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Ryegrass-derived pyrogenic organic matter changes organic carbon and nitrogen mineralization in a temperate forest soil

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Abstract:

Pyrogenic organic matter (PyOM) is considered as a technique to improve soil fertility and store carbon (C) in soil. However, little is known regarding soil organic C and nitrogen (N) mineralization in PyOM-amended soils. To investigate the relationship between the C and N mineralization rates and the possible consequences in terms of C storage and N availability, we incubated ryegrass-derived PyOM (pyrolyzed at 450°C) enriched in ^{13}C (4.33 atom %) in a forest Cambisol for 158 days with and without mineral N addition. We determined PyOM and native soil organic C mineralization, NH_4^+ and NO_3^- contents in the soil, gross N mineralization, phenol-oxidase and protease activities, and microbial biomass throughout the incubation experiment and the incorporation of PyOM in microbial biomass at the end of the experiment (158 days). We determined that 4.3% of the initial PyOM-C was mineralized after 158 days. Moreover, PyOM induced a strongly positive priming effect within the first 18 days; a negative priming effect was observed from days 18 to 158. The initial increase in organic matter mineralization corresponded to a higher gross N mineralization and NH_4^+ content in the PyOM-treated soil than in the untreated soil. Ammonium was rapidly transformed into nitrate and stored in this form until the end of the experiment. We conclude that the presence of PyOM affected the mineralization pattern of native soil organic matter mineralization and increased mineral N content, while N addition did not influence PyOM or soil organic matter mineralization.

1. Introduction:

Pyrogenic organic matter (PyOM), the product of incomplete combustion of biomass (Goldberg, 1985), plays an important role in the terrestrial C cycle because it can constitute up to 45% of soil organic carbon (Schmidt et al. 1999). PyOM has a turnover time of several centuries (Singh et al. 2012), a magnitude longer than any other class of soil organic compounds (Schmidt et al. 2011). Despite several recent developments in the assessment of PyOM stability (Bruun et al. 2008; Major et al. 2010; Santos et al. 2012), many uncertainties remain regarding its fate in the soil. In particular, little is known concerning the interaction between PyOM and the mineralization of native soil organic matter. Understanding this interaction is crucial for assessing the effect of PyOM on the soil C cycle because it may significantly modify the long-term C balance (Woolf and Lehmann, 2012). We define the *priming effect* to be the change in the native organic matter mineralization rate due to the addition of an organic substrate (Bingeman et al. 1953). Specifically, we used the term positive priming effect when mineralization of the native organic matter is increased and negative priming effect when mineralization is decreased. PyOM has been observed in previous studies either to induce a positive priming effect (Wardle et al. 2008; Major et al. 2010; Novak et al. 2010; Keith et al. 2011; Luo et al. 2011; Zimmerman et al. 2011), a negative priming effect (Liang et al. 2010; Cross and Sohi, 2011; Jones et al. 2011), or no priming effect (Kuzyakov et al. 2009; Abiven and Andreoli, 2010; Cross and Sohi, 2011; Santos et al. 2012).

Changes in N mineralization were often found to follow C fluxes (Booth et al. 2005; Herrmann and Witter, 2008) because they are bound in the same organic compound. In fact, as for soil organic C mineralization, PyOM was found to exert a broad range of effects on the N cycle. This variability results from the differences in PyOM feedstock, pyrolysis temperature, and soil characteristics. Nelissen et al. (2012) found that a C-rich maize-derived PyOM increased gross short-term N mineralization in loamy soil. They suggested that

60 microbes were “mining” soil organic matter to acquire N (Craine et al. 2007). DeLuca et al.
61 (2002, 2006) observed that PyOM produced during wildfires increased nitrification in boreal
62 and temperate forests and explained this as the result of sorption of phenols, which are known
63 for being nitrification inhibitors, on PyOM surfaces (DeLuca and Sala, 2006; Ball et al. 2010).
64 Moreover, Wang et al. (2012) observed an increase in nitrate content in a fertilized plot one
65 year after the addition of rice husk-derived PyOM. Across three different soil types, Kolb et
66 al. (2009) found that increasing the rate of PyOM addition, derived from a mix of manure and
67 wood, reduced the amount of available N because of increasing microbial N demand. A
68 similar conclusion was drawn using pecan-shell derived PyOM by Novak et al. (2010), while
69 Bruun et al. (2012) found a relation between pyrolysis duration and the C:N ratio of the
70 resulting PyOM, which was in turn affecting the quantity of N immobilized in the soil
71 amended with PyOM. In contrast, no PyOM effect on the N cycle was observed by Zavalloni
72 et al. (2011) and Zhang et al. (2011) using wood-derived PyOM and wheat straw-derived
73 PyOM, respectively.

74 While many studies investigated the PyOM effects on mineral N, very little is known about
75 the effect of mineral N on PyOM decomposition. Santos et al. (2012) found no effect of N
76 addition on PyOM mineralization. However, Maestrini et al. (personal communication) found
77 a decrease in PyOM mineralization. We hypothesized that N addition may decrease the
78 PyOM decomposition because increased N deposition depresses the activity of phenol-
79 oxidase (Sinsabaugh et al. 2002; Grandy et al. 2008), which is responsible for the
80 decomposition of aromatic compounds. Moreover, we hypothesized that increased N
81 availability will decrease microbial decomposition of the more recalcitrant fraction of PyOM,
82 which is generally thought to be more rich in N, as proposed by the *nitrogen mining theory*
83 (Craine et al. 2007). Similarly, Brodowski et al. (2005) suggested that microbes may
84 decompose PyOM to have access to the N adsorbed on their surfaces. Changes in N fluxes

85 due to increased microbial decomposition may be related to microbial biomass dynamics and
86 thus can give an indication of both PyOM-C and mineral N stored by soil microflora
87 (Nannipieri and Eldor, 2009).

88 To our knowledge, this study was the first to couple C fluxes and gross N mineralization in a
89 PyOM-amended soil. The present paper is aimed to investigate if PyOM affects organic
90 matter mineralization and if changes in C fluxes due to priming are reflected in N
91 mineralization. We also hypothesize that N addition may reduce PyOM decomposition. To
92 investigate the mechanisms responsible for the alteration of C and N fluxes, we used a holistic
93 approach: we divided the system into pools (native soil organic matter, PyOM, microbial
94 biomass, and mineral N) and related the C and N fluxes to the changes in the size of the pools
95 and to the activity of enzymes targeting aromatic molecules, such as PyOM (phenol-oxidase)
96 and N-rich compounds (protease). We believe that the holistic approach is the most efficient
97 and well-adapted method for studying soil functionality compared to approaches based on the
98 inference of C and N dynamics from microbial taxonomy and functional characterization
99 (Nannipieri et al. 2003).

100 We incubated ^{13}C-labeled PyOM (4.33 atom %) for 158 days in a mineral forest soil with and
101 without mineral N addition. We measured SOC mineralization, gross N mineralization, NH_4^+
102 and NO_3^- content, incorporation of PyOM derived C into microbial biomass and potential
103 enzymatic activity of phenol-oxidase and protease over the course of the period.

104 Our research questions were the following: (i) Does ryegrass-derived PyOM increase native
105 soil organic matter mineralization, gross N mineralization and net nitrification in a Cambisol?
106 (ii) If so, can these changes be explained by the phenol oxidase and protease activity and
107 microbial biomass-C and N? Lastly, (iii) does N addition affect mineralization of ryegrass-
108 derived PyOM?

