Distribution of black carbon and black nitrogen in physical
soil fractions from soils seven years after an intense forest
fire and their role as C sink

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Abstract

After vegetation fires, incorporation of pyrogenic organic matter (PyOM) into
soil organic matter (SOM) shifts its composition toward higher aromaticity and
to an increase of N-heterocyclic constituents, formerly introduced as black
nitrogen (BN). To investigate the medium-term impact of these shifts on the
quality of SOM and its role as an important C sink, the A horizon from soils of
the fire-prone Sierra de Aznalcóllar (Southern Spain) were sampled 4 weeks

and 7 years after a severe fire. The solid-state ^{13}C and ^{15}N nuclear magnetic resonance (NMR) spectra of the samples obtained 4 weeks after the fire indicated quick incorporation of PyOM into SOM. Correspondingly, pyrrole-type N dominated the organic N fraction. Seven years after the fire, the aryl C contribution decreased from 46% to 23% of organic C, although it was still higher at the burnt sites than in the unburnt reference soil (16%). This fast loss of PyOM may be due to erosion, transport into deeper soils or microbial decomposition. The contribution of the latter is in line with former incubation experiments with burnt soils from the same area. Comparably, in the recovered soil, BN was almost completely substituted by amide N. Studying the partitioning of PyOM among the density and particle size fractions of the soils, indicated that after medium-term recuperation, most PyOM occurred in the free and occluded particulate OM fractions (fPOM and oPOM). The low protection against microbial degradation and the low density of these fractions may explain the high PyOM losses from the studied soils either by decomposition or by transport. We suggested that formation of PyOM-soil mineral associates represents an important step for reducing losses of fire-derived charcoal due to biochemical mineralization and thus to its sequestration in soils.

Keywords: particulate organic matter, particle size fractionation, solid-state NMR, biochemical recalcitrance of pyrogenic organic matter.

Highlights:

- After fire, pyrogenic organic matter (PyOM) occurs preferentially as particulate OM.
- Interaction with clay seems to be important for the survival of PyOM in A horizons.
- Only the PyOM in the clay fraction contributes to increased C sequestration in A horizons.
- Aged PyOM in soils represents only small fraction of the original charcoal input.

Graphical Abstract:

Introduction

During a wildfire event, the vegetation and its litter suffer structural change such as condensation and cyclization of organic structures (Almendros et al., 2003). The new mixture of heat-altered biomacromolecules belongs to a continuum (Masiello, 2004) of pyrogenic organic matter (PyOM), the structure and properties of which depends on the combustion conditions and its source (Knicker, 2010). In soils, PyOM is commonly seen as an important C sink due to its highly aromatic nature. This was supported by Vasilyeva et al. (2011) who observed that, after 55 years in fallow Chernozems, the quantity and quality of PyOM did not change. On the other hand, recent laboratory incubation studies have reported mean resident times of PyOM in soils in the range of decades to centuries (Hilscher et al., 2009; Santos et al., 2012; Bird et al., 2015) but not millennia as previously claimed (Saldarriaga and West, 1986; Kuzyakov et al., 2009). However such long MRTs were observed for biochars, produced via pyrolysis at temperatures above 550°C (Zimmerman, 2010). Incubation experiments by Nocentini et al. (2010) and De la Rosa et al. (2018) showed that charcoal can be mineralized by microorganisms. Further indications for biochemical degradation of PyOM were given by Hilscher and Knicker (2011) who performed laboratory incubation experiments with grass-derived charcoal and found evidence of microbial attack of the aromatic network, with a concomitant increase in carboxyl C. In fact, analysis of deep

horizons of Ferralsols under Cerrado, revealed that these oxidized aromatic ring structures can be transported into deeper soil horizons where they accumulate (Velasco-Molina et al., 2016). Abiven et al. (2011) reported that aged charcoal (10 years) released 40-55 times more benzene polycarboxylic acids (BPCAs) than fresh charcoal, possibly due to the higher oxidation state of that aged sample. Evidence Biochemical degradation of PyOM in natural soils has been obtained for soils from a fire-prone region in the Sierra de Aznalcóllar in Southern Spain. Here, the turnover time of PyOM was only slightly higher than those determined for their humified SOM (Knicker et al., 2013). Analysis of the soils 7 years after the last fire confirmed rapid recuperation of most soil parameters to the pre-fire values. Comparably, the aromaticity was only slightly higher in the fire-affected soils than in the unburnt counterparts, indicating that little pyrogenic organic C (PyOC) survived the postfire recovery time (López-Martín et al., 2016b).

Only recently, the PyOM research community has recognized that the so called black nitrogen (BN), composed of N-containing heterocyclic aromatic structures represents an important fraction of the black carbon (BC) pool (Knicker et al., 2008; Knicker, 2010). It is formed by cyclization of biogenic organic-N such as peptides and amino sugars into pyrrole-type components. Whereas early studies assumed significant recalcitrance of these structures (Knicker and Skjemstad, 2000), newer incubation studies with ¹⁵N-enriched material

provided evidence for their biochemical “degradability” and the subsequent use of the mobilized N for plant growth (de la Rosa and Knicker, 2011). Experiments using ¹⁵N-enriched PyOM as a soil amendment in pot experiments further evidenced the incorporation of BN-derived N into the peptide pool of SOM (López-Martín et al., 2016a). Whereas our understanding of how and when BC is degraded in soils is still insufficient, even less is known about the fate and stability of BN during aging of PyOM. The latter is assumed to provide low biochemical accessibility mainly due to its highly aromatic structure. However, comparable to SOM, it may also be protected from decomposition with physical mechanisms (Skjemstad et al., 1996; Sollins et al., 1996) either by, encapsulation within a hydrophobic network (Knicker and Hatcher, 1997), adsorption to the mineral phase entrapment into micropores (Mayer, 1994) or occlusion within aggregates (Skjemstad et al., 1996; Sollins et al., 1996). Based on the latter concepts, density and physical fractionation are powerful methods for distinguishing OM pools that differ in their degradability (Baldock et al., 1992; von Lützow et al., 2007).

