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3   Black humic acid dynamics during natural reforestation of Japanese

4   pampas grass (*Miscanthus sinensis*)

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21   **ABSTRACT**

22

23   The dynamics of the polyaromatic structures of black humic acids (HAs), which are

24   presumably derived from charred materials, are of significant interest for the global

25   carbon cycle. However, the details of those dynamics are not yet well understood. We

26   investigated differences in the degree of darkness (A_{600}/C values), isotopic ratios ($\delta^{13}C$,

27    $\delta^{15}N$, and $\Delta^{14}C$ values), and ^{13}C NMR spectra of size-separated black HAs extracted

28   from Japanese volcanic ash soils in order to estimate the variations in the polyaromatic

29   structures of black HAs during ca. 100 years of natural reforestation of Japanese

30   pampas grassland. For several hundred years, all the study sites were managed similarly

31   as grassland by burning. Subsequently, their management differed: at site G

32   (*Miscanthus sinensis*: C4 plant), maintenance as of the time of this study was still

33   performed by mowing, while at sites P (*Pinus densiflora*: C3 plant) and Q (*Quercus*

34   *crispula*: C3 plant), maintenance was discontinued ca. 30 and 100 years ago,

35   respectively. Thus, the sites range from grassland (site G) to coniferous forest (site P) to

36   broad-leaved forest (site Q). For all HA size fractions at all sites, we found that $\delta^{13}C$

37   values correlate positively with $\delta^{15}N$ values, although the gradients are much lower for

38   fractions of small to medium molecular size than for fractions of medium to large

39   molecular size (denoted as *lower-size* and *higher-size* fractions, respectively). Overall,

40   for the lower-size fractions, the contribution ratio of C4-plant-derived carbon shows a

41   significant positive correlation with A_{600}/C values and a negative correlation with $\Delta^{14}C$

42   values, and their aromatic characteristics are greater than those of higher-size fractions

43   within the same black HA. Furthermore, the relative proportion of lower-size fractions

44 decreases with reforestation, especially from site P to Q. The δ^{13}C values for all size

45 fractions are similar for sites G and P, but are relatively low for site Q. The aryl C

46 contents of the lower-size fractions are lower and the *O*-alkyl C contents and the

47 aliphaticity (alkyl C:*O*-alkyl ratio) are clearly higher for sites P and Q than for site G.

48 These results strongly suggest that stimulation of HA biodegradation might be

49 achievable by continuous input of new plant litter during reforestation, even for

50 lower-size HA polyaromatic structures, despite the fact that lower-size HAs biodegrade

51 more slowly than higher-size HAs.

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53 Key words: black humic acids; ^{13}C NMR spectroscopy; natural reforestation; stable

54 isotope ratio; polyaromatic structures; radiocarbon concentration; biodegradation.

1. Introduction

Japan is a typical volcanic country, where volcanic ash soil formed from deposits of volcanic material such as ash is widely distributed (Shindo et al., 2004). Many such soils have very thick and dark A horizons with significant amounts of black humic acids (HAs) that are major components of the humic material (Kumada, 1987; Shindo and Honma, 2001). Black HAs are often characterized by extremely high aromatic and stable structures (turbostratic structure; Yanagi et al., 2002, 2003), such as black carbon (Kumada, 1987; Shindo et al., 1986; Shindo and Honma, 2001). The mechanisms of their formation and accumulation have been discussed extensively (Hiradate et al., 2004).

Golchin et al. (1997) reported an interesting phenomenon that occurs in typical Japanese volcanic ash soil. During ca. 100 years of natural reforestation from pampas grassland to deciduous broad-leaved forest via pine forest, dark A horizons were decolorized. In addition, ^{13}C cross-polarization/magic-angle-spinning nuclear magnetic resonance (CPMAS NMR) spectroscopy analyses of bulk soil and HAs show that the aromatic C peak area decreases while the alkyl C peak area increases. We also performed quantitative analysis and characterization of humic fractions using liquid-state ^{13}C NMR spectroscopy at the same study site as that used by Golchin et al. (1997). We found that the change in quantity and quality of soil organic matter (SOM) that occurs with natural reforestation is caused specifically by the transformation of HA fractions (Iimura et al., 2010). However, the detailed physicochemical properties and isotopic ratios of HAs in such situations and the causes of transformation have not yet

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78   been well studied.

79   HAs are heterogeneous mixtures of natural organic macromolecules. Thus, to gain

80   further insight into the chemical characteristics of soil HAs, fractionation techniques

81   such as size-exclusion chromatography (SEC) are necessary (Piccolo et al., 2002; Kuráň

82   et al., 2008). Recent studies show that high-performance SEC (HPSEC) techniques such

83   as preparative HPSEC (prep HPSEC) enable faster- and finer-size fractionation of soil

84   HAs with high reproducibility (Asakawa et al., 2011). Prep HPSEC is also reportedly

85   advantageous for HA characterization by spectroscopic methods such as ^{13}C NMR

86   spectroscopy (Conte et al., 2006).

87   The isotopic ratio of C (δ^{13}C value) in soil HAs is stable, and so is useful for

88   studying HA plant origins and estimating the rates at which natural succession from

89   grassland to forest occurs, with a resulting shift in photosynthetic strategy as the

90   dominant plant species changes from C4 to C3 (Balesdent et al., 1987; Vitorello et al.,

91   1989; López-Ulloa et al., 2005). The δ^{13}C value for atmospheric CO_2 is around −7‰;

92   the value for plant organic carbon depends on the CO_2 fixation system, with the average

93   values for carbon derived from C4 and C3 plants reported as −13‰ and −27‰,

94   respectively (Yoneyama et al., 2001). The natural radioisotope ^{14}C is useful for studying

95   HA dynamics in terrestrial environments. Radiocarbon concentration (Δ^{14}C) is known

96   to directly reflect the modern C concentration in HAs (Nagao et al., 2004), making it

97   possible to consider the presence of the initial recalcitrant C in black HAs.

