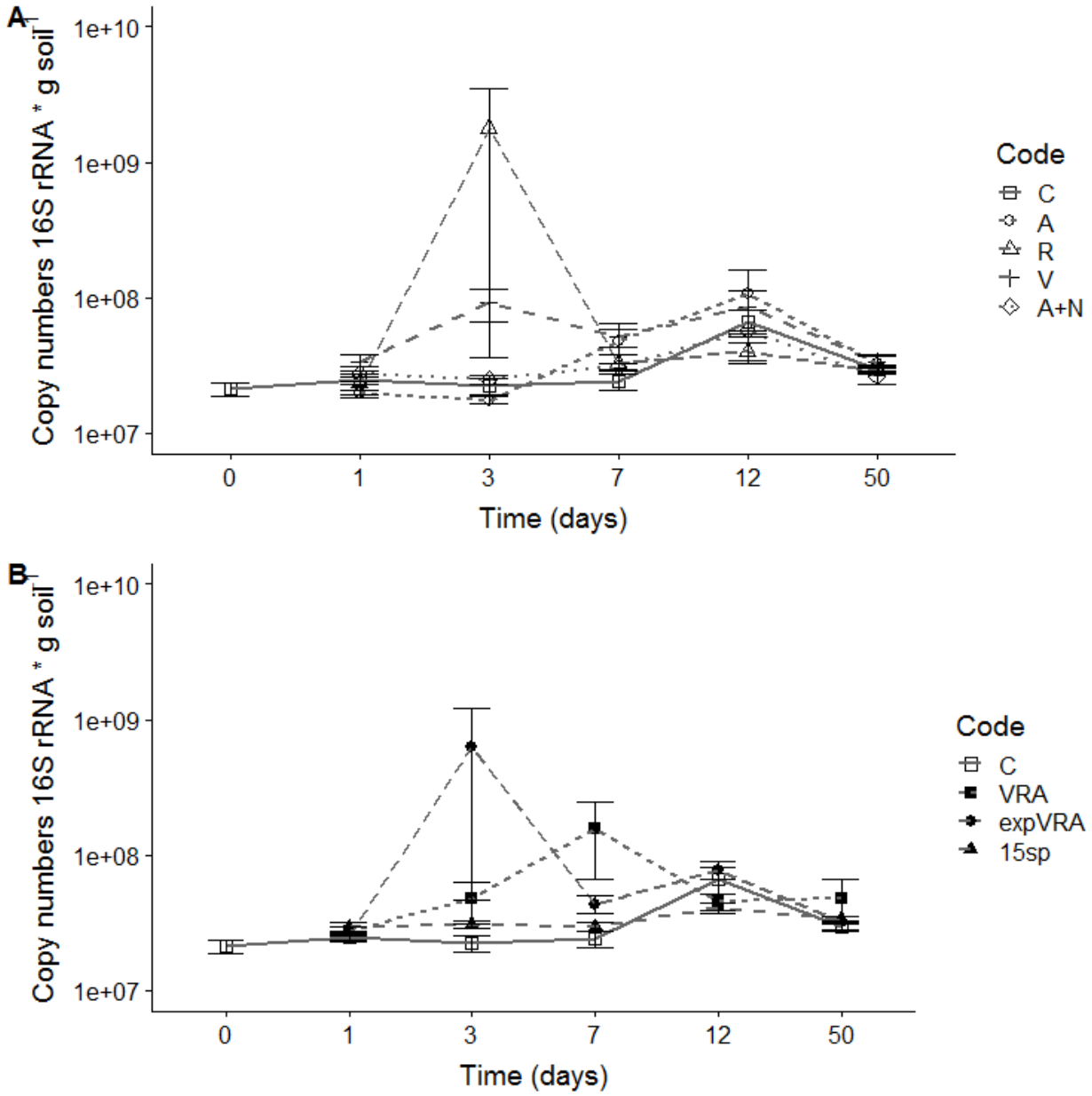
	N	**C**	**C:N**	**K**	**P**	**S**
	($g*kg^{-1}$)	($g*kg^{-1}$)		($g*kg^{-1}$)	($g*kg^{-1}$)	($g*kg^{-1}$)
***V. sativa* (V)**	39.7	327.6	8.26	21.23	5.87	3.21
***R. sativus* (R)**	24.5	382.7	15.64	12.17	5.49	3.14
***A. strigosa* (A)**	12.0	373.0	31.16	2.79	1.91	1.16
3 species (VRA)	25.4	361.1	14.23	12.06	4.42	2.51
15 species (15sp)	18.1	364.6	20.16	9.56	3.31	2.32



691

692 Figure 1

693



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695 Figure 2

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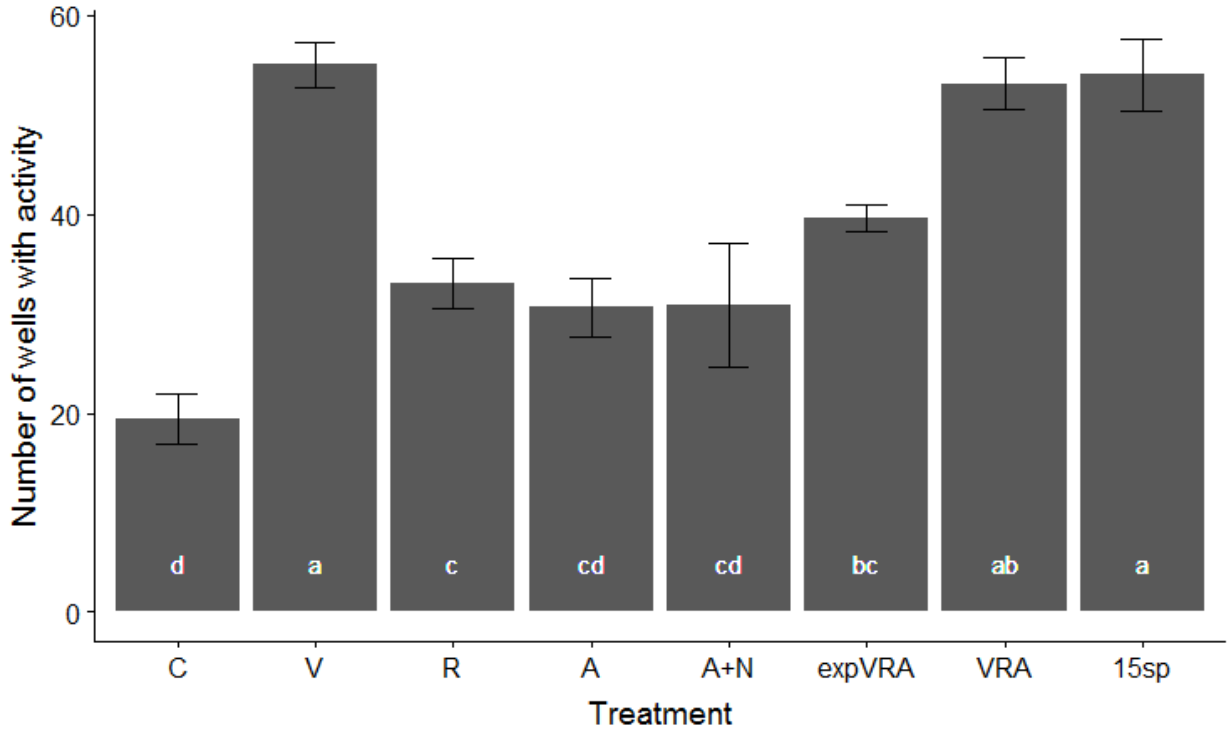
697