Accordingly, the free particulate OM (fPOM) is only very slightly degraded and does not interact with the mineral phase (unprotected C pool) (Sohi et al., 2001; Sollins et al., 2006; Cerli et al., 2012). The occluded particulate OM (oPOM) shows a greater degree of degradation and is protected within macroaggregates (Kölbl and Kögel-Knabner, 2004; Cerli et al., 2012). Whereas, both the fPOM

and oPOM represent labile pools with turnover times from days to years, turnover times from years to centuries were observed for material associated with the fine fractions (Wagai et al., 2015). However, it has to be kept in mind that fresh microbial material can also contribute to the fine fractions (Clemente et al., 2011; Plaza et al., 2013).

Studies of Australian soils demonstrated that PyOM is preferentially associated with the fine fraction (Skjemstad et al., 1999), which may protect PyOM from degradation or loss by transport due to their association with minerals. On the other hand Singh et al. (2014) report that 10 months after PyOM addition to a soil, the fPOM separate still contains the highest yields of PyOM. Heckman et al. (2014) found preferential accumulation of PyOM into the oPOM fraction. Comparably, during a short term incubation experiment, Hilscher and Knicker (2011) using dual-isotopically (^{15}N , ^{13}C) labelled PyOM, recovered most of the label in the POM fraction, although, in spite of the short timespan of the experiment, some of the PyOM residues were already incorporated into the mineral-associated phase. However, Liang et al. (2008) evidenced physical protection by way of superficial interaction between PyOM and minerals, but suggested that chemical recalcitrance was more important.

In order to bring some more light on the stability of BC, and in particular BN in the A horizon of burnt soils, we followed the alteration of the composition of SOM from topsoils of a fire-prone region in Southern Spain during post-fire

recuperation. Soil samples were taken 4 weeks and 7 years after the last fire event, and characterized by solid-state ^{13}C and ^{15}N NMR spectroscopy. By studying the partitioning of BC and BN among the density and particle size fractions of the soils sampled after 7 years of recuperation, we hoped to obtain more information about the impact of SOM-mineral interactions on PyOM stabilization.

1. Material and methods

1.1. Site description

The study site is in the National Park of Sierra Aznalcóllar in Southern Spain, which was affected by wildfires in 1996 and 2004. The latter destroyed ca. 30,000 ha of forest. A more detailed description of the site, the fire history of the region and the studied samples is given in López-Martín et al. (2016b). Here, the impact of spatial inhomogeneity of the sampling area on the variability of soil parameter and SOM composition is discussed. Note that for consistency reasons, the sample codes used in the former publication were maintained.

The soils are classified as Leptosols and Cambisols (IUSS Working Group WRB 2014). Before the last fire, the covering vegetation was pine (*Pinus pinaster* and *Pinus pinea*) and *Quercus* (*suber* and *ilex*) forests, but the 2004 fire largely

destroyed the vegetation. The first sample set was used to describe the short-term impact of fire on the SOM decomposition of bulk soils and derived from a sampling campaign conducted 4 weeks after the fire event in August 2004. The respective samples are marked with the code 04. The material was taken from the first 5 cm of a fire-unaffected (sample code U) mineral soil (A horizon) under pine (sample code P; sample AZ04PU) and of the burnt (sample code B) mineral soils under *Quercus* (sample code Q; AZ04QB), respectively, after removal of the litter or ash layer (Knicker et al., 2013). A further composite sample derived from a doubly burnt (sample code DB) Cambisol close to a pine which survived the fires in 1996 and in 2004 (AZ04PDB). In June 2011, these locations were visited again. At each site, a square with a side length of 15 m was randomly chosen and material was taken from the first 5 cm of the A horizon from the four corners and the center after removing the litter layer (López-Martín et al., 2016b). At the site under oak, the trees, which survived the fire in 2004 had recovered, together with a dense shrub cover. For the present study, out of the five samples only the two with the biggest difference with respect to quality and quantity of the bulk SOM were selected for physical size fractionation.

One derived from a place under a shrub (AZQBd) and the second from a scarcely covered space (AZQBa). At the doubly burnt site, most of the burnt trees were removed and maquia (*Cistus ladanifer*, *Retama sphaerocarpa*) had developed. Here again, based on pre-experiments (López-Martín et al., 2016b),

3 of the 5 samples taken from the corner and the center of the square (AZPDBc,d,e) were chosen for further analysis according to detectable differences with respect to OM quality and quantity.

At a further sampling site, formerly under pine (AZPB), the remaining tree trunks and their roots were removed after the fire and the area was terraced for restoration. Again, from the five samples collected at the corners and the center of a square, only three were used for the present study. At this site, considerable amounts of charcoal particles were visually detected during sampling. Most likely, they originate from an unregistered fire, which occurred between 2004 and 2011, or from charred wooden hunks that had disintegrated and were incorporated into the soil during the restoration work.

A third sampling was performed in December 2011. Here, composite samples of sites under *Quercus*, which were burnt only once in 2004 (AZQB1) and twice in 1996 and 2004 (AZQDB1), were probed. A composite sample from an unburnt soil under *Quercus* (AZQU3) was used as a reference.

The soils from the first sampling in 2004 were air-dried, whereas those taken in 2011 were dried at 40 °C. All samples were sieved through a mesh of $\phi < 2$ mm to provide the fine earth and were stored in the dark until further analysis. The soil texture was determined with the Bouyoucos hydrometer method (Bouyoucos, 1936) and the textural classification was carried out (Table 1) according to the world reference base for soil resources 2006 (FAO, 2006).

1.2. Soil fractionation

1.2.1. Density fractionation

In order to reveal the medium-term fate of BC and BN, the topsoil samples taken in 2011 were subjected to a density fractionation procedure to separate the fPOM, oPOM and the mineral associated organic fraction (MAF) (Golchin et al., 1994b; Sohi et al., 2001). Briefly, 30 g fine dry earth ($\phi < 2$ mm) were weighted in a glass recipient and slowly covered with 200 ml sodium polytungstate (SPT; $3\text{Na}_2\text{WO}_4 \cdot 9\text{WO}_3 \cdot \text{H}_2\text{O}$) solution with a density of 1.8 g/cm^3 using a Pasteur pipette. After standing overnight, the floating fPOM was separated from the sediment, comprising MAF, by soaking and collected it in a side-arm flask using a vacuum system. Immediately afterwards, the fPOM was washed with deionized water ($\leq 2 \text{ }\mu\text{S/cm}$) using ultrafiltration through a glass fiber filter (142 mm diameter, $0.22 \text{ }\mu\text{m}$, Millipore) until the solution reached an electrical conductivity of $\leq 5 \text{ }\mu\text{S/cm}$.