2. Materials and Methods

2.1 PyOM characteristics

Two different sets of ryegrass *(Lolium perenne L.*) were grown under controlled conditions in labeling growth-chambers. One set was grown under an atmosphere enriched in ^{13}C-CO_2 (*6* atom %); the other was grown under an ambient atmosphere. Edaphic, light, and air temperature conditions were identical for the two setups. Ryegrass was harvested after 1 month in both cases.

Labeled and not labeled grasses were pyrolyzed in a quartz tube oven (Montanaro manufacturer, Glattbrugg, CH) at 450°C under a N_2 stream of 1 l min^{-1} (equivalent to 0.45 times the volume of the oven per minute) for 4 hours as described in Hammes et al, (2006). The recovery of PyOM after pyrolysis was approximately 33% (weight %) of the initial material. Characteristics of the ^{13}C-labeled PyOM are summarized in Table 1. The set of ryegrass grown under enriched ^{13}C-CO_2 conditions had slightly higher C and N contents (30 vs. 34% C and 3.2 vs. 3.6% N, $p<0.05$, t-test, $n = 4$), compared to the one grown under unlabeled conditions. However, the C:N ratios of the two sets did not significantly differ. The PyOM had a low C content (34%) and a high O (28.0%) and ash contents (53% residual after ignition at 550 °C for two hours). The H:C atomic ratio was 0.67±0.02, which is similar to values reported by Hammes et al. (2006) and Keiluweit et al. (2010) for grass-derived PyOM. This indicates that the PyOM had a relatively low C content due to a high content in microelements (resulting in high ash content). However, the aromaticity level, indicated by the H:C ratio, did not differ from other grass-derived PyOM. The low C content of our PyOM agrees with findings from Knicker, (2010), who also observed a C content of 30% for ryegrass-derived PyOM due to the low thermal stability of cellulose, a major component of grass, as also observed by Chatterjee et al. (2012). Our PyOM was characterized by a narrow C:N ratio, smaller than 10, and a very high ash content (Table 1), values similar to C:N ratio

and ash content of PyOM derived from ryegrass obtained in another study (Knicker, 2010) this indicates that characteristics of ryegrass-derived PyOM maybe similar. In contrast Keiluweit et al. (2010), using a different grass species, found a higher value. The main explanation for the low value is the higher level of N incorporation in the pyrolysis products compared to C. In the study from Knicker, (2010), N was observed to occur mostly in heterocyclic forms, like pyrroles. High ash content may also result from low thermal stability of cellulose.

The ^{13}C-labeled PyOM had a ^{13}C value of 4.33 atom % (Table 1); we have assumed that ^{13}C was uniformly distributed within the plant because it was grown in an atmosphere enriched in ^{13}C-CO_2 from the first emergence of a leaf.

2.2 Incubation setup

We sampled the top 10 cm of a Cambisol in a clearance of a temperate forest on Laegeren Mountain (NW of Zurich, Swiss Plateau, 800 m asl., Ruehr et al. 2009). The characteristics of the soil are summarized in Table 1. The soil was sieved fresh through a 2-mm mesh. The equivalent of 80 g dry soil was weighed into crystallizing dishes (Duran, Germany) 70 mm in diameter and placed inside a sealed 1.8-liter jar (Korken, IKEA). In the vessels the soil had a bulk density of 0.7 g cm^{-3}, and no effect of PyOM was observed on bulk density. The soil was pre-incubated at 27 °C for 23 days prior to the beginning of the incubation. The temperature and soil moisture were kept constant throughout the entire incubation period at 27°C and 70% of the water holding capacity, respectively. The soil moisture content was periodically adjusted (fluctuations in the soil moisture content were therefore generally lower than 1% weight). A bottle containing 20 ml of water was placed inside the jar to maintain the humidity saturation of the air. The incubation consisted of a 2x2 factorial experiment with the following treatments: soil control, soil + PyOM, soil + mineral N, soil +PyOM + mineral N. Nitrogen

158 treatment corresponds to an addition of 25 µg N-NH_4NO_3 g^{-1} dry soil at the beginning of the
159 incubation. This quantity is equivalent (considering the top 15 cm of the soil) to 53 kg N ha^{-1},
160 which is in the range applied yearly in two well-known field experiments on N deposition
161 (Aber et al. 1998; Egli et al. 1998). N was added from an aqueous solution containing
162 approximately 181.32 mg N-NH_4NO_3 l^{-1}. We added an equivalent amount of water to the
163 control soils.

164 At the beginning of the incubation we added the equivalent of 13 mg PyOM g^{-1} dry soil to
165 PyOM-treated vessels and all samples were mixed thoroughly. This quantity was equivalent
166 to an addition rate of 27 t ha^{-1}, considering an application to the first 15 cm of the soil and a
167 bulk density of 1.4 g cm^{-3}. Unlabeled PyOM was added to vessels to be extracted after 4, 18,
168 46 and 88 days whereas ^{13}C-labelled PyOM was added to the vessels to be extracted on the
169 last sampling date, i.e., after 158 days. On days 4, 18, 46, 88, and 158 after incubation started,
170 soils were sampled for analysis of mineral N content (NH_4^+ and NO_3^-), gross N mineralization
171 (see section 2.4) and microbial biomass (see section 2.5). Phenol-oxidase and protease
172 activities and soil pH were measured on days 4, 46, and 158 (see section 2.5).

173 ## 2.3 CO_2 efflux and partitioning

174 CO_2 efflux and ^{13}C-CO_2 were monitored throughout the incubation experiment. CO_2 efflux
175 from the soil was trapped in bottles containing 20 ml of 1 M NaOH and subsequently placed
176 in the jars. The amount of CO_2 trapped as sodium carbonate (Na_2CO_3) was estimated by
177 measuring the decrease in conductivity using the linear model described by Wollum and
178 Gomez, (1987) and recently applied by Abiven and Andreoli, (2010). A set of blanks (n=4)
179 was also measured to account for the CO_2 initially present in the container; both the quantity
180 of CO_2 emitted and the isotopic signal were accordingly corrected. The jars were opened only
181 at the reported sampling dates. After measuring the conductivity, the NaOH vials were

182 removed and substituted with new ones so that on each date we could measure the cumulative
183 CO_2 emitted from the sample.

184 Briefly, the ^{13}C-CO_2 was measured by precipitating trapped CO_2 with $BaCl_2$ as described in
185 Gaillard et al. (2003). An aliquot of 5 ml of NaOH solution was added to 10 ml 1 M $BaCl_2$,
186 and subsequently filtered (<0.45 µm cellulose acetate filter paper, *GVS*, *Bologna, Italy*). The
187 precipitates remaining on the filter were then dried, crushed with a spatula, and an aliquot of
188 approximately 5 mg was used for the ^{13}C analysis using an isotope mass ratio spectrometer
189 (Delta S, Thermo Finnigan, USA). To partition the origin of the trapped CO_2 between the
190 native soil organic matter and PyOM, we used a two-source isotope mixing model equation:

191 $$f = 1 - (^{13}C_{mix} - {}^{13}C_{PyOM}) / (^{13}C_{control} - {}^{13}C_{PyOM}), \quad [1]$$

192 where f is the fraction of CO_2 derived from PyOM, $^{13}C_{mix}$ is the ^{13}C content of the trapped
193 CO_2, $^{13}C_{PyOM}$ represents the ^{13}C content of PyOM, i.e., 4.33%, and $^{13}C_{control}$ is the isotopic
194 signature of soil CO_2 in the corresponding control treatment.