698

699 Figure 3

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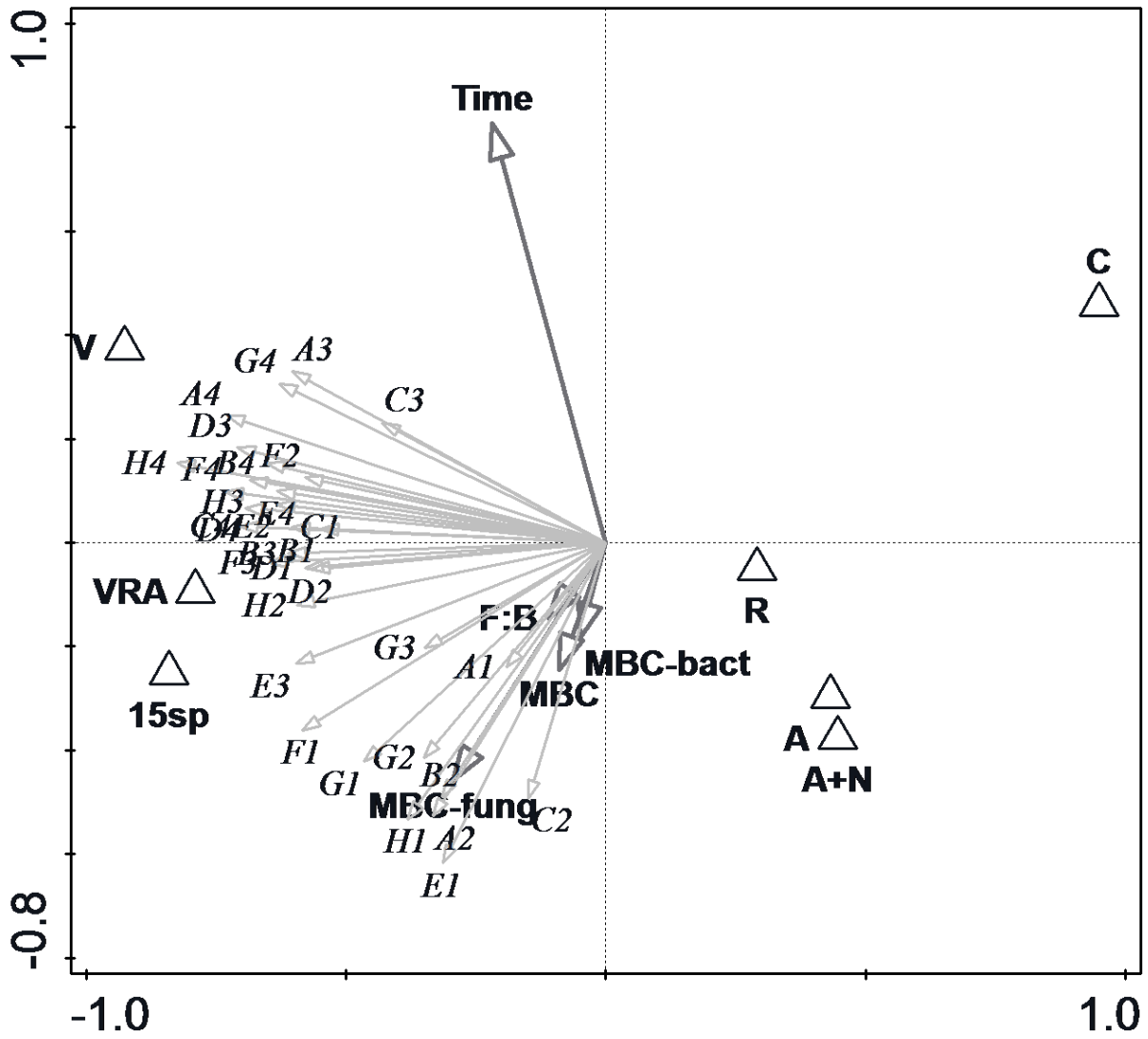