The sediment was resuspended in a polyethylene bottle with SPT with a density of 1.8 g/cm^3 . The suspension was subjected to sonication with a probe, submerged to 15 mm depth in order to disrupt the aggregates and separate the oPOM from the mineral phase. The applied energy input was 400 J/ml by ultrasound (UP 400S ultrasonic processor, Hielscher Ultrasound Technology) and was adjusted according to calibration with a clay dispersion curve. The bottle was submerged in an ice bath to avoid thermal alteration of the OM. The

output of ultrasonic energy was carried out by calorimetric calibration and fixed at 26.28 J/s. The needed time, t , was calculated according to the volume of the sample, V (ml), as follows:

$$t = V \times (440 \text{ J/ml}) / (26.28 \text{ J/s})$$

After dispersing, the oPOM floating on SPT was recovered after centrifugation for 10 min at 4000 rpm and subsequent decanting the liquid phase together with the floating OM. Cleaning of the oPOM was achieved by vacuum filtration through a glass fiber filter and rinsing with deionized water until the solution reached 5 $\mu\text{S/cm}$. Lastly, the sediment remaining in the polyethylene bottle represents the MAF. In order to remove residual SPT, this fraction was repeatedly washed with deionized water until the electrical conductivity reached 50 $\mu\text{S/cm}$. All fractions were freeze-dried and stored in the dark for further analysis.

1.2.2. Particle size fractionation of MAF

The recovered MAF was separated into sand-size (2000-63 μm), coarse silt-size (63- 20 μm) and fine (< 20 μm) fractions. Therefore, 10 g dry MAF were suspended for 1 h in 50 ml deionized water and then wet-sieved.

1.3. C and N analysis

The bulk soils, fPOM, oPOM, MAF and the particle size fractions were analyzed for their total C and N content in triplicate using an elemental analyzer (Carlo-Erba EA-1108-CHNS). All samples were free of carbonate, so the total C concentration was taken as the organic carbon (C_{org}).

1.4. Soil demineralization

Prior to solid-state ^{13}C and ^{15}N NMR spectroscopy, the bulk soils and the particle size fractions were demineralized 4 times with 10% (v/v) of hydrofluoric acid (HF) according to Gonçalves et al. (2003) in order to concentrate the SOM and remove paramagnetic ions. Finally, the samples were freeze-dried. Earlier analysis of the impact of this treatment on the SOM composition in soils including those of the studied area, clearly evidenced that no preferential extraction is induced by this demineralization technique (Knicker, 2011). Differences between the intensity distribution of the solid-state ^{13}C NMR spectra before and after the demineralization were shown to be caused by the selective impact of paramagnetics on the cross polarization efficiency of certain C groups in the untreated samples. For most of the bulk soils, the C-loss due to HF-treatment was lower than 10%.

1.5. Solid-state ^{13}C and ^{15}N NMR spectroscopy

The cross polarization (CP) magic angle spinning (MAS) ^{13}C NMR spectra of the HF-treated bulk soils and physical particle size fractions were acquired

with a Varian 7.05 T Unity Inova 300 MHz and a Bruker Avance III HD 400 MHz wide bore NMR spectrometer. The first was equipped with a broadband double resonance probe for rotors with a diameter of 6 mm, the later with a triple resonance probe for rotors with a diameter of 4 mm. In both cases, a ramped ^1H pulse was applied during the contact time of 1 ms, using a pulse delay time of 300 ms. The spinning speeds of the rotors were 8 and 14 kHz, respectively. For quantification, the following five chemical shift regions were integrated: alkyl C (45 to 0 ppm), *N*-alkyl C (60 to 45 ppm), *O*-alkyl C (110 to 60 ppm), aryl C (160 to 110 ppm), carbonyl C (220 to 160 ppm) (Knicker, 2011). The intensity of the spinning side bands of the aryl C region between 325 and 300 ppm and 0 and -50 ppm were added to that of the aryl C (Knicker et al., 2005). The CPMAS ^{15}N NMR spectra were obtained with a widebore Bruker Avance III 600 MHz NMR spectrometer and spinning the 4-mm rotor at a speed of 15 kHz, a widebore Bruker Avance III HD 400 MHz equipped with a double resonance probehead for 7 mm rotors and a widebore Varian 7.05 T Unity Inova 300 MHz with a rotor of 6 mm. For the latter, the spinning speeds were 6 and 8 kHz, respectively. In the first two cases, a pulse delay of 200 ms and a contact time of 0.7 ms were applied. In the experiment with the Varian instrument, the pulse delay time was 400 ms and the contact time 1 ms. The ^{15}N NMR spectra were not quantified due to the low signal/noise ratios.

3. Results and discussion

3.1. Composition of bulk soils

A detailed description and a statistical evaluation of the impact of spatial inhomogeneity of the study area on the intensity distribution of the solid-state ^{13}C NMR spectra of the bulk soils has been reported earlier (Knicker et al., 2013; López-Martín et al., 2016b). In brief, the spectra of the unburnt soil under pine, sampled in 2004 (AZ04PU) and of the unburnt soil under *Quercus* taken in 2011 (AZQU3) are dominated by the signal intensity in the chemical shift region of *O*-alkyl C, which derives mainly from carbohydrate of biomass residues (Fig. 1). The high alkyl C content (45 to 0 ppm) of > 27% (pine) and 30% (*Quercus*) of the total ^{13}C intensity indicates advanced humification of the SOM. Here, the term humification is defined according to the International Humic Substance Society (IHSS, 2007). The proportion of aryl C (160 and 110 ppm) of 21% (pine) and 16% (*Quercus*) is in the range expected for soils without or with low contribution of charred material (Knicker et al., 2007). The clear signals in the region between 160 and 140 ppm (*O/N*-aryl C) indicate the presence of lignin residues. Inspecting the respective ^{15}N NMR spectrum allows the assignment of almost the entire organic N to amide N, which confirms a mainly biogenic origin of the SOM (Fig. 1).