195 The priming effect induced by PyOM on native soil organic C mineralization was calculated
196 using

197 $$PE = (SR_{PyOM} * (1-f) - SR_{control}) / SR_{control} * 100, \quad [2]$$

198 where SR_{PyOM} and $SR_{control}$ are soil respiration in PyOM and the control soil, respectively, and
199 f is the fraction of soil respiration derived from PyOM mineralization using equation 1. In
200 equation 2, PE is expressed as the percentage of soil respiration in the control treatment.To
201 calculate mean residence time based on the cumulative PyOM mineralization data, we used a
202 two-pool parallel exponential decay model (Manzoni and Porporato, 2009; Minderman, 1968
203 equation 3):

204 $$C_t = C_0 * fr * \exp(-k_1 * t) + C_0 * (1-fr) * \exp(-k_2 * t), \quad [3]$$

where C_t is PyOM at time t and C_0 is the initial quantity of PyOM added. The fitted parameters were fr, k_1 and k_2, which represent the fast pool fraction (dimensionless), and the PyOM mineralization rate, expressed as % of PyOM-C lost per day, of the fast (k_1) and slow (k_2) pools, respectively; t is the time in years. Parameters where refined by successive iterations to minimize the residual sum-of-squares. From the mineralization rates (k_1 and k_2) we derived the mean residence time (MRT) of the corresponding pool using

$$MRT = 1/k_{1,2}, \quad [4]$$

where k_1 corresponds to the MRT of the fast turning pool and k_2 refers to the slow turning pool.

2.4 Mineral N content and gross N mineralization

Total mineral N was extracted using a 1 M KCl solution (1 hour of shaking, 180 rpm, 1:4 soil:solution ratio). Nitrate and ammonium concentrations were determined using spectrophotometry (San^{++}, Skalar, Netherlands). To measure gross N mineralization, we used the ^{15}N pool isotope dilution technique (Murphy et al. 2003). 40 g of dry soils were amended with 2 ml of a 100 mg N-$(NH_4)_2SO_4$ l^{-1} solution labelled with ^{15}N (2.7 atom %), giving a 5 µg N-$(NH_4)_2SO_4$ g^{-1} dry soil. The solution was added drop-wise onto the soil surface after which the soil samples were thoroughly mixed to homogenize added N distribution. After 4, 24, and 72 hours, an aliquot of 10 g of fresh soil was extracted (using 1 M KCl) and measured for total NH_4^+ content and ^{15}N-NH_4^+ using the diffusion technique described by Herrmann et al. (2007). Briefly, 15 ml of KCl soil extract was filled into a 20 ml scintillation vial and approximately 200 mg MgO was added to generate NH_3 for the determination of the atom % ^{15}N of the NH_4^+ pool. The evolved NH_3 was trapped onto an acidified paper disk which was placed between a double layer of polytetrafluoroethylene (PTFE) tape and stretched over the top of the scintillation vials which were then capped. All samples were gently shaken for 72

hours to transform NH_4^+ into NH_3. To prevent the introduction of sulphur in the isotopic ratio mass spectrometer the method was modified according to Schleppi et al. (2006), i.e., using citric acid instead of sulphuric acid. The isotopic signature of the ^{15}N-NH_4^+ trapped on the acid filters was then measured using an isotope ratio mass spectrometer (Delta S, Thermo Finnigan, USA*)*. To calculate gross N mineralization fluxes, we used the formula from Khirkham and Bartholomew, (1949), as reported in Smith et al. (1992):

gross mineralization =

$$=\{[(AT_1 - AT_2)/\Delta t] * [\log (AL_1 * AT_2) / \log (AL_2 * AT_1)]\} / \log (AT_1/AT2),$$

where AT is the total amount of NH_4^+ (μg N g^{-1} dry soil), AL is the amount of recovered ^{15}N-NH_4^+ (μg N g^{-1} dry soil), and Δt is the time between subsequent extractions (hours). In our study two times intervals were considered: (i) 20 hours, i.e. KCl extraction 4 and 24 hours after ^{15}N addition, and (ii) 48 hours, i.e. KCl extraction 24 and the 72 hours after ^{15}N addition. The subscripts indicate the extraction time. Estimated gross N mineralization rates were similar in the two time intervals, i.e., 2-24 and 24-72 hours (paired t-test, $p>0.05$). Therefore, the assumption of zero-order kinetics of gross N mineralization was met in the present experiment and we calculated an average value of gross N mineralization across the two time intervals.

2.5 Enzyme activities, pH and microbial biomass

Protease activity was measured using casein as substrate as described by Alef and Nannipieri, (1995); phenol-oxidase was measured using a di-phenol (3,4-diidrossi-l-fenilalanina, also named L-DOPA) substrate as described by Carreiro et al. (2000). Although other substrates have been proposed to assess phenol-oxidase, e.g., guaiacol, a mono-phenol (Nannipieri et al. 1991), and others (Baldrian, 2006), we used L-DOPA because it is the most adopted substrate in environmental studies and has a very high sensitivity (Sinsabaugh, 2010). Both reactions

253 were performed at pH 8.2. Soil pH was measured in a 1:5 soil:water (fresh weight:weight)
254 mixture after shaking and subsequent sedimentation for 12 hours.

255 Microbial C and N were measured by the fumigation-extraction method (Vance et al. 1987) in
256 which 10 g of fresh soil were fumigated with alcohol free chloroform in a desiccator for 24
257 hours. The samples (both fumigated and non-fumigated) were then extracted using a 1 M KCl
258 solution. The total organic C (TOC) and total N in the fumigated extracts were analyzed using
259 a TOC-TN analyzer (TOC-V, Shimadzu Corporation, Japan). Microbial C and N
260 concentrations were determined by subtracting C and N in the non-fumigated treatment from
261 C and N in the fumigated treatment and multiplying by a factor of 2.64 (Vance et al, 1987).
262 We also determined the fraction of the microbial biomass derived from labeled PyOM on the
263 last sampling date (158 days). An aliquot of 10 ml from the fumigated and non-fumigated
264 extracts was freeze-dried and the resulting material was measured for ^{13}C content with an
265 isotope mass ratio spectrometer (Delta S, Thermo-Finnigan, *USA*). The ^{13}C signature of the
266 microbial biomass was then estimated using equation 5 (Dawson et al. 2002):

267 $$^{13}C_{mb} = ((^{13}C_{fum} * C_{.fum}) - (^{13}C_{non\text{-}fum} * C_{non\text{-}fum})) / (C_{fum} - C_{non\text{-}fum}), \quad [5]$$

268 where C_{fum} and $C_{non\text{-}fum}$ are the amounts of C extracted from the fumigated and non-fumigated
269 samples (μg C g^{-1} dry soil) and $^{13}C_{fum}$ and $^{13}C_{non\text{-}fum}$ were the ^{13}C contents of the fumigated
270 and non-fumigated extracts (atom %). To quantify the portion of microbial C derived from the
271 added PyOM, we used equation 1, substituting $^{13}C_{mix}$ with $^{13}C_{mb}$ and ^{13}C with the $^{13}C_{mb}$ in the
272 control treatment. Liang et al. (2010) pointed out that the use of chloroform fumigation
273 extraction in soils rich in PyOM, might underestimate microbial biomass due to the
274 readsorption of lysed cells on PyOM walls. Nevertheless, we believe that even if such
275 underestimation may be pronounced, it is of minor importance in our experiment, as the ratio
276 between the PyOM-C and soil organic carbon ratio was 7 times lower than reported in Liang

et al. (2010). Therefore, we expect that the underestimation of the microbial biomass due to PyOM sorption of lysed cells will have a minor effect.