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703 Figure 4

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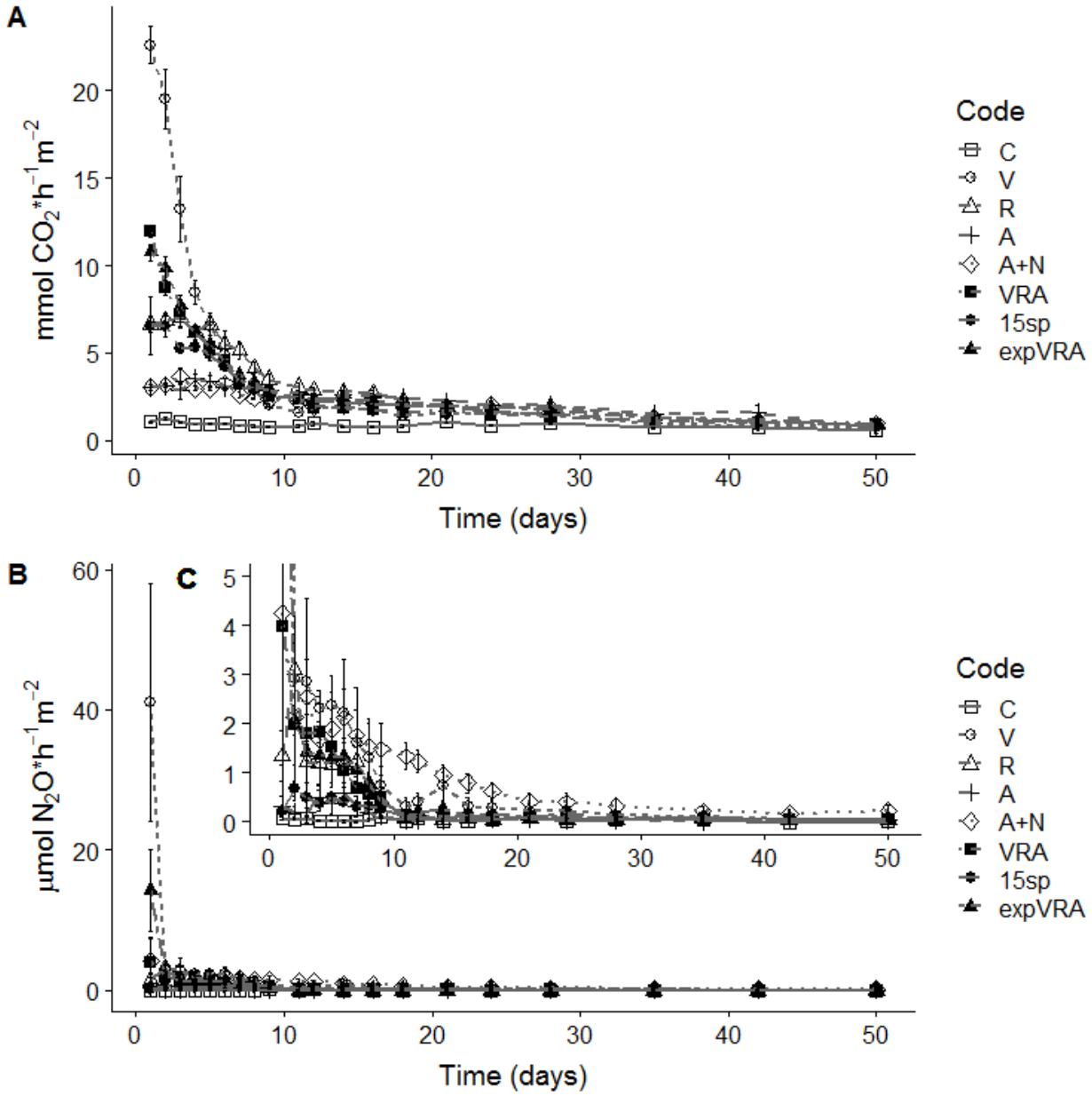

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706 Figure 5

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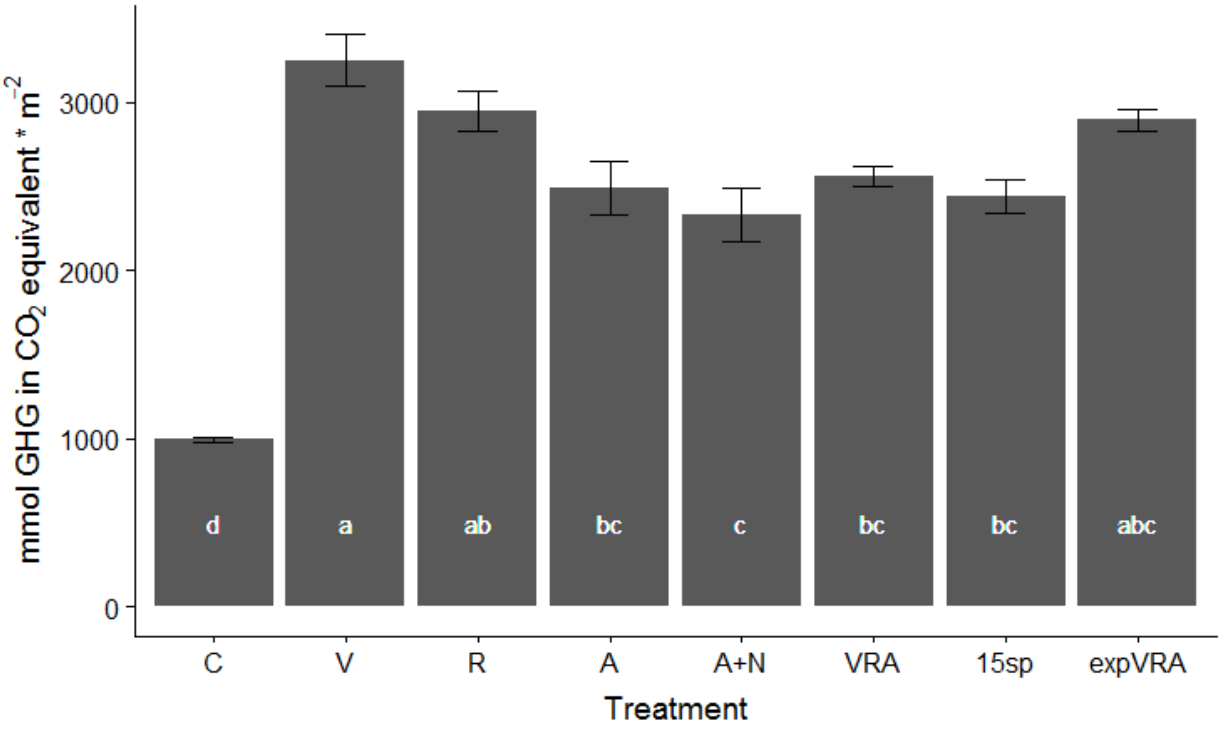