At the sampled site, the wildfire in 2004 resulted in a considerably increase in aromaticity, both for C_{org} and N_t . The latter is revealed by the notable contribution of ^{15}N intensity in the region assigned to pyrrolic N (-150 to - 250 ppm) in the ^{15}N NMR spectra of AZ04QB and AZ04PDB (Fig. 1). The high aromaticity of the fire-affected soil sampled 4 weeks after the fire event is best explained by a high input of charred material, although the ash layer was removed prior to sampling. The presence of PyOM was confirmed by NMR spectra obtained after subjecting the sample to strong chemical oxidation with acidic $K_2Cr_2O_7$ (Knicker et al., 2007; López-Martín et al., 2016b). Tentatively, the fast incorporation of PyOM was facilitated by the high porosity of the soils caused by the high content of gravel and stones. Seven years after the fire, the composition of the bulk soils changed considerably which is clearly expressed in a reduction in aromaticity with a concomitant increase of *O/N*-alkyl C. This indicates a substantial loss of PyOM, either by way of degradation or erosion, during the 7 years recuperation after the fire event. Another cause may be vertical transport as it was observed recently for burnt soils of the Cerrado in Brazil (Velasco-Molina et al., 2016). Rapid biochemical degradation of PyOM is in line with laboratory respiration studies indicating, under controlled conditions at 25 °C, a medium residence time of PyOM in the soil of approximately 40 years (Knicker et al., 2013). Input of fresh OM from litter and microbial residues during recuperation contributes to the masking of the remaining PyOM. However, compared with the unburnt soils, an elevation of

aromaticity in the soils with fire history was confirmed by the application of a more detailed statistical analysis (López-Martín et al., 2016b). For the samples included in the present study, the aryl C content increased from 16% of the total organic carbon of the unburnt soil under *Quercus* to 22 – 28% in the soils 7 years after fire. The respective augmentation for the soils under pine is from 21% in the unburnt soil taken in 2004 to 22-32% in the doubly burnt soils (AZPDB) (Fig. 1 and 2). The soils (AZPBc,d,e) obtained from the site at which charcoal residues were found, yielded solid-state ^{13}C NMR spectra with comparable intensity distribution to those of the soils studied 4 weeks after the intense fire. Obviously, degradation of charcoal and masking by input of fresh litter had not yet occurred to a greater extend. Thus, in contrast to the other samples, the partitioning pattern of PyOM in physical soil fractions is more likely to describe short-term effects.

With respect to the N fraction, recovery of burnt soils leads to a decrease in the intensity of the typical resonance line for BN between -145 and -250 ppm (Knicker, 2010) in favor of the amide N signal between -250 and -285 ppm (Fig. 1). Only in the ^{15}N NMR spectrum of AZPDBe, a clear shoulder can still be discerned in the region of pyrrole N. This is in line with rapid degradation of N-heterocyclic aromatics in PyOM, as recently observed in pot experiments (de la Rosa and Knicker, 2011). Note that, in spite of the high aromaticity of the samples AZPBc,d,e, only low intensity in the pyrrole N region is apparent in

their ^{15}N NMR spectra. This may be an indication that the charred residues derive from N-poor sources such as wood char rather than from charred OM of N-rich sources such as the humus layer or leaf residues.

3.2. Mass, C_{org} and N_t recovery after density and particle size fractionation

After density separation, for all soils > 90% of the mass was recovered, most of which was assignable to the mineral fraction (Table 2). An exception is AZPDBe, of which only 81% of the total mass was recovered, probably due to loss of clay during the fractionation procedure. After the particle size fractionation, the highest mass recovery was in the fraction $\phi < 20\ \mu\text{m}$ with values > 39% with the exception of AZPDBe and AZQDB1, with highest recovery (57 and 62%) in the sand size fraction. The recovery of both N_t and C_{org} after density fractionation was > 73% except for AZQBd and AZQU3 where the C_{org} recovery was 59 and 65%, respectively. The yield after particle size fractionation exceed 71% for both C_{org} and N_t with the exception of AZQDB1, with a recovery of 64% for N_t .

3.3. Distribution of C_{org} , N_t among the fractions as C_f and N_f and the respective C/N ratio

For the soils from the sites formerly under pine, most of the initial total organic carbon and total nitrogen of the bulk soil is associated with the fPOM fraction (Table 3). The relative amount of C and N recovered from C_{org} and N_t of the bulk soils as C_f and N_f in the different fractions is fairly comparable between those sites. However, lower amounts of C_f and N_f were recovered in the oPOM fraction of the doubly burnt site than in that of the singly and recently burnt soil. The opposite is true for MAF. Note that the C/N values of the fPOM and oPOM fractions of the singly burnt sites under pine are clearly higher than those of the fractions of the doubly burnt soils under pine. This may indicate that the SOM at the doubly burnt site was at a more developed degradation state. Alternatively, the singly burnt site may have received a greater input from a low-N source. Separating the MAF into sand (2000-63 μm), coarse silt (63-20 μm) and fine fraction (< 20 μm) showed that the latter represents the largest part with respect to the contribution of organic C (C_{iMAF}) and N (N_{iMAF}) in MAF (Table 4), in line with de Junet et al. (2013). The low C/N values (Table 4) of the mineral fractions are in the range expected for microbial biomass or well-humified SOM. The very low C/N ratios below 7 cannot be explained by the presence of inorganic N, since the latter should have been removed with the aqueous solution during the fractionation procedure. It is more likely that they are related to the fact that for samples with C/N values below 10 and low C and N contents even small experimental errors can notably change the calculated C/N ratios.

Compared with the soils under pine, for the soils under *Quercus* less C_f and N_f were recovered with fPOM; however, a clear trend with respect to fire history was not revealed. Whereas the different fractions from AZQBa,d show comparable C/N values to those of the unburnt composite soil AZQU3, those from AZQB1 and AZQDB1 were clearly higher. In line with the observation for the pine stand, after particle size fractionation, between 79 and 92% of the total C_{iMAF} and N_{iMAF} were recovered in the fine fraction. Thus, this fraction mainly determined the composition of MAF. The coarse silt fraction comprises < 4% and 8% of N_{iMAT} and C_{iMAT} , respectively. Comparably, the sand fraction contains < 17% of C_{iMAT} and between 8 and 16% of N_{iMAT} . A trend with fire history or stand was not observed.