2.6 Statistical analyses

The effects of PyOM and N addition were tested using a two-way analysis of variance (ANOVA) for all variables, except for CO_2 effluxes, where repeated measures two-way ANOVA was adopted. Two-way ANOVA were also performed separately for each sampling date. When data were not normally distributed according to the Shapiro normality test ($p>0.05$), the data were log transformed. The Kruskal-Wallis test was adopted instead of ANOVA, if also log-transformed data were not normally distributed. When time was a significant factor, we performed a Tukey-post-hoc test to determine which sampling dates were significantly different. All computations were performed using the statistical software *R*. The "agricolae" package was used to perform the Tukey test; the "ezANOVA" package was used for the repeated measures ANOVA.

3. Results

3.1 Soil respiration, native and pyrogenic organic matter (PyOM) mineralization

Soil respiration was significantly influenced by time ($p<0.05$), the presence of PyOM ($p<0.05$) and the interactions between PyOM and time ($p<0.05$). Particularly, the presence of PyOM increased the total soil respiration within the first 18 days and decreased it afterwards (Figure 1 a). Neither PyOM nor N addition altered the total net cumulative soil respiration over 158 days of incubation (Table 2). After 158 days, PyOM-C losses as CO_2 were 4.3±0.1 and 4.4±0.1% of the added PyOM-C, with and without N addition, respectively. Most of the PyOM mineralization occurred within the first 4 days (approximately 2.9% of the initial PyOM-C). The PyOM mineralization was not influenced by N addition at any sampling date

during the incubation; the cumulative mineralization at the end of the experiment was also not affected (Table 2). We fitted a two-pool exponential decay model to our PyOM mineralization data (Equation 3) and found no significant differences in the mean residence times (Table 3) between N addition treatments. The fast pool represented 3.3% of the initial PyOM-C and had an MRT of 2 days; the slow pool had an MRT of 40 years (Table 3). Over the 158-day period, the presence of PyOM inhibited cumulative native organic matter mineralization ($p<0.05$, Table 2), i.e., it induced a negative priming effect equivalent to 10.09 ± 3.08 or 13.53 ± 3.11% of the soil respiration in the control treatment with or without N treatment, respectively. However, the priming effect direction changed over time. Within the first 18 days, PyOM induced a positive priming effect; a negative effect occurred from day 18 to day 158 (Kruskal-test or ANOVA test on individual dates, $p<0.05$, Figure 1 c). The N addition did affect the priming effect.

3.2 Microbial biomass

Over the entire incubation period, the PyOM addition increased the microbial biomass C ($p<0.05$, Figure 1 d) in comparison with control treatments. Within treatments, the microbial biomass C decreased over time (Tukey post-hoc test between dates, $p<0.05$), while the N addition did not alter the microbial biomass C. The increase in soil microbial biomass C due to PyOM addition was only significantly different on days 4, 18, and 88 ($p<0.05$). We did not find an effect of PyOM or N addition on microbial biomass N and the microbial C:N ratio (Table 1, supplementary material). The fraction of PyOM-derived C recovered in the microbial biomass after 158 days was 0.45±0.03 and 0.47±0.02% (t-test, $p<0.05$, Table 2) with and without N addition, respectively, corresponding to 0.07± 0.01 and 0.08 ± 0.01% of the initially added PyOM-C, with no significant difference between the N addition treatments.

3.3 Nitrogen cycling

Mineral N content in the soil increased significantly after PyOM addition ($p<0.05$), mineral N addition ($p<0.05$) and over time ($p<0.05$, Figure 2 a). PyOM increased the NH_4^+ content after 4 days (Kruskal-test, $p<0.05$, Figure 2 b), while we found almost no NH_4^+ on day 18 in both treatments. After 18 days, the NH_4^+ content increased again. However, we could not observe a clear trend in NH_4^+ content for all treatments. For individual dates, PyOM addition affected significantly the gross N mineralization on days 4 ($p<0.05$) and 158 (Kruskal test, $p<0.05$). However, on sampling days 18 and 46, the NH_4^+ contents in the extracts after 72, and sometimes even 24 hours after ^{15}N addition, were extremely low. More specifically, NH_4^+ was not detectable in some PyOM-amended soils. Therefore, measurements from those dates are unreliable. This decrease of NH_4^+ from the mineral N pool in soil amended with PyOM may be directly related to PyOM sorption capacities (Jones et al. 2012). Overall net nitrification rates (Figure 2 d) were not affected by PyOM addition. However, it was affected by N addition ($p<0.05$) and time ($p<0.05$). For individual dates, N addition increased nitrification from day 0 to day 4 ($p<0.05$), very likely a result of the nitrification of NH_4^+ added as NH_4NO_3 at the beginning of the experiment. In comparison, PyOM increased the net nitrification from day 4 to 18 ($p<0.05$). No differences in nitrification were observed after day 18 due to the addition of PyOM or N.

3.4 Enzyme activities and soil pH

PyOM addition decreased the activity of phenol-oxidase ($p<0.05$, Figure 3) compared to the control treatment; we did not observe an N addition effect. In contrast no PyOM addition, N addition or time effects were observed on protease activity. PyOM addition significantly increased pH (Kruskal-Wallis test, $p<0.05$, Figure 1 supplementary material) for the entire duration of the experiment. Soil pH decreased for all sampling dates for the PyOM treatment and only between the first and second sampling dates for the control treatment (pairwise Wilcox-test, $p<0.05$). Moreover, for the first sampling date, the control with N had a lower pH

than the control without N (Wilcox-test, $p<0.05$). This could be attributed to the initial nitrification of the added NH_4^+.

4. Discussion

4.1 PyOM mineralization, soil respiration and phenol oxidase activity

After five months of incubation, 4.3% of PyOM-C was mineralized (Table 2). Our results are comparable to previous findings on ryegrass-derived PyOM decomposition from Hilscher et al. (2009). They found a decomposition ranging between 2.5 and 3.2% of the initial PyOM-C after 52 days, depending on the duration of the pyrolysis process, with longer durations delivering more resistant PyOM. Using our model (Figure 1b) we found that 3.7% of PyOM-C was decomposed after 52 days. We believe that the difference between the two studies is due to the different edaphic conditions. Specifically, they incubated PyOM in a B horizon poorer in organic C (3.4 mg C g^{-1} soil), and most likely also less microbial biomass compared to our study. Also the PyOM characteristics may have played a role. In fact their PyOM was produced at a lower temperature and was characterized by a higher C:N ratio. Hamer et al. (2004) incubated ryegrass-derived PyOM and microbial inocula in quartz sand and found that only 0.8% of PyOM-C was decomposed after 60 days. This confirmed that soil characteristics, e.g., microbial structure, and aggregation play a crucial role in determining PyOM stability. We fitted a two-pool decomposition model to our PyOM mineralization data (Figure 1 b) and observed that PyOM had a fast pool with a turnover time of 2 days, equivalent to 3.3% of PyOM-C, and a slower pool, with a turnover time of 40 years (Table 3). These values are in agreement with previously reported PyOM turnover times determined from a meta-analysis for grass-derived PyOM in incubation studies by Singh et al. (2012). Several authors observed that pyrolysis may increase carbonate content of the pyrolysis product (Lehmann and Joseph, 2009). Therefore it is likely that part of the initial high PyOM-C losses derives from PyOM-inorganic C, i.e., carbonates (Jones et al. 2011; Bruun et al.

2013). The release of CO_2 from carbonates is also reflected in the pH decrease over time (Figure 1, supplementary materials), decreasing as PyOM-carbonates were consumed. Using the two-pool model, we predict that the quantity of PyOM remaining in the soil after 100 years (which is the minimum permanence requested by many C reduction schemes) will be 8% of the initial PyOM-C. Such a relatively fast decomposition rate would represent a challenge for the use of PyOM as a tool to store C in the soil. Nevertheless, caution is necessary when using exponential decomposition models to predict the long-term stability of PyOM. In fact, models calibrated on short-term experiments capture only the initial fast decomposition rate of PyOM and therefore they may overestimate PyOM decomposition (Singh et al. 2012).