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709 Figure 6

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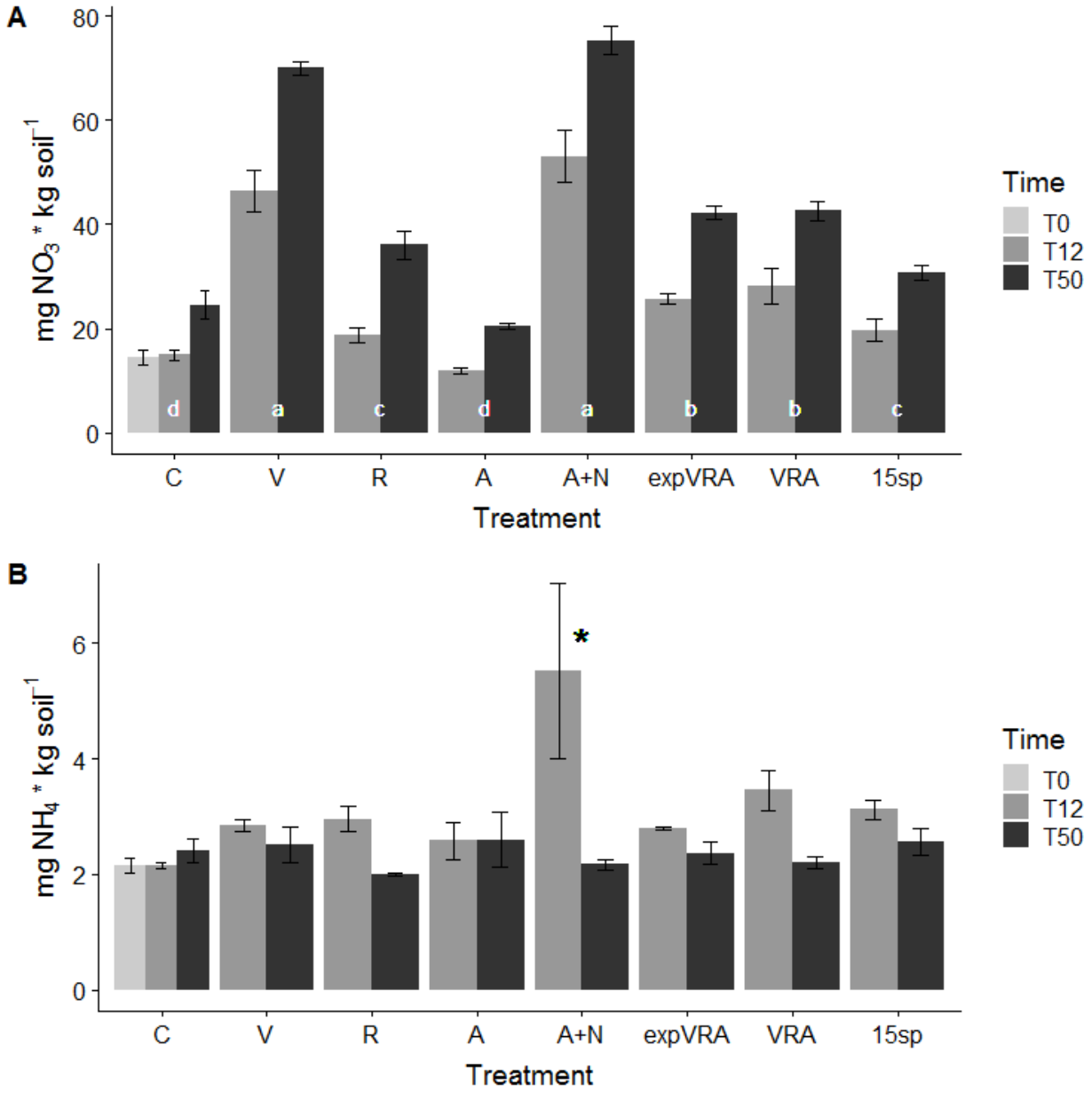

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712 Figure 7

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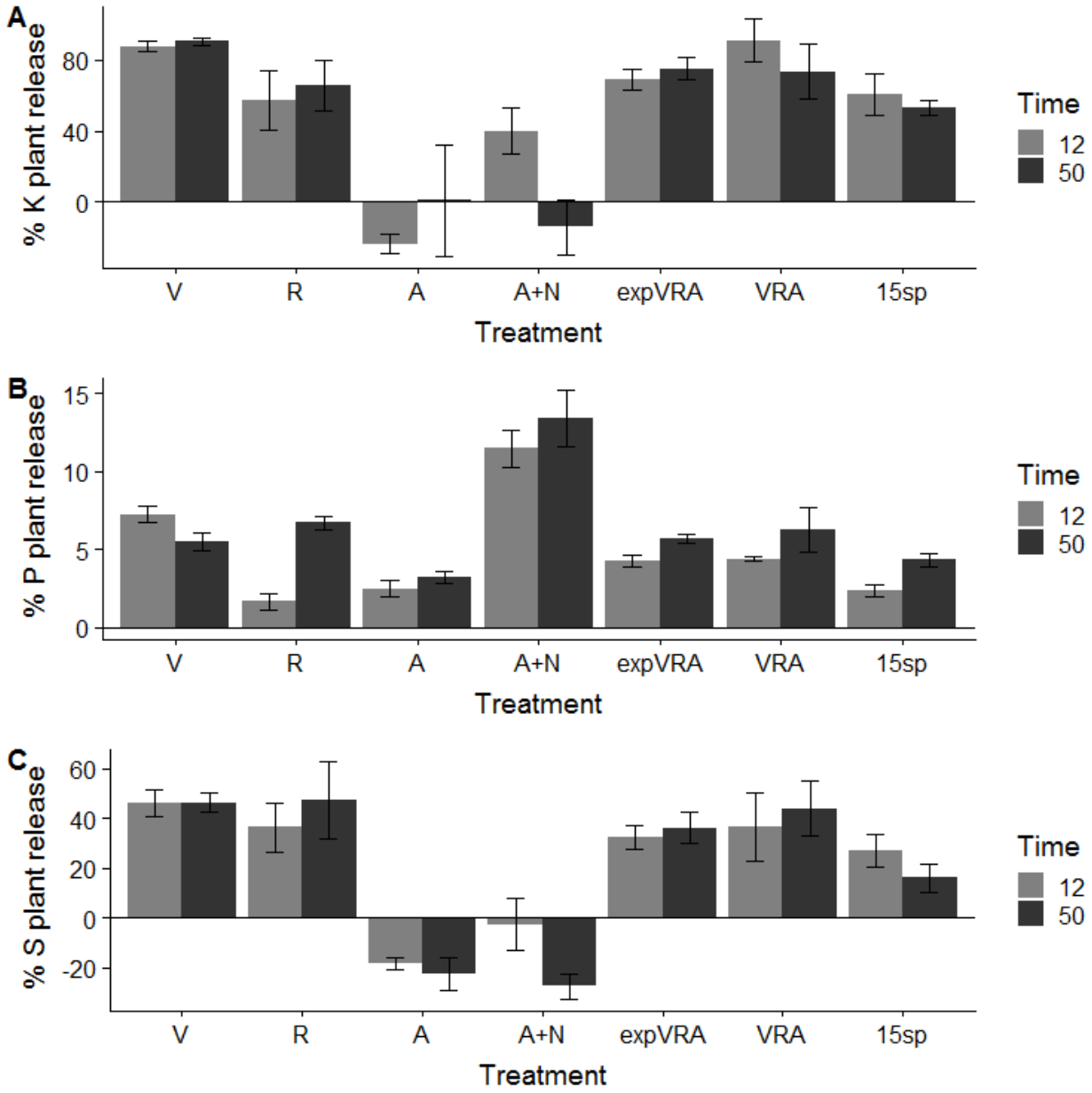