3.4. Characterization of SOM fractions

The solid-state ^{13}C NMR spectra of the fPOM of almost all soils from stands under *Quercus* are dominated by signals from *O*-alkyl C, alkyl C followed by aryl C, as found by Plaza et al. (2012) and Golchin et al. (1994b) for soils with different properties and climate conditions (Figs. 2 and 3). Compared with the bulk soils, the fPOM fractions contain slightly more *O*-alkyl C and the oPOM slightly less *O*-alkyl C. This is in agreement with the fact that fPOM is composed mainly of plants debris, which is only partly degraded (Golchin et al., 1994a), whereas the material in oPOM underwent advanced microbial

degradation (Christensen, 2001; Dorodnikov et al., 2011). The alkyl C / *O*-alkyl C ratio (Table 5) suggested by (Golchin et al., 1994a; Baldock et al., 1997) as a degradation index is in line with this interpretation. Calculating the alkyl C / carboxyl C ratio points toward an increase in average chain length from fPOM to oPOM, in line with Plaza et al. (2012) who revealed an enrichment in unsubstituted aliphatic material such as lipid structures (cutin and suberin) in oPOM. Note that these patterns were observed for all the samples sets irrespective of site and fire history. Comparably, the ¹⁵N NMR spectra of all the POM fractions show no major difference (Fig. 4). The only signal which can clearly be discerned from the noise is attributed to amide N, indicating that BN did indeed not survive to a greater extent in these fractions. The C in MAF at particle sizes < 20 µm is composed mainly of *O*-alkyl C. A comparable finding was reported by Sohi et al. (2001) and Boeni et al. (2014). Comparing the relative intensity distribution in the ¹³C NMR spectra of the fine fractions with those of the oPOM fractions, a greater contribution of *O*-alkyl C was observed for all sampling sites. A relationship with stand or fire history is not evident. The alkyl C / *O*-alkyl C ratio increased in the following order fPOM, < 20 µm and oPOM (Table 5). This is in contrast to the general assumption that the most degraded SOM is associated with fine particle-size fractions. On the other hand, it is in line with Bimüller et al. (2014) who stated that, in spite of the enrichment in polysaccharides, the SOM in the fine fractions is expected to be

less bioavailable or/and accessible for microbial degradation due to the interaction of the OM with the mineral surfaces.

In the fine particle-size fractions, the carboxyl C contributes < 10% to C_f , which is in accord with the assumption that their OM has not experienced extensive microbial degradation but has a contribution from microbial residues (Six et al., 2002; Clemente et al., 2011; Clemente et al., 2012). The fact that the intensity of the methoxyl C / *N*-alkyl C (60 to 45 ppm) increased slightly, whereas the clear signals assignable to *O*-aryl C of lignin between 160 and 140 ppm almost vanished, underlines an accumulation of peptides in the mineral fraction. This is supported by their narrow C/N ratio and the dominance of the signal in the amide N region between -250 to -285 ppm of their ^{15}N NMR spectrum (Fig. 4). Accordingly, Zhou et al. (2014) suggested that the clay fraction adsorbs and protects N-rich OM such as peptides.

With respect to the contribution of aryl C, a clear impact of the fire history was observed for the samples from the stands under *Quercus* and pine (Fig. 2). Although for the bulk soils the aromaticity decreased considerably during the 7 years of recuperation (Fig. 1), the fPOM and oPOM fractions of the singly and doubly burnt site 7 years after the fire clearly showed a greater aryl C contribution than for the unburnt site AZQU3. With the exception of the AZPBc,d,e samples, the aromaticity of oPOM from the soils from the burnt sites collected in 2011 was higher than that of fPOM, but no difference between

double and single burning was apparent (Fig. 2). This is in contrast to studies of Singh et al. (2014) who found the highest PyOM contribution in the fPOM fraction. Between 62 and 86% of the aryl C of the bulk samples (C_{arylbulk}) was recovered with both POM fractions (Table 6). A preference for oPOM or fPOM was not apparent for the samples derived from the sites under *Quercus*, whereas those from the stands under pine indicated a preferential accumulation in the fPOM fraction. A closer analysis of the aryl C region of the ^{13}C NMR spectrum of the $< 20 \mu\text{m}$ fraction of the unburnt soils under *Quercus* (Fig. 2) shows an almost complete disappearance of the signal in the region between 160 and 140 ppm assignable to *O*-aryl C, as occurs in lignin. This is in accord with other reports showing that lignin residues rarely enter this fraction (Dorodnikov et al., 2011; Heckman et al., 2014) and the aromatic contribution must originate from other sources. Alternative compounds are aromatic amino acids or olefinic structures in unsaturated lipids or a charcoal contribution. Conclusively, in the case of the burnt soils, the greater aryl C contribution is best explained by way of fine PyOM residues. However, their contribution is $< 4\%$ of C_{org} in the bulk soils and $< 20\%$ of aryl C in the bulk soils (C_{arylbulk}) (Table 6). Less than 30% of C_{arylbulk} has been calculated to be associated to the coarse mineral-associated fraction.

For the soils from the pine stands, even lower amounts of C_{org} were recovered as aryl C in the $< 20 \mu\text{m}$ fractions. Note that here too, a clear signal for lignin is

not apparent in the respective ^{13}C NMR spectra, and the respective ^{15}N NMR spectra show most of their intensity in the amide-N region (Fig. 4) Between 59 and 69% of the $\text{C}_{\text{arylbulk}}$ was recovered with the fPOM fraction, leaving 15 to 18% associated with oPOM. In samples with a recent charcoal input (AZPBc,d,e) these values change to 47 to 57% for fPOM and 19 to 24% for oPOM. Less than 30% of $\text{C}_{\text{arylbulk}}$ can be attributed to the coarse mineral-associated fraction (2000-20 μm). However, note that at both pine sites, 60 to 70% of C_{org} of the bulk soils was recovered with fPOM. Our results are in accord with Soucémarianadin et al. (2014), showing that in fire affected soil the charcoal can be found in the fPOM fraction. Heckman et al. (2014), on the other hand, identified the greatest charcoal contribution in the oPOM fraction of soils derived from a temperate forest ecosystem under a conifer stand. They concluded that fire was the main driver there of the formation of the occluded fraction and that the selective preservation of PyOM was the main C stabilization mechanism in the oPOM fraction. Other studies report a preferential accumulation of BC in the light fraction ($\rho < 2 \text{ mg/m}^3$) of agriculturally used top soils (Brodowski et al., 2007; Vasilyeva et al., 2011). They showed that with increasing soil depth, the BC content of the light fraction decreased, whereas that of the more dense fraction remained constant, possibly because the latter experienced better protection due to interaction with the mineral phase. Protection by means of mineral-BC interaction was also suggested by Vasilyeva et al. (2011) and Rovira et al. (2009). Liang et al.