N addition did not affect PyOM-C losses over time, confirming previous findings by Santos et al. (2012). Because our soil was not N-limited and the net N mineralization was positive throughout the incubation period, it was unlikely that N addition would play an important role. Surprisingly, the activity of phenol-oxidase was inhibited by PyOM addition but not by N addition, which is not in agreement with previous observations that N addition may inhibit phenol-oxidase (Sinsabaugh, 2010). DeLuca et al. (2002) observed that PyOM has the capacity to absorb phenols. This may lead to a decrease in the concentration of the assay, resulting in a lower availability of the assay for the targeted enzymes and therefore in a decrease of enzymatic activity. A decrease in phenol oxidation due to sorption on mineral surfaces was also observed by Scott et al. (1983). It is important to consider that the assay method used in the present study (L-DOPA, an o-diphenol), although widely used, is only one of the plethora of assays that have been employed to measure phenol-oxidase activity. It is most likely that it does not cover the entire range of enzymes involved in the oxidation of phenols or related molecules, e.g., the aromatic structures forming PyOM.

4.2 Microbial biomass

Microbial biomass and soil respiration decreased over time (Figure 1 d), as it has been shown in many models using microbial biomass to predict soil respiration (Fang et al. 2005; Fontaine and Barot, 2005). PyOM significantly increased the microbial biomass amount on days 4, 18 and 88, confirming previous observations by Steiner et al. (2008) and Kolb et al. (2009). The increase may be explained by the easily decomposable fraction initially present in the PyOM (Lehmann et al. 2011) and by the PyOM capacity to host a microbial community (Pietikäinen et al. 2000). Moreover, the increase in soil pH following PyOM addition to the soil may have contributed to increased microbial biomass (Badalucco et al. 1992). The fraction of PyOM incorporated into microbial biomass after 158 days was very low, 0.4% of added PyOM-C, confirming findings by Singh et al. (personal communication), Bruun et al. (2008), and Santos et al. (2012). In contrast, Kuzyakov et al. (2009) observed that, 1.5 % of added PyOM, was incorporated into microbial biomass after nearly 20 months incubation. This finding implies that incorporation of PyOM into microbial biomass may be time dependent.

Jones et al. (2012) found that the microbial community of a soil containing PyOM was characterized by a lower microbial efficiency, and hypothesized that this was due to a relative increase in bacteria instead of fungi in the microbial community. In fact, bacteria are known for being characterized by a lower efficiency. This hypothesis was not confirmed in the present study where a significant change in microbial biomass C:N was not observed (Table 1, supplementary material), a commonly used indicator of the fungal:bacterial composition of microbial biomass (Fierer et al. 2009).

4.3 Temporal mineralization pattern of native soil organic matter

The presence of PyOM promoted the mineralization of native soil organic matter, i.e., induced a positive priming effect, in the first 18 days and inhibited mineralization from day 18 until the end of the incubation, i.e., induced a negative priming effect (Figure 1 c). Our findings are similar to those by Zimmerman et al. (2011) who hypothesized that in an initial phase, the

425 organic matter promoted the decomposition of PyOM and in a second phase, PyOM sorbed
426 the organic matter and protected it from decomposition. Such a priming effect pattern was
427 also used in the process-based model developed by Woolf and Lehmann, (2012) to evaluate
428 the impact of yearly PyOM addition on soil C storage in a maize crop ecosystem over 100
429 years. In our experiment, the partitioning of soil respiration between PyOM and soil organic
430 matter derived-C indicated that the mineralization of native organic matter was also promoted
431 in the short term, confirming the findings of Keith et al. (2011).

432 Several processes that may be simultaneous, sequential or mutually exclusive may have
433 occurred to explain these observations. One hypothesis for the initial positive priming effect is
434 that the labile fraction present in PyOM triggers soil microflora. Several authors distinguished
435 two processes occurring in the priming effect that are induced by labile substrates: apparent
436 and real priming effects (Blagodatsky et al. 2010; Kuzyakov, 2010). The apparent priming
437 effect is an increase in the CO_2 efflux resulting from the activation of the dormant biomass
438 due to the addition of available substrates. This results in an increase in the maintenance
439 respiration of the total soil microflora (Blagodatsky and Richter, 1998; Blagodatsky et al.
440 2010). The real priming effect appears in a second phase and is the result of increased
441 mineralization of the soil organic matter by some of the activated microbes (Kuzyakov, 2010)
442 or by the K-strategist microorganisms. The latter take advantage of the enzymes released by
443 the activated one (Fontaine et al. 2003). The apparent priming effect has often been observed
444 as a result of the addition of labile substrates, e.g., glucose (Wu et al, 1993; Conde et al, 2005;
445 Blagodatsky et al, 2010; Blagodatskaya et al, 2011). Although PyOM is often treated as a
446 homogeneous recalcitrant compound it may contain a fraction of easily decomposable
447 substances which have the potential to trigger microbial biomass activity. In our study, the
448 two-pool decomposition model indicated that PyOM also contained a fast pool corresponding
449 to 3.3% of the total PyOM-C (Table 3). The presence of a readily available fraction was

450 confirmed by Hilscher et al. (2009) who observed that ryegrass-derived PyOM contained a
451 fraction of water-soluble C equivalent to 3.9% of PyOM-C. The occurrence of an apparent
452 priming effect in the first four days of the incubation is supported by the following indicators:
453 (i) the easily decomposable fraction of PyOM is lower than the initial microbial biomass
454 (approximately 13% of microbial biomass C) and thus considered to be an insufficient
455 quantity to induce a real priming effect (Blagodatskaya et al. 2011), and (ii) the quantity of
456 primed C after 4 days was lower than the amount of microbial C in the soil (8% of microbial
457 C). This lower level is also assumed to be an indicator of an apparent priming effect
458 (Kuzyakov, 2010).

459 The positive priming effect may also result from the pH change induced by PyOM addition
460 (Figure 1 supplementary materials). Luo et al. (2011) found that the increase in native organic
461 matter mineralization promoted by the presence of PyOM was proportionally higher in acidic
462 than in alkaline soils, suggesting that liming could play a role in determining the magnitude of
463 the positive priming effect. It is well known that liming in acid soils may cause a short-term
464 increase in soil respiration (Badalucco et al. 1992; Haynes and Naidu, 1998; Haynes, 1984).
465 Jones et al. (2011) suggested that PyOM may change the soil pH towards the optimum for
466 extracellular enzymes. In our study, the presence of PyOM increased the pH of soil
467 throughout the entire incubation period (Figure 1 supplementary material). However, we
468 observed a change in the direction of priming after 18 days. Therefore, we can only speculate
469 that the change in soil pH was not the prevailing factor responsible for the change in the
470 native soil organic matter mineralization after 18 days. The most often cited explanation for
471 the negative priming effect is that PyOM adsorbs organic matter on its surfaces (Liang et al.
472 2010; Cross and Sohi, 2011; Zimmerman et al. 2011). Alternatively, the negative priming
473 effect could be explained by a depletion in the available substrate (Bingeman et al. 1953).
474 However, this explanation is unlikely in our soil, which had a very high C content, and

475 therefore was not likely to be C-limited. Moreover, Hamer and Marschner (2005) did not
476 observe a limitation in the availability of soil organic C due to the priming effect in a
477 Cambisol incubation in which different substrates were added. We also observed that PyOM
478 caused a decrease in the phenol-oxidase activity (Figure 3). This could have contributed to a
479 decrease in mineralization of more condensed compounds. However, such decrease was
480 already observed in the first sampling date when the positive priming effect was observed.
481 Moreover, we believe that such a decrease was more likely an artifact of PyOM sorption of
482 the assay (see section 4.1). Thus, we can only speculate that the reduction in phenol-oxidase
483 may have contributed to the negative priming effect in the second part of the experiment.