714

715 Figure 8

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717

Supplementary Tables

Table S1: Result statistical test of ergosterol (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	2.1826	0.311802	8.0318	**5.353e-08**
Time	5	0.1946	0.038924	1.0026	0.4192
Time*Treatment	7	1.4066	0.050236	1.2940	0.1710
Residuals	146	4.6973	0.038821		

Table S2: Result statistical test of the copy numbers of the 16S rRNA gene (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	1.3584e-13	1.9405e-14	7.0563	**4.473e-07**
Time	5	1.8413e-13	3.6826e-14	13.3909	**2.017e-10**
Time*Treatment	28	1.6517e-13	5.8990e-15	2.1450	**0.002356**
Residuals	123	3.3826e-13	2.7500e-15		

Table S3: Calculated microbial biomass C (MBC) in mg C per kg soil and fungi:bacteria ratio (F:B) based on 16S and ergosterol content in the soil over time. The results of the statistical test are shown in Table S4 and S5.

Time		C	V	R	A	A+N	VRA	exp VRA	15sp
0	MBC	0.45±0.07							
	F:B	0.34±0.09							
1	MBC	0.51±0.04	0.49±0.07	0.51±0.11	0.73±0.13	0.69±0.15	0.60±0.04	0.58±0.06	0.61±0.09
	F:B	0.29±0.07	0.56±0.29	0.43±0.28	0.39±0.13	0.58±0.19	0.30±0.09	0.42±0.09	0.30±0.06
3	MBC	0.47±0.09	0.56±0.11	28.8±56.3	1.59±0.83	0.53±0.08	0.94±0.59	10.3±18.8	0.70±0.22
	F:B	0.34±0.09	1.00±0.34	0.16±0.13	0.10±0.05	0.32±0.06	0.26±0.13	0.18±0.13	0.41±0.35
7	MBC	0.50±0.11	1.00±0.74	0.66±0.19	0.97±0.28	0.72±0.13	2.67±2.89	0.88±0.28	0.71±0.16

	F:B	0.29±0.08	0.38±0.20	0.26±0.07	0.18±0.10	0.47±0.37	0.19±0.17	0.24±0.05	0.51±0.39
12	MBC	1.15±0.44	1.90±1.68	0.80±0.28	1.65±0.87	1.12±0.80	0.99±0.26	1.45±0.41	0.88±0.11
	F:B	0.10±0.07	0.17±0.09	0.23±0.09	0.32±0.35	0.27±0.11	0.38±0.30	0.17±0.03	0.36±0.24
50	MBC	0.57±0.44	0.67±1.68	0.60±0.28	0.71±0.87	0.61±0.80	0.94±0.26	0.66±0.41	0.70±0.11
	F:B	0.20±0.07	0.26±0.09	0.28±0.09	0.34±0.35	0.48±0.11	0.32±0.30	0.28±0.03	0.28±0.24

729

730

731 **Table S4**: Result statistical test of the soil microbial biomass C (MBC) (with Anova). Df is degrees of
732 freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P-value**
Treatment	7	1.9754	0.28220	5.7023	1.031e-05
Time	5	2.4063	0.48127	9.7249	7.923e-08
Time*Treatment	28	1.9986	0.07138	1.4423	0.09049
Residuals	121	5.9881	0.04949		

733

734 **Table S5**: Result statistical test of the fungi:bacteria ratio (with Anova). Df is degrees of freedom, Sum
735 Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P-value**
Treatment	7	1.0004	0.142911	4.0883	0.0004628
Time	5	0.4695	0.093897	2.6861	0.0244125
Time*Treatment	28	2.4501	0.087502	2.5032	0.0003126
Residuals	121	4.2297	0.034956		

736

737 **Table S6**: Result statistical test of the biolog ECO plates (with Anova). Df is degrees of freedom, Sum
738 Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P-value**
Treatment	7	11389.5	1627.08	20.9820	**5.525e-13**
Time	2	83.8	41.92	0.5406	0.58571
Time*Treatment	7	1321.4	188.77	2.4343	**0.03126**

Residuals	51	3954.9	77.55

Table S7: Summary table RDA biolog ECO plates.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.3413	0.0727	0.0145	0.0099
Explained variation (cumulative)	34.13	41.4	42.85	43.84
Pseudo-canonical correlation	0.8497	0.8269	0.7102	0.6944
Explained fitted variation (cumulative)	75.17	91.18	94.37	96.55

Table S8: Analysis table of the RDA biolog ECO plates to identify significant contributors to the RDA plot. P-adjusted is P value corrected with Bonferroni correction.

Name	Explains %	pseudo-F	P-value	P-adjusted
Treatment: C	15.5	10.5	**0.002**	**0.024**
Treatment: V	9.6	6	**0.002**	**0.024**
Treatment: 15sp	8.2	5.1	**0.004**	**0.048**
Treatment: VRA	6.9	4.2	**0.014**	0.168
Time	6.6	4.1	**0.006**	0.072
MBC-fungus	5.2	3.1	**0.022**	0.264
Treatment: A+N	2.7	1.6	0.148	1
Treatment: A	2.5	1.5	0.162	1
MBC	1.5	0.8	0.436	1
Treatment: R	1.4	0.8	0.476	1
F:B	1.2	0.7	0.64	1
MBC-bacteria	1.1	0.6	0.702	1

745 **Table S9**: Substrates in Biolog ECO plates as carbon sources for the microbial community.

Code	Substrate	Substrate group
A1	Water	Blank
A2	β-Methyl-D-glucoside	Carbohydrate
A3	D-Galactonic acid γ-lactone	Carbohydrate
A4	L-Arginine	Amino acid
B1	Methylpyruvate (Pyruvic Acid Methyl Ester)	Ester
B2	D-Xylose	Carbohydrate
B3	D-Galacturonic acid	Carboxylic acid/sugar acid
B4	L-Asparagine	Amino acid
C1	Tween 40	Polymer
C2	i-Erythritol	Carbohydrate
C3	2-Hydroxybenzoate	phenolic compound
C4	L-Phenylalanine	Amino acid
D1	Tween 80	Polymer
D2	D-Mannitol	Carbohydrate
D3	4-Hydroxybenzoate	phenolic compound
D4	L-Serine	Amino acid
E1	α-Cyclodextrin	Polymer
E2	N-Acetyl-D-glucosamine	Carbohydrate
E3	γ-Hydroxy-butyric acid	Carboxylic acid
E4	L-Threonine	Amino acid
F1	Glycogen	Polymer
F2	D-Glucosaminic acid	Carboxylic acid
F3	Itaconic acid	Carboxylic acid
F4	Glycyl-L-glutamic acid	Amino acid
G1	D-Cellobiose	Carbohydrate
G2	Glucose-1-phosphate	Phosphorylated compound
G3	α-Ketobutyric acid	Carboxylic acid
G4	Phenyl-ethylamine	Amide/amine

H1	α-D-Lactose	Carbohydrate
H2	D,L-α-Glycerol phosphate	Phosphorylated compound
H3	D-Malic acid	Carboxylic acid
H4	Putrescine	Amide/amine

Table S10: Correlation table between the environmental variables used for the RDA analysis. Lower left part of the table are the correlation coefficients. Upper right panel are the P-values of the corresponding correlation.