(2008), examining the stability of BC in an Anthrosol proposed further that chemical recalcitrance might have been more important than physical protection at their study site. Stabilization of PyOM via mineral-BC interaction in the fine size particles cannot be confirmed by our study (Table 6) since < 20% of C_{arylbulk} was recovered with the fraction < 20 μm (clay, fine and medium silt). It seems that the majority of char particles introduced into the soil after the fire was too large or too unreactive to enter the medium and fine silt fraction. This may have increased their vulnerability to erosion, vertical transport or biochemical degradation. Considering that, for PyOM in these soils, a relative short mean residence time in the range of centuries and less was determined (Knicker et al., 2013) ongoing recuperation of the ecosystem may lead to further degradation and thus disintegration of PyOM associated with POM. On one hand, the degradation and the loss of POM would lead to a relative enrichment of mineral-associated PyOM, but also to the formation of smaller units, which can enter the fine particle-size fractions and contain sufficient functional groups for providing adsorption sites for interaction with the mineral phase.

4. Conclusions

This investigation confirmed low longevity for PyOM in fire-affected soils from the Sierra de Aznalcóllar. However, after 7 years of recuperation from the last fire, the presence of PyOM was still revealed by way of higher aryl C content

in the SOM of burnt soils than of unburnt soils. The observation that most aromatic structures were recovered in the POM may be evidence for significant chemical recalcitrance of the remaining charred residues since POM is only weakly protected by mineral interaction and is assumed to be rapidly degraded by microorganism. On the other hand, the fast loss of aromaticity during the 7 years of recuperation argues against this interpretation of chemical recalcitrance. Possibly, the lack of mineral-PyOM association may have allowed rapid PyOM degradation or loss due to transport. However, the functional groups of some partially oxidized PyOM interacted with the mineral surface before being completely metabolized or lost. Physically protected by this interaction, the PyOM may be able to survive in the A horizon on a long-term scale. Following this argument, the BC detected in the fine fraction of Chernozems (Vasilyeva et al., 2011) and North American (Glaser and Amelung, 2003) and Australian grassland soils (Skjemstad et al., 1999) would be formed by such residual PyOM, and represent only a very small fraction of the original charcoal input which accumulated with each fire occurring over thousands of years.

A further interesting conclusion is based on the observation that there was no indication for the survival of heterocyclic N in soils 7 years after the fire, with the exception of the bulk soils AZPDBe and AZPBc. Thus, although BC was still identified, the relative contribution of BN to the N_t diminished under those discernable from the noise in the respective solid-state ^{15}N NMR

spectra, whereas the proportion of amide N increased. Possibly, charged N groups in pyrrole-type structures are better accessible to enzymes and microorganism than condensed polybenzene rings. Consequently, aromatic N would decrease below its detection level whereas aryl C from N-free aromatic structures still contribute to the aryl C intensity in the ^{13}C NMR spectra. However, the question to which extent the content of BN in PyOM determines the stability of the latter in soil and thus its role for C sequestration has to be answered in future research.

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742

743 FIGURE CAPTIONS

744 **Fig. 1.** Solid-state ^{13}C NMR and ^{15}N NMR spectra of HF-treated unburnt (U),
745 burnt (B) and doubly burnt (DB) Leptosol and Cambisol sampled in 2004 (4
746 weeks after fire) and in 2011 (7 years after last fire). The soils were formerly
747 under pine forest (AZ04PU, AZ04PDB), AZPDBc,d,e and AZPBc,d,e) and under
748 *Quercus* stands (AZ04QB, AZQU3, AZQB1, AZQDB1 and AZQa,b).

749 **Fig. 2.** Intensity distribution of the solid-state ^{13}C NMR spectra of HF-treated
750 bulk soils from Cambisols, and free and occluded particulate organic matter
751 (fPOM, oPOM) obtained after density fractionation (1.8 g/cm^3) and their fine-
752 particle size fraction ($< 20\text{ }\mu\text{m}$). The soils were sampled 7 years after a severe
753 fire event and were located under *Quercus* stands which were unburnt
754 (AZQU3), singly burnt (AZQB1, AZQa,d) and doubly burnt (AZQDB1) and from
755 soils under pine before they were burnt twice (AZPDBc,d,e) or once
756 (AZPBc,d,e).

757 **Fig. 3.** Solid-state ^{13}C NMR spectra of HF-treated fine particle-size fractions ($<$
758 $20\text{ }\mu\text{m}$) and the free and occluded POM (fPOM, oPOM) after density
759 fractionation (1.8 g/cm^3) from Cambisols sampled 7 years after a fire. The latter
760 were located under *Quercus* forest which were unburnt (AZQU3), burnt once
761 (AZQB1, AZQa,d) and doubly burnt (AZQDB1). Further material was obtained

from soils under pine before they were burnt once (AZPBc,d,e) or twice (AZPDBc,d,e).

Fig. 4. Solid-state ^{15}N NMR spectra of free and occluded POM fractions (fPOM, oPOM) and HF-treated fine particle-size fraction ($< 20\ \mu\text{m}$) from Cambisols. They were sampled in 2011, 7 years after a wildfire of a singly (AZQB1, AZQBd) and doubly (AZQDB1) burnt *Quercus* forest and a pine forest (AZPBc).

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Table 1

Texture, organic C (C_{org}) and total N (N_t) content of the A horizon from Leptosols under an unburnt pine (AZ04PU) and a burnt (AZ04QB) *Quercus* forest, collected in 2004, 4 weeks after an intense fire, and topsoils derived from an unburnt (AZQU3), singly (AZQB1, AZQBa,d) and doubly (AZQDB1) burnt Cambisol under *Quercus* and a singly burnt Cambisol under pine (AZPBc,d,e). These soils are from the Sierra de Aznalcóllar, Southern Spain and were obtained in 2011, 7 years after a severe fire (nd, no data).