484 **4.4 N dynamics**

485 PyOM only altered the NH_4^+ content of the soil up to day 4 of the incubation period (Figure 2
486 b and c). NH_4^+ content of PyOM (Table 1) can only explain 26% of the additional NH^{4+} that
487 was recovered on day 4. Therefore, we concluded that the remaining 74% mineral NH_4^+ was
488 derived from the increased mineralization of the native organic matter (i.e., priming effect)
489 and PyOM mineralization. Moreover, PyOM addition increased gross N mineralization on
490 day 4 (Kruskal test, $p<0.05$, Figure 2 c). This confirms the findings of Nelissen et al. (2012)
491 who observed an increase in gross N mineralization in the first week after PyOM addition. By
492 modeling N fluxes using ^{15}N tracer, they found that increased gross N mineralization was
493 mostly derived from the recalcitrant pool of organic matter. We hypothesize that the increase
494 in gross N mineralization is mainly derived from increased microbial activity, therefore we
495 favor the microflora triggering explanation for priming effect over the pH change one, as
496 liming does affect neither gross (Cheng et al. 2013) nor net (Dancer et al. 1972) N
497 mineralization. Gross N mineralization in the PyOM treatment was also slightly higher than in
498 the control treatment in the fifth sampling date, i.e., after 158 days. The higher N
499 mineralization rate at the end of the incubation period could be due to the mineral N derived

500 from the PyOM decomposition, which in the present study, was shown to have a very low
501 C:N ratio and was therefore a source of N. Moreover, the adsorption of added labelled NH_4^+
502 onto PyOM surfaces (Jones et al. 2012) may have also reduced the content of labelled NH_4^+
503 recovered in the extract. This would result in a bias when interpreting gross mineralization
504 data, i.e. the observed NH_4^+ decrease in mineral N pool would be interpreted as an increase of
505 gross mineralization, but low amounts of NH_4^+ were due to its adsorption onto PyOM
506 surfaces and not because of an increase in mineralization per se. The NH_4^+ mineralized within
507 the first few days was rapidly transferred to the NO_3^- pool and remained in this form until the
508 end of the incubation period. Nitrification was very high in our soil. On day 4, the initial N
509 addition induced a higher nitrification rate, which was probably derived from the nitrification
510 of NH_4^+ from the N added as unlabeled NH_4NO_3 at the start of the experiment. From day 4 to
511 18, we found a higher nitrification in the PyOM treatments compared to the control soils
512 likely because of the transformation of the NH_4^+ derived from the strong initial priming effect.
513 Our results disagree with the findings of DeLuca and Sala, (2006) who observed higher
514 nitrification rates in burned forest soil compared to unburned. They suggested that PyOM
515 removed nitrification inhibitors, e.g., phenols, derived from shrubs growing in the understory.
516 In the present study, nitrification seemed to be limited by its substrate NH_4^+ rather than by the
517 presence of phenols. Bruun et al. (2012) found that PyOM induced a net N immobilization,
518 while in our study we observed that PyOM induced a net N mineralization. This discrepancy
519 can be explained by the different C:N ratio of the two PyOM (40 and 47, Bruun et al. 2012
520 versus 9 in the present study). These findings confirm the importance of C:N to predict N
521 mineralization in soils amended with PyOM or other substrates (Mary et al. 1996). The
522 increased N mineralization was not accompanied by an increase in protease activity (Table 2).
523 This is in agreement with the N mining theory that postulates that higher N availability
524 decreases the decomposition of the recalcitrant fraction of a substrate only when it is poor in
525 N (Craine et al. 2007), which was obviously not the case for the PyOM in our study (Table 1).

Moreover, the unchanged protease activity in the presence of PyOM might also be because casein is an assay representative of high molecular weight compounds, while the organic matter decomposed at the beginning of the experiment was more likely composed of soluble low-weight N molecules rather than relatively less soluble large molecules.

5. Conclusions

We incubated ryegrass-derived ^{13}C-labeled PyOM for five months in the topsoil of a Cambisol with and without additional N amendments. The PyOM was characterized by a narrow C:N ratio, and mineralized relatively fast. Therefore its efficiency as C-sink in soil system would be rather limited. PyOM promoted native organic matter mineralization during the first 18 days and inhibited it afterward. We suggest that the positive priming effect resulted from an increase in the activity of soil microflora or from the shift in pH following PyOM addition. While negative priming effect may follow from depletion of available C or from the adsorption of organic matter on PyOM surfaces. Our initial hypothesis that N addition may decrease PyOM decomposition via depressing phenol-oxidase activity was not confirmed. On the contrary, PyOM decreased the potential activity of the enzyme, most likely by partly adsorbing the assay. The initial positive priming effect was concurrent to an increase in gross N mineralization and NH_4^+ content. The latter was rapidly nitrified in our soil system. We believe that our results were strongly influenced by the characteristics of the PyOM used, which was characterized by a notably narrow C:N ratio and by the presence of an easily decomposable C-pool. Therefore, we conclude that special attention needs to be paid to PyOM characteristics when evaluating the effect of PyOM on soil C and N dynamics.

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Contributions

B. M. developed the experimental setup, performed the analysis, and wrote the article. S. A. contributed to the development of the experimental setup, data analysis, elaboration of the manuscript, and successfully applied for funding the project. A. M. H. contributed to the setup of the ^{15}N pool dilution technique and data analysis, and P. N. contributed to the setup for the measurement of the enzymatic activity. All authors provided input and drafting to the final version of the manuscript.

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Figure captions:

Figure 1: Cumulative total soil respiration (a), PyOM remaining in the soil as measured and modeled according to equation 3 (b), cumulative mineralization of native soil organic matter (c) and (d) microbial C dynamics throughout the incubation period. Full symbols represent the experiment without N addition treatment: empty symbols are for the N addition treatment. The circles represent the control treatments and triangles are for PyOM addition treatments. The dashed line represents the modeled PyOM mineralization with N treatment; the continuous line is for the PyOM mineralization without N treatment. In all figures, the error bars represent the standard error of the mean (n = 4).

Figure 2: Mineral N dynamics in the soil along the incubation period: (a) soil mineral N content, (b) soil NH_4^+ content (c) gross mineralization and, (d) nitrification. Full symbols represent without N addition treatment, empty symbols represent with N addition treatment, circles are for control treatments and triangles are for PyOM addition treatments. Error bars represent the standard error of the mean (n = 4). On sampling days 18 and 46, the measurement of gross N mineralization failed because the NH_4^+ contents in the extracts after 72, and sometimes even 24 hours after ^{15}N addition, were extremely low, sometimes below the detection limit.

Figure 3: Potential phenol-oxidase activity (using L-DOPA as substrate). Error bars represent the standard error of the mean (n = 4). Within each sampling date, the bars are in the following order: control without N addition, control with N addition, PyOM without N addition and PyOM with N addition.