	Time	**Fungal biomass**	**Bacterial biomass**	**MBC**	**F:B ratio**
Time		0.30	0.031	0.024	0.37
Fungal biomass	-0.14		0.49	0.082	<0.001
Bacterial biomass	-0.28	0.09		<0.001	<0.001
MBC	-0.29	0.23	0.99		0.014
F:B ratio	0.12	0.72	-0.43	-0.32	

Table S11: Result statistical test of the CO_2 emisisons over time (with Kruskal-Wallis rank sum test). Df is degrees of freedom.

	Chi-squared	**Df**	**P-value**
Treatment	185.76	7	**< 2.2e-16**
Time	356.99	19	**< 2.2e-16**

Table S12: Result statistical test of the N_2O emisisons over time (with Kruskal-Wallis rank sum test). Df is degrees of freedom.

	Chi-squared	**Df**	**P-value**
Treatment	186.29	7	**< 2.2e-16**
Time	246.08	19	**< 2.2e-16**

Table S13: Result statistical test of the cumulative GHG fluxes (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	12855295	1836471	35.216	**4.041e-11**
Residuals	24	1251563	52148		

Table S14: Result statistical test of the NO_3 content in the soil (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	15.3757	2.19653	102.2832	**<2e-16**
Time	2	3.9127	1.95637	91.0997	**<2e-16**
Time*Treatment	7	0.1087	0.01552	0.7229	0.6532
Residuals	51	1.0952	0.02148		

Table S15: Result statistical test of the NH_4 content in the soil (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	0.045870	0.006553	2.2178	**0.04784**
Time	2	0.085545	0.042772	14.4764	**1.049e-05**
Time*Treatment	7	0.057577	0.008225	2.7838	**0.01568**
Residuals	51	0.150686	0.002955		

Table S16: Result statistical test of the K content in the soil (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	3.0723e-05	4.389e-06	74.3937	**< 2.2e-16**
Time	2	1.3819e-06	6.910e-07	11.7119	**6.522e-05**
Time*Treatment	7	3.0570e-07	4.370e-08	0.7402	0.6391
Residuals	51	3.0089e-06	5.900e-08		

Table S17: Result statistical test of the P content in the soil (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	3.7357	0.53366	50.7854	**< 2.2e-16**
Time	2	0.0354	0.01769	1.6831	0.196
Time*Treatment	7	0.6375	0.09108	8.6673	**5.745e-07**
Residuals	51	0.5359	0.01051		

Table S18: Result statistical test of the S content in the soil (with Anova). Df is degrees of freedom, Sum Sq is sum of squares, Mean Sq is mean sum of squares.

	Df	Sum Sq	Mean Sq	F value	P-value
Treatment	7	121.870	17.4100	26.7582	**5.524e-15**
Time	2	26.083	13.0415	20.0440	**3.774e-07**
Time*Treatment	7	2.493	0.3561	0.5473	0.7945
Residuals	7	121.870	17.4100	26.7582	**5.524e-15**