	Texture	C_{org} (mg/g)	N_t (mg/g)
AZ04PU	Clay-loam	$22 \pm \text{nd}$	$1.5 \pm \text{nd}$
AZ04QB	Loam	$70 \pm \text{nd}$	$3.6 \pm \text{nd}$
AZ04PDB	Clay-loam	$51 \pm \text{nd}$	$3.7 \pm \text{nd}$
AZQU3	Sandy-loam	69.9 ± 0.1	3.9 ± 0.0
AZQB1	Sandy-loam	58.7 ± 0.0	2.6 ± 0.0
AZQDB1	Sandy-loam	23.1 ± 0.0	0.8 ± 0.0
AZQBa	Sandy-loam	15.7 ± 0.1	1.8 ± 0.0
AZQBd	Loam	63.4 ± 0.3	3.2 ± 0.0
AZPDBc	nd	54.4 ± 0.2	2.6 ± 0.0
AZPDBd	Loam	70.6 ± 0.0	3.8 ± 0.0
AZPDBe	Sandy-loam	53.3 ± 0.4	2.3 ± 0.0
AZPBc	nd	127.5 ± 0.1	4.6 ± 0.0
AZPBd	Clay-loam	101.5 ± 0.2	1.9 ± 0.1
AZPBe	Loam	34.7 ± 0.1	1.2 ± 0.0

Table 2

Recovery after density fractionation (sodium polytungstate at 1.8 g/cm³) from unburnt (AZQU3), singly (AZQB1, AZQBa,b) and doubly burnt (AZQDB1) soils under *Quercus* and under doubly (AZPDBc,d,e) and singly (AZPBc,d,e) burnt Cambisol under pine before a severe fire event. The soils were sampled after 7 years of the last fire. The recoveries of the particle-size fractions are given relative to the yields of the mineral associated fraction (MAF). The particulate organic matter (POM) fractions are composed of the free POM (fPOM) and occluded POM (oPOM).

Sample	Recovery (%) ^a					
	Density fractionation			Particle-size fraction		
	fPOM	oPOM	MAF	(µm)		
				2000-63	63-20	< 20
AZQU3	8	5	82	38	10	52
AZQB1	7	7	83	38	6	55
AZQDB1	3	4	90	62	13	23
AZQBa	4	2	94	37	6	55
AZQBd	9	2	80	43	5	49
AZPDBc	16	2	80	33	4	62
AZPDBd	14	2	79	32	6	39
AZPDBe	15	7	60	57	9	34
AZPBc	31	10	55	20	7	73
AZPBd	16	5	71	28	6	65
AZPBe	6	2	89	27	6	65

^a Recovery of particle-size fractions given relative to yield of MAF.

Table 3 Recovery of organic carbon (C_{org}) and total N (N_t) as carbon (C_f) and nitrogen (N_f) of the free (fPOM), occluded (oPOM) particle organic matter fractions and the mineral-associated organic fraction (MAF) from unburnt (AZQU3), singly (AZQB1, AZQBa,b) and doubly burnt (AZQDB1) Cambisols under *Quercus* and from doubly (AZPDBc,d,e) and singly (AZPBc,d,e) burnt Cambisols under pine sampled 7 years after the fire.

Sample	fPOM			oPOM			MAF		
	C_f of C_{org} (%)	N_f of N_t (%)	C/N	C_f of C_{org} (%)	N_f of N_t (%)	C/N	C_f of C_{org} (%)	N_f of N_t (%)	C/N
AZQU3	46.0 ± 0.3	28.7 ± 4.1	24 ± 3	33.8 ± 1.0	25.3 ± 2.5	20 ± 1	21.2 ± 1.0	46.7 ± 5.9	7 ± 1
AZQB1	31.7 ± 1.6	17.9 ± 3.2	36 ± 6	48.3 ± 7.2	28.1 ± 5.5	35 ± 3	20.0 ± 0.2	54.0 ± 6.6	7 ± 1
AZQDB1	32.7 ± 1.4	16.1 ± 0.0	39 ± 5	43.9 ± 0.6	26.3 ± 1.3	34 ± 4	24.1 ± 1.5	57.6 ± 6.8	11 ± 2
AZQBa	41.8 ± 4.0	17.1 ± 2.9	26 ± 3	26.9 ± 1.5	11.4 ± 1.7	26 ± 2	31.3 ± 0.0	71.6 ± 12.1	5 ± 1
AZQBd	58.5 ± 6.4	30.4 ± 5.0	26 ± 1	11.4 ± 0.4	6.8 ± 0.9	24 ± 2	30.1 ± 3.2	62.7 ± 6.5	7 ± 1
AZPDBc	67.7 ± 1.7	40.5 ± 5.7	29 ± 4	12.9 ± 0.3	8.5 ± 0.9	27 ± 3	19.4 ± 0.3	51.1 ± 3.6	7 ± 1
AZPDBd	63.1 ± 1.1	36.5 ± 7.4	31 ± 5	15.0 ± 0.4	9.9 ± 1.1	33 ± 3	21.9 ± 1.6	55.6 ± 7.5	7 ± 0
AZPDBe	71.4 ± 2.1	47.3 ± 7.2	34 ± 5	16.7 ± 0.2	12.1 ± 2.3	32 ± 5	11.9 ± 2.0	40.7 ± 3.8	7 ± 1
AZPBc	65.6 ± 1.7	50.2 ± 7.0	40 ± 5	26.5 ± 2.4	21.7 ± 5.3	38 ± 5	7.9 ± 0.2	28.1 ± 3.2	9 ± 1
AZPBd	62.0 ± 1.6	39.8 ± 5.1	47 ± 5	26.9 ± 0.5	17.8 ± 2.1	45 ± 4	11.1 ± 0.6	42.4 ± 3.9	8 ± 3
AZPBe	60.5 ± 2.3	26.6 ± 4.9	47 ± 6	25.3 ± 0.8	9.5 ± 1.1	55 ± 5	14.2 ± 1.0	63.9 ± 12.1	5 ± 1

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Table 4

Recovery of organic carbon (C_f) and nitrogen (N_f) from the organic carbon (C_{iMAF}) and total nitrogen (N_{iMAF}) of MAF in the sand-size (2000-63 mm), coarse silt-size (63-20 mm) and fine-size ($< 20 \mu\text{m}$) fractions from unburnt (AZQU3), singly (AZQB1, AZQBa,b) and doubly burnt (AZQDB1) soils under *Quercus* and from Cambisols under pine after two (AZPDBc,d,e) and one (AZPBc,d,e) forest fires and C/N for the different fractions. The