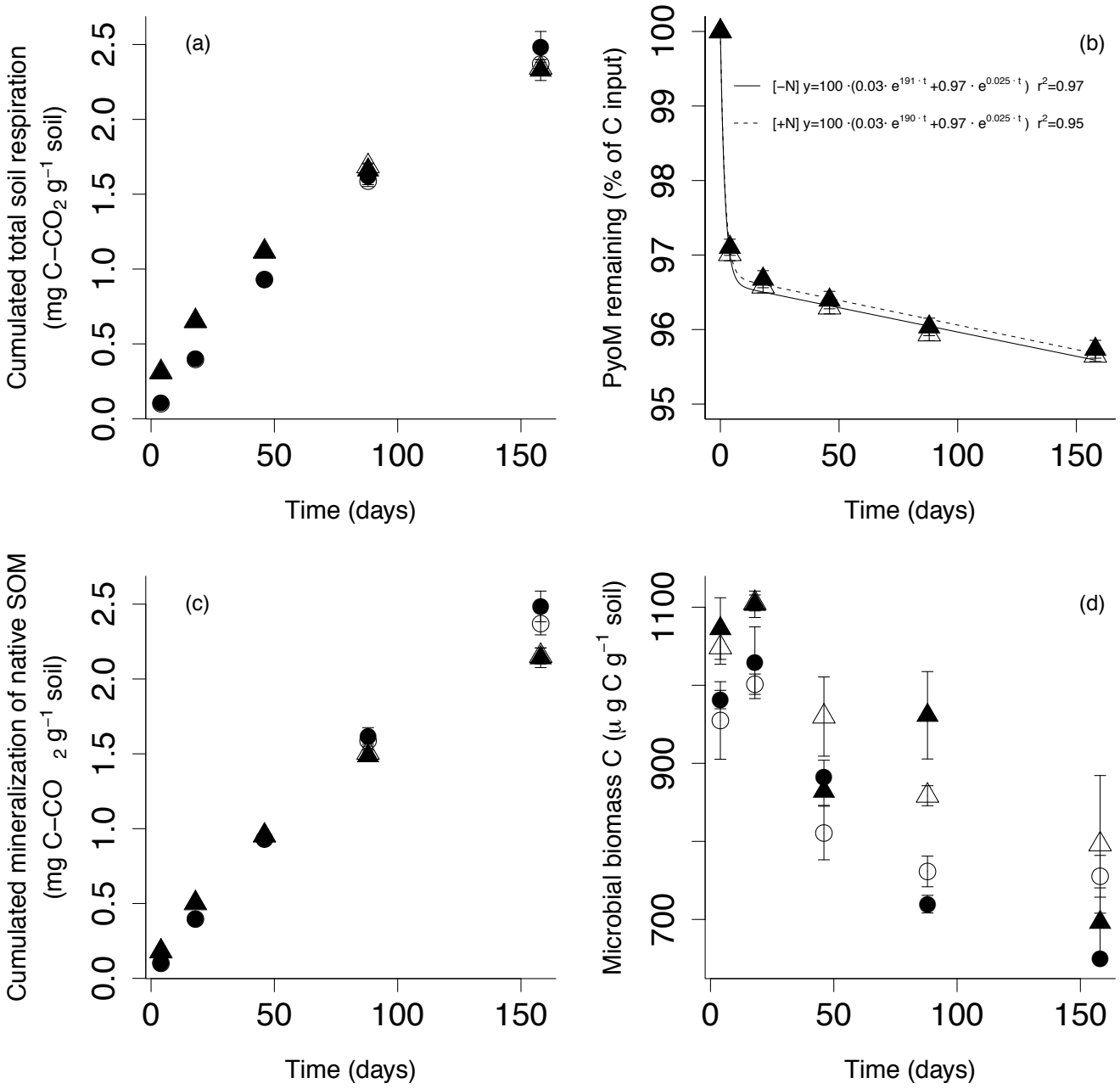
(a)
Cumulated total soil respiration
(mg C–CO2 g−1 soil)
0.0
0.5
1.0
1.5
2.0
2.5
0
50
100
150
Time (days)
(b)
PyoM remaining (% of C input)
95
96
97
98
99
100
[−N] y=100 ·(0.03· e191 · t +0.97 · e0.025 · t) r2=0.97
[+N] y=100 ·(0.03· e190 · t +0.97 · e0.025 · t) r2=0.95
0
50
100
150
Time (days)
(c)
Cumulated mineralization of native SOM
(mg C–CO 2 g−1 soil)
0.0
0.5
1.0
1.5
2.0
2.5
0
50
100
150
Time (days)
(d)
Microbial biomass C (µ g C g−1 soil)
700
900
1100
0
50
100
150
Time (days)

Figure 1

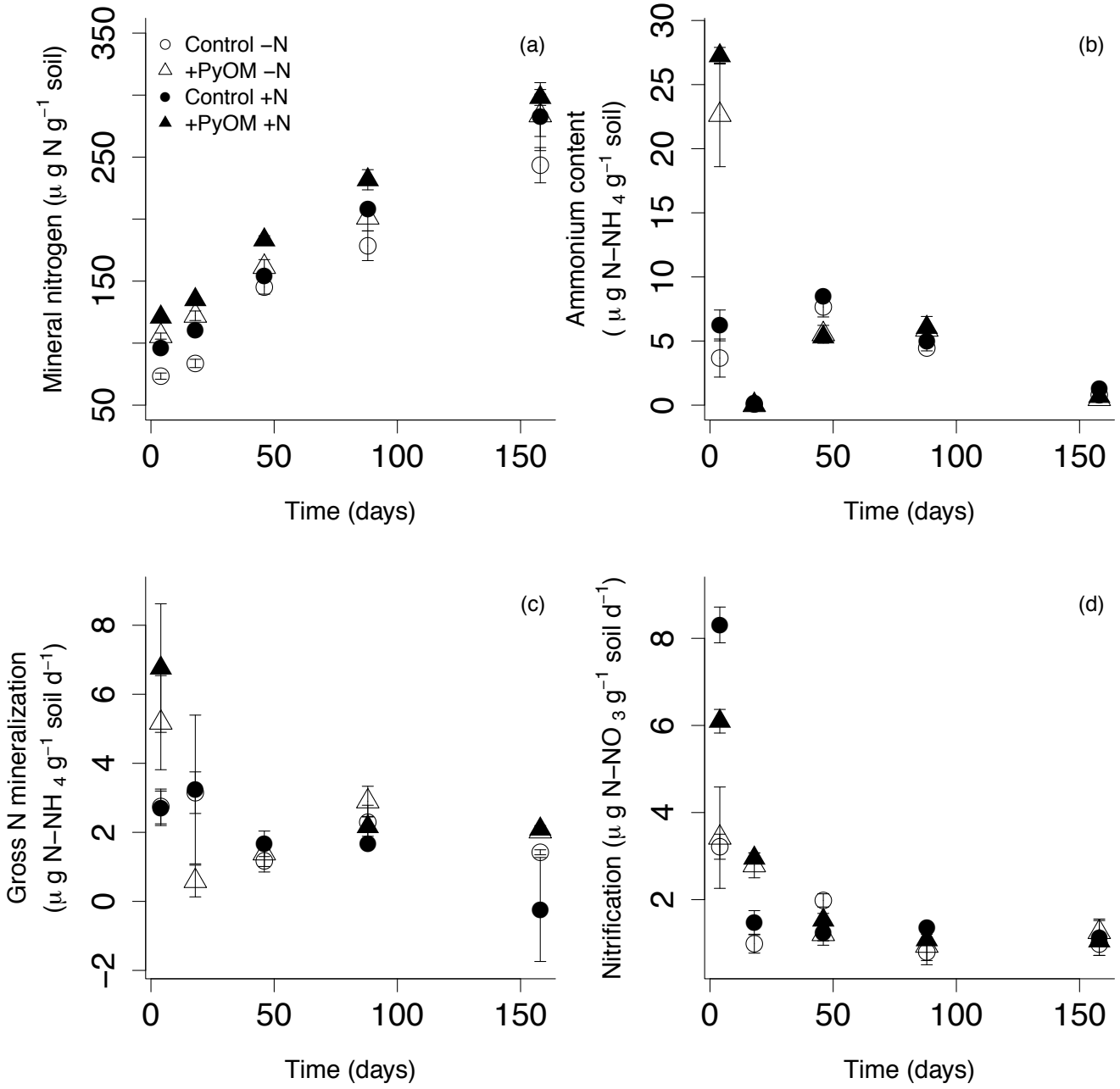
Control –N
+PyOM –N
Control +N
+PyOM +N
(a)
Mineral nitrogen (μ g N g^{-1} soil)
Time (days)
(b)
Ammonium content
(μ g N–NH $_4$ g^{-1} soil)
Time (days)
(c)
Gross N mineralization
(μ g N–NH $_4$ g^{-1} soil d^{-1})
Time (days)
(d)
Nitrification (μ g N–NO $_3$ g^{-1} soil d^{-1})
Time (days)