SUPPLEMENTARY

Supplementary Figures

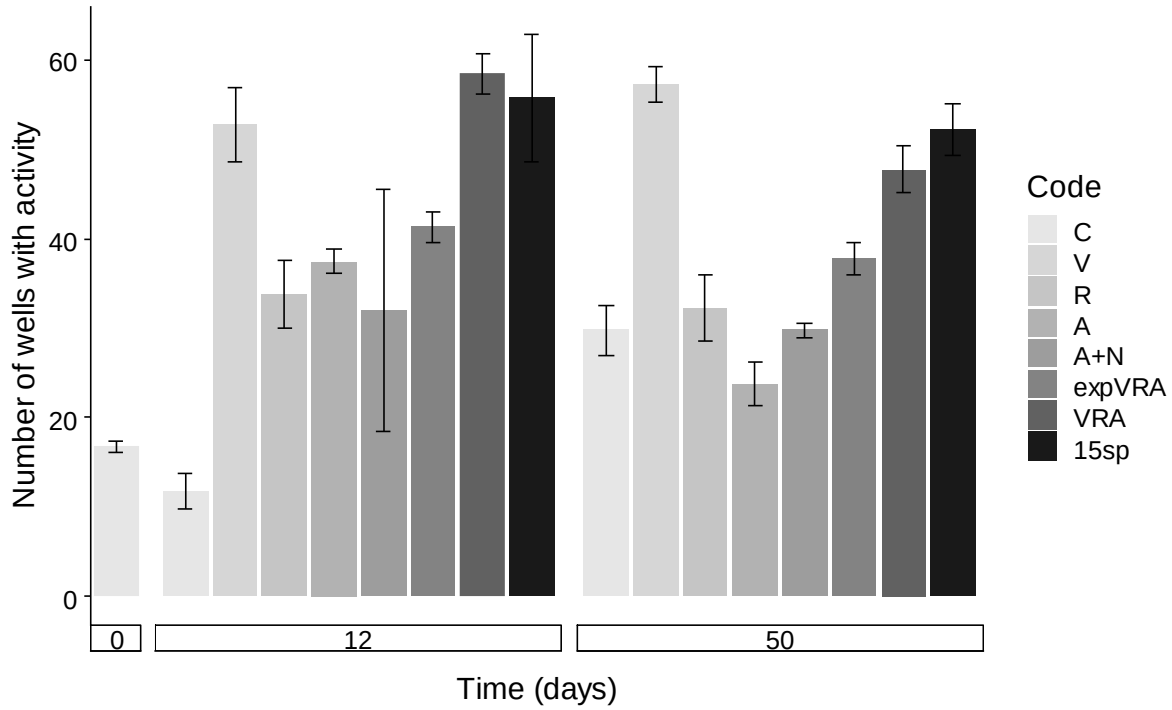
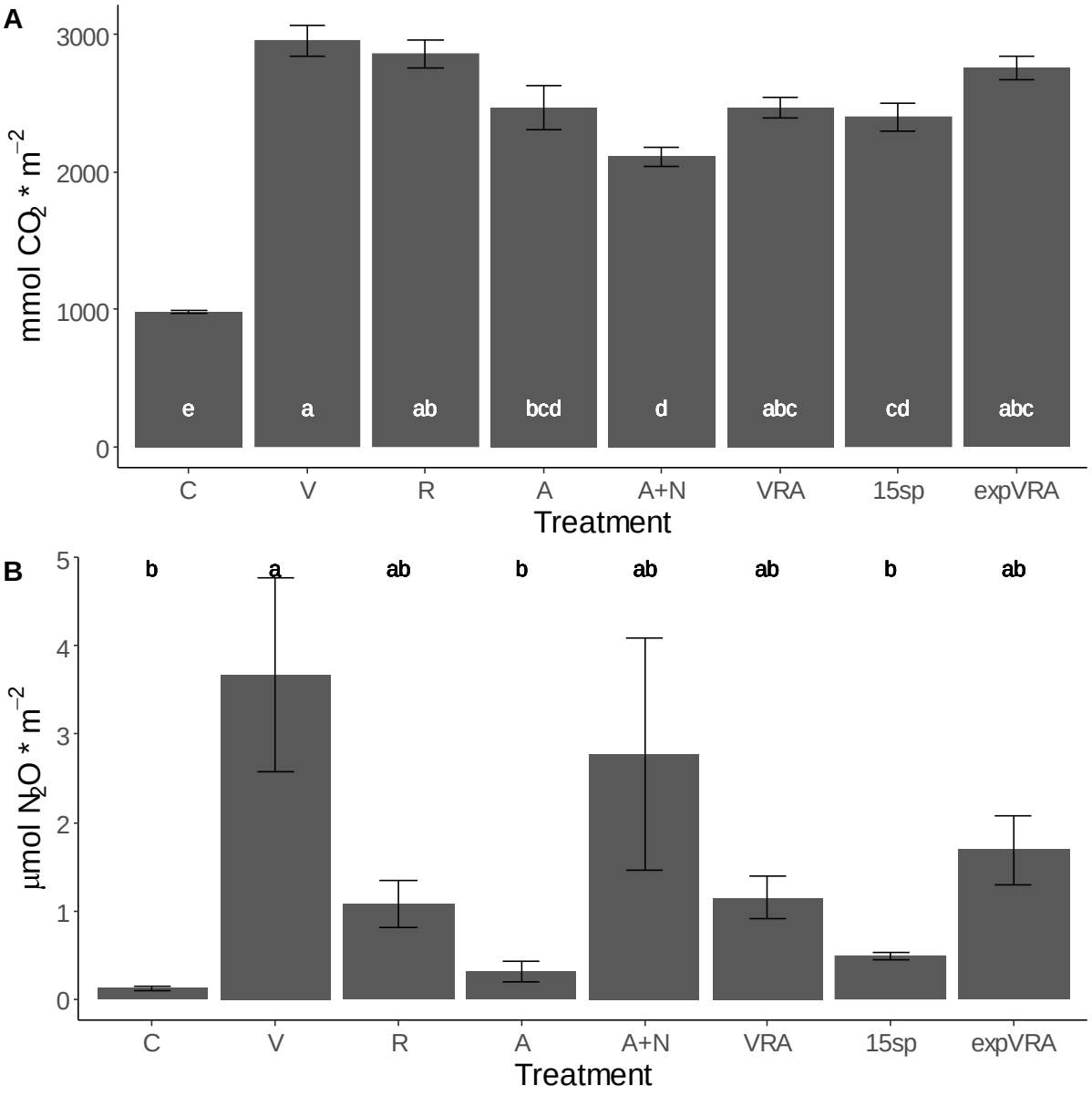


Figure S1: Microbial functional diversity: number of wells that showed a positive response in Biolog ECO plates after incubation of 7 days (mean ± SE; n = 4).



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786 **Figure S2**: Cumulative greenhouse gas fluxes of CO_2 (A) and N_2O (B) (mean ± SE; n = 4). The letters

787 indicate significant differences between the treatments.