Sample	2000-63 mm			63-20 mm			< 20 μm		
	C_f of C_{iMAF} (%)	N_f of N_{iMAF} (%)	C/N	C_f of C_{iMAF} (%)	N_f of N_{iMAF} (%)	C/N	C_f of C_{iMAF} (%)	N_f of N_{iMAF} (%)	C/N
AZQU3	6.3 ± 0.8	8.4 ± 0.0	5 ± 0	2.9 ± 0.1	3.0 ± 0.4	7 ± 1	89.2 ± 6.6	91.6 ± 2.6	7 ± 0.3
AZQB1	13.2 ± 1.7	11.7 ± 2.9	10 ± 3	8.4 ± 1.2	4.4 ± 0.5	17 ± 0	78.6 ± 3.8	83.9 ± 2.0	8 ± 0.3
AZQDB1	16.8 ± 2.8	14.6 ± 7.5	13 ± 1	7.9 ± 1.4	4.0 ± 1.7	27 ± 8	74.9 ± 1.9	86.6 ± 0.0	11 ± 0.3
AZQBa	13.8 ± 1.5	15.5 ± 3.5	5 ± 1	5.8 ± 0.2	3.9 ± 0.0	8 ± 0	80.1 ± 7.1	79.1 ± 14.7	6 ± 0.9
AZQBd	11.2 ± 1.3	16.0 ± 4.6	6 ± 2	2.5 ± 0.1	2.9 ± 0.0	7 ± 0	86.6 ± 1.2	80.9 ± 3.7	9 ± 0.3
AZPDBc	9.4 ± 0.2	10.6 ± 0.0	6 ± 0	4.0 ± 0.5	1.8 ± 0.2	15 ± 1	86.1 ± 5.3	87.8 ± 5.6	7 ± 0.0
AZPDBd	10.4 ± 2.5	14.6 ± 4.3	6 ± 2	4.0 ± 0.3	1.8 ± 0.7	11 ± 3	83.2 ± 3.9	82.6 ± 9.0	8 ± 0.6
AZPDBe	26.4 ± 3.0	30.4 ± 8.5	7 ± 1	8.0 ± 0.1	5.2 ± 0.0	12 ± 0	66.1 ± 0.6	64.5 ± 2.1	8 ± 0.2
AZPBc	5.3 ± 0.6	4.8 ± 0.6	8 ± 1	3.4 ± 0.3	3.2 ± 0.3	8 ± 0	91.7 ± 5.5	92.0 ± 5.9	8 ± 0.0
AZPBd	6.9 ± 0.3	8.2 ± 1.0	7 ± 1	3.2 ± 0.3	1.5 ± 2.2	8 ± 0	89.5 ± 7.7	90.3 ± 6.4	8 ± 0.1
AZPBe	12.7 ± 3.6	8.2 ± 1.1	7 ± 3	3.1 ± 0.1	3.4 ± 0.4	4 ± 0	84.5 ± 0.0	88.4 ± 0.0	4 ± 0.0

samples were taken 7 years after the last fire.

Table 5

Alkyl C/O-alkyl C and alkyl C/carboxyl C ratios of free (fPOM), occluded (oPOM) particle organic matter and fine fraction (< 20 µm) of the mineral associated organic matter derived from unburnt (AZQU3), single (AZQB1, AZQBa,b) and double burnt (AZQDB1) soils under *Quercus* and from double (AZPDBc,d,e) and single (AZPBc,d,e) burnt Cambisols under pine. All soils were sampled seven years after the last fire from the Sierra de Aznalcóllar, Southern Spain.

Samples	Alkyl C/O-alkyl C			Alkyl C/carboxyl C		
	fPOM	oPOM	< 20 µm	fPOM	oPOM	< 20 µm
AZQU3	0.8	1.0	0.7	4.2	3.7	4.0
AZQB1	0.6	1.3	0.8	3.7	4.7	4.4
AZQDB1	0.7	1.3	0.8	3.7	4.8	4.3
AZQBa	0.8	1.5	1.0	3.2	3.4	3.1
AZQBd	0.8	1.0	0.7	2.9	2.9	3.4
AZPDBc	0.7	1.1	0.8	3.6	3.4	2.5
AZPDBd	0.7	1.5	0.8	3.5	3.9	2.9
AZPDBe	1.3	1.4	1.4	2.8	1.8	2.6
AZPBc	0.6	1.1	0.8	2.0	2.4	2.6
AZPBd	0.7	1.3	0.9	2.7	3.6	3.4
AZPBe	0.7	1.1	0.9	3.3	3.8	2.7

Table 6 Recovery of organic C of the bulk soil as aryl carbon (C_{aryl}) in the free (fPOM), occluded (oPOM) particle organic matter, fine fraction ($< 20 \mu\text{m}$) and coarse fraction ($2000 - 20 \mu\text{m}$) of unburnt (AZQU3), single (AZQB1, AZQBa,b) and double burnt (AZQDB1) soils under *Quercus* and from double (AZPDBc,d,e) and single (AZPBc,d,e) burnt Cambisol under pine sampled 7 years after a fire in the Sierra de Aznalcóllar, Southern Spain. C_{arylbulk} corresponds to the contribution of aryl C in each fraction to the total aryl C content in the bulk soil.

Samples	fPOM		oPOM		$< 20 \mu\text{m}$		2000-20 μm
	C_{aryl} (%)	C_{arylbulk} (%)	C_{aryl} (%)	C_{arylbulk} (%)	C_{aryl} (%)	C_{arylbulk} (%)	C_{arylbulk} (%)
AZQU3	6.8	43.0	5.4	34.6	1.8	11.5	11
AZQB1	7.0	24.5	10.6	37.3	2.4	8.4	30
AZQDB1	6.7	26.1	9.3	36.4	2.2	8.4	29
AZQBa	8.5	39.3	6.2	28.9	4.3	20.1	12
AZQBd	12.5	54.4	2.8	12.4	4.1	17.9	15
AZPBc	23.8	47.8	9.6	19.2	1.5	3.0	30
AZPBd	23.7	57.1	9.8	23.6	2.4	5.8	14
AZPBe	21.4	47.0	10.9	24.0	2.8	6.2	23
AZPDBc	17.4	68.8	3.8	14.8	3.6	14.2	2
AZPDBd	12.8	59.2	3.4	15.8	2.2	10.1	15
AZPDBe	22.1	68.2	5.9	18.1	2.4	7.4	6

960 Figure

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Figure

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965 Figure 4