Figure 2

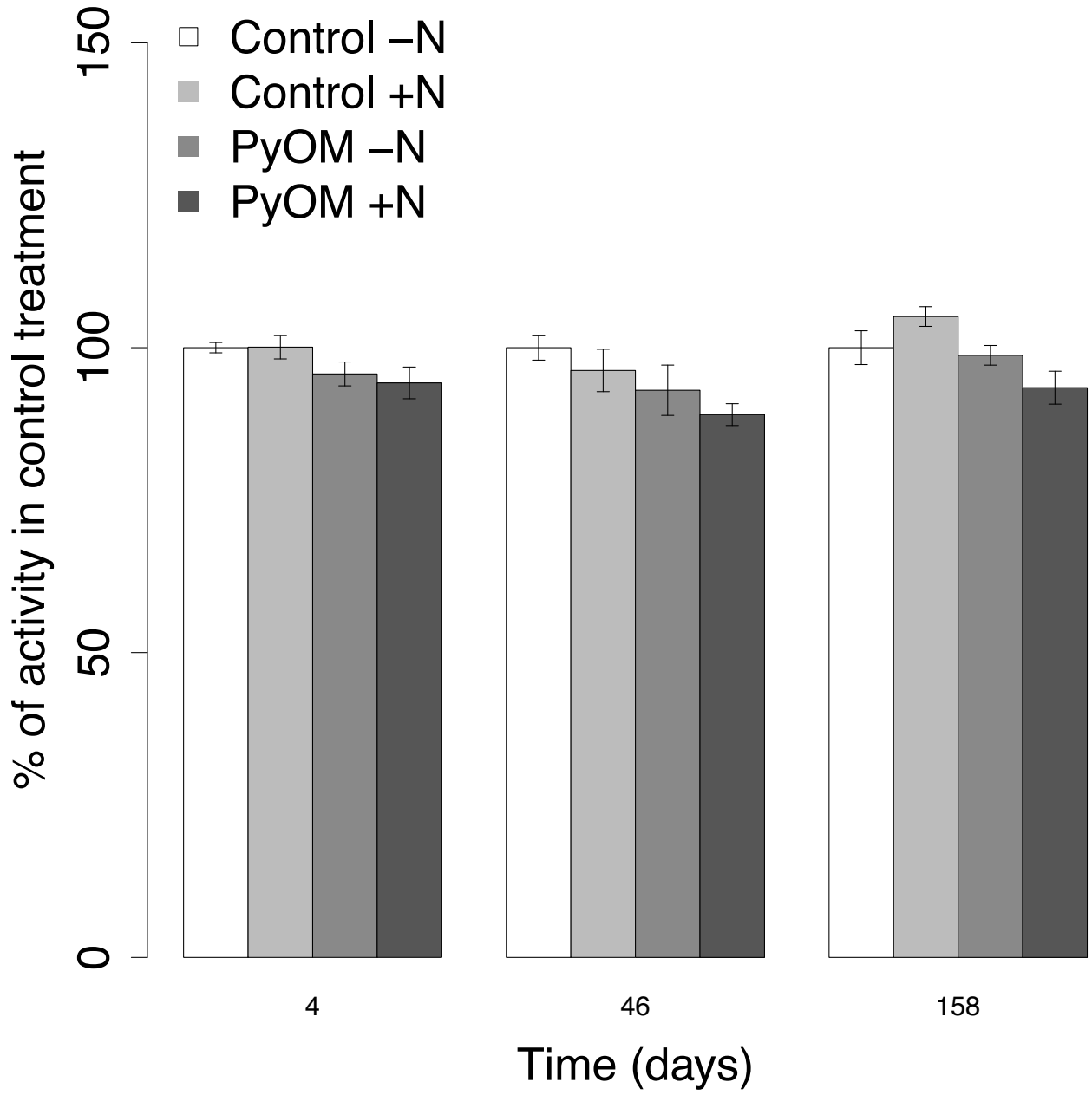
Control –N
Control +N
PyOM –N
PyOM +N
% of activity in control treatment
0
50
100
150
4
46
158
Time (days)

Figure 3

Table 1: Characteristics of the soil and PyOM, values are the average of 4 replicates ± standard error.

	pH	**Texture**			**C content**	**N content**	**C/N**	**Ashes (n=2)**	**^{15}N**	**^{13}C**	**Bulk density (in the field)**	**NO_3^-**	**NH_4^+**
		(mass %)			mg C g^{-1} dry soil	mg N g^{-1} dry soil	(w/w)	(mg g^{-1})	(atom %)		g cm^{-3}	µg N g^{-1} soil	
		Sand	Silt	Clay									
Soil	5.72±0.04	45.5±3.5	24.2±4.4	31.5±2.4	35.6±0.01	2.93±0.03	12.1		0.3664±0.0001	1.0761±0.0001	1.4	56.85±0.76	1.53±0.65
					mg C g^{-1} PyOM	mg N g^{-1} PyOM						mg N g^{-1} PyOM	
PyOM	10.02±0.01				344±3	36.87±0.06	9.3	530.4±2	0.36888±0.00005	4.33±0.01		0.05±0.02	0.4±0.1

Table 2: Total soil respiration, cumulative native organic matter mineralization, potential protease activity (substrate caseine), and cumulative PyOM mineralization. Values are average of four replicates ± standard error of the mean.

	Cumulated Soil respiration (after 158 days)	**Cumulated native organic matter decomposition (after 158 days)**	**Cumulative PyOM decomposition (after 158 days)**	**Mean Protease activity**	**Fraction of microbial biomass C derived from PyOM (after 158 days)**
	(mg C-CO_2 g^{-1} soil)	(mg C-CO_2 g^{-1} soil)	(% of initial input)	(µg tyrosine g^{-1} soil $hour^{-1}$)	%
Control -N	2.37±0.08	2.37±0.08		1.73±0.12	
PyOM input -N	2.34±0.05	2.15±0.05	4.4±0.18	1.76±0.08	0.47 ± 0.02
Control +N	2.48±0.10	2.48±0.10		1.70±0.07	
PyOM input +N	2.33±0.07	2.14±0.06	4.3±0.1	1.92±0.12	0.45 ± 0.03

Table 3: Mean residence time (MRT) calculated with the two-pool exponential decay model fitted to the mineralization dynamics corresponding to the treatments without and with N addition. Values are average of four replicates ± standard error.

	MRT labile	**MRT resistant**	**fast pool fraction**
	(days)	(years)	(% of initial PyOM-C)
without N	1.92 ± 0.03	40.35 ± 0.31	3.4 ±0.1
with N	1.92 ± 0.02	39.79 ± 0.33	3.3 ± 0.1