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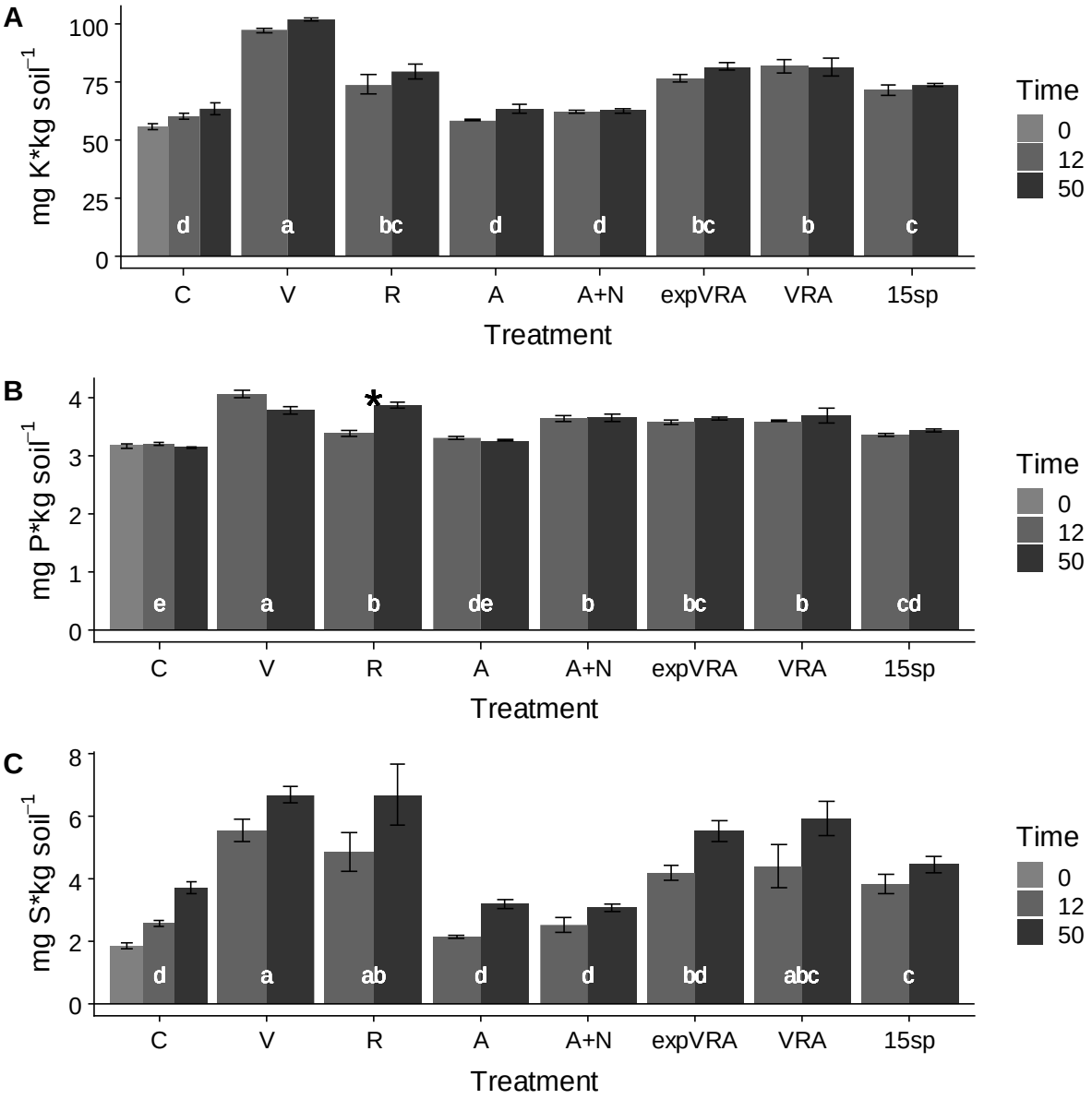
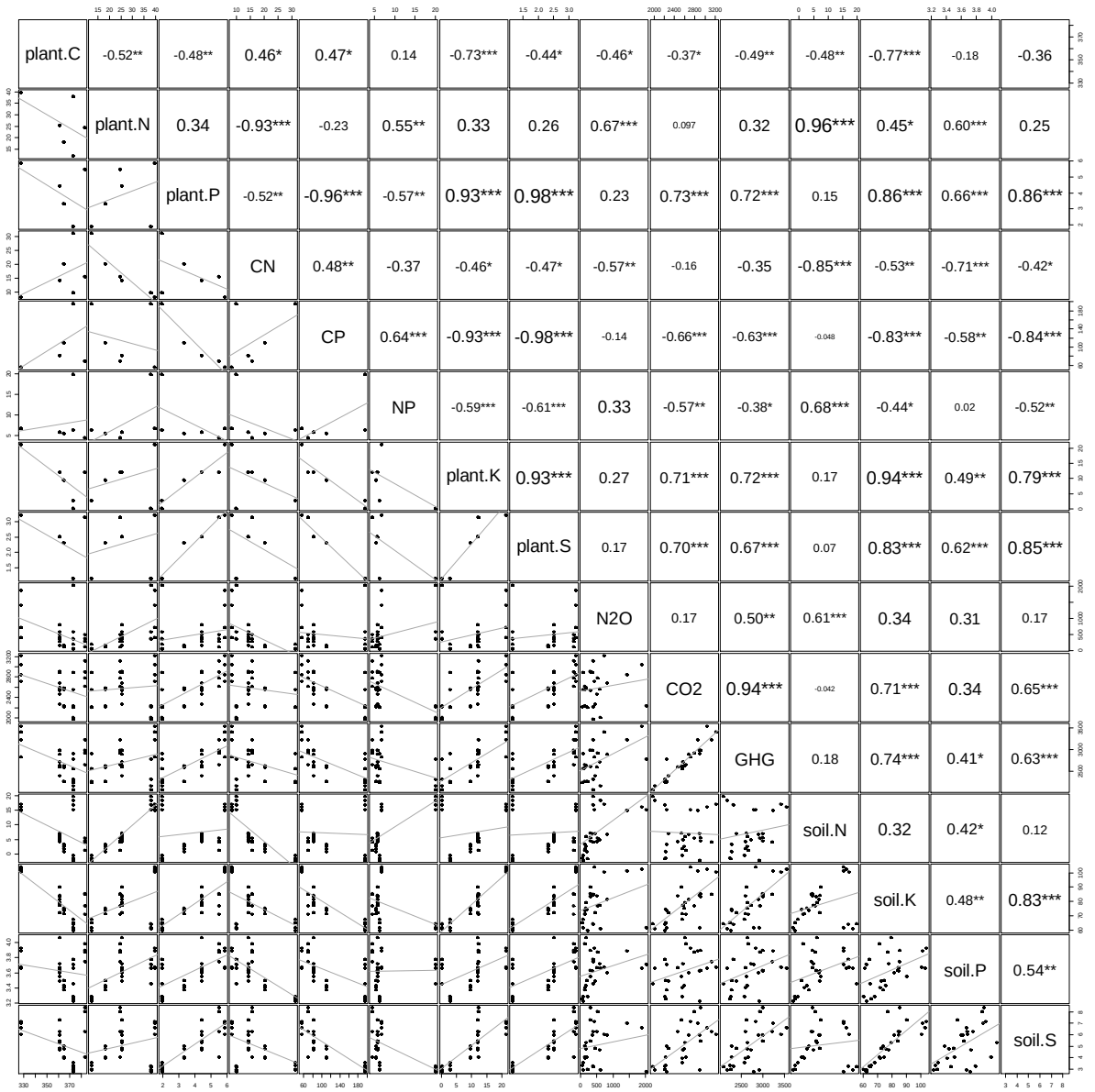


Figure S3: Nutrient content (mg per kg of dry soil) in the soil at the different time points (in days) (mean ± SE; n = 4). A: K; B: P; C: S. Letters indicate significant differences between the treatments. The asterisk in B indicate significant difference between T12 and T50 of R. In both A and C, all treatments show similar increase over time. The results of the statistical test are shown in Table S16, Table S17 and Table S18.



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797 **Figure S4**: Correlation table between plant residue nutrient content (C, N, P, C:N, C:P, N:P, K and S),
798 cumulative greenhouse gas emissions (N_2O, CO_2 and GHG based on GWP) and soil nutrient content
799 (N, K, P and S) after 50 days of incubation. The numbers in the right panel are the correlation values
800 between the parameters (Pearson correlation). If this correlation is significant, this is shown with
801 asterisks: * if 0.01<p<0.05, ** if 0.001<p<0.01 and *** if p<0.001. The size of the values indicate stronger
802 correlation.

803

804