98   In this study, we investigated the chemical characteristics (^{13}C NMR spectroscopy),

99   degree of darkness (A_{600}/C values), and isotopic ratios (δ^{13}C, δ^{15}N, and Δ^{14}C values) of

100   size-separated HA fractions obtained by the prep HPSEC technique; this investigation

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101   was conducted on samples from Japanese volcanic ash soil retrieved from the same

102   ecological succession series—from grassland to deciduous broad-leaved forest via pine

103   forest—which occurred over the last ca. 100 years; the investigation focused on the

104   variation in black HA polyaromatic structures during long-term natural reforestation of

105   Japanese pampas grassland.

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107   **2. Material and methods**

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109   *2.1. Study sites and samples*

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111   The study area is situated in the campus of the Sugadaira Montane Research Center

112   of Tsukuba University (SMRC), Nagano Prefecture, Japan. The study area is 1315 m

113   above sea level at N 36°31′25″, E 138°20′50″. The mean annual precipitation is 1226

114   mm, and the mean annual temperature is 6.5 °C. Three sites within the study area were

115   selected: sites G, P, and Q, all of which were managed as grassland for several hundred

116   years by burning. Maintenance at site G, as of the time of this study, is performed by

117   mowing; maintenance at sites P and Q was discontinued in the eastern area of the

118   grassland ca. 30 and 100 years ago, respectively. Thus, the three sites collectively

119   represent a progression in ecological succession from grassland (site G) to coniferous

120   forest (site P) to broad-leaved forest (site Q). The dominant vegetation cover is

121   *Miscanthus sinensis* (C4 plant) at site G, *Pinus densiflora* (C3 plant) with an understory

122   of *Sasa* spp. (C3 plant) at site P, and *Quercus crispula* (C3 plant) with an understory of

123   *Sasa* spp. at site Q. A few C3 plant species (*Pteridium aquilinum,* etc.) are also present

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124   at site G as the minor vegetation cover. Detailed soil profiles, site sketches, aerial

125   photographs, and natural-succession histories for each site from 1950 to the present

126   were previously reported (Iimura et al., 2010).

127   Soil samples at each site were collected from the surface mineral horizon (0–20 cm)

128   after researching on soil profiles. Soil sampling points were determined after

129   preliminary research by soil auger at various points. Table 1 lists several soil

130   characteristics for each site. HA samples were the same as those purified by Iimura et al.

131   (2010).

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133   *2.2. Mp, Mw, and polydispersity*

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135   Prep HPSEC was performed according to the methods of Asakawa et al. (2011). For

136   estimates of molecular weight distribution, aliquots of solutions of size-separated HA

137   fractions were diluted (50×) with eluent and analyzed by analytical HPSEC (Asakawa et

138   al., 2008). Molecular weight at peak maximum (Mp), weight-averaged molecular

139   weight (Mw), and polydispersity were calculated with Waters Millenium 32

140   Chromatography Manager version 3.06 software. For other analyses, evaporated

141   solutions of each size fraction were acidified to pH 1.0 with 6 M HCl, dialyzed in

142   deionized water (Spectra/Por CE membrane, M_W cutoff 500 Da, Spectrum, Houston,

143   TEX, USA), and freeze dried.

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145   *2.3. Degree of darkness*

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147   The degree of darkness (A_{600}/C values) of the size-separated HA fractions was

148   determined according to the methods of Ikeya and Watanabe (2003), where A_{600} is the

149   absorbance at 600 nm and C is the organic C concentration. A_{600} was measured on a

150   UV–vis spectrophotometer (Jusco V-530). C was measured on a TOC analyzer

151   (Shimadzu TOC-V), with samples prepared as follows: HAs were dissolved in 0.1M

152   NaOH for determine the absorbance at 600 nm, the solution was adjusted to about pH 4

153   by dilution (5×) with 0.066 M kH_2PO_4 and dissolved CO_2 was removed by bubbling

154   with N_2 for determine the organic C concentration.

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156   *2.4. Elemental composition and $\delta^{13}C$, $\delta^{15}N$, $\Delta^{14}C$ values*

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158   Elemental analysis of the size-separated HA fractions was performed on a CHNS/O

159   analyzer (PerkinElmer 2400II) using 2 mg of dry sample per measurement. Ash content

160   was determined after combustion of 10 mg of dry sample at 550 °C in a muffle furnace

161   for 4 h.

162   Isotopic analysis on samples were accomplished with an elemental analyzer coupled

163   with an IsoPrime EA stable isotope ratio mass spectrometer (GV Instruments, UK).

164    $\delta^{13}C$ and $\delta^{15}N$ values were calculated as follows:

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166    $\delta^{13}C$ or $\delta^{15}N = [R_{sample}/R_{standard} - 1] \times 1000,$

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168   where R is the $^{13}C{:}^{12}C$ or $^{15}N{:}^{14}N$ ratio in the sample and the standard. Reference

169   material USGS 40 (L-glutamic acid) with $\delta^{13}C = -26.4$ ‰ and $\delta^{15}N = -4.5$ ‰ was used

170   as a calibrated standard, and results were reported relative to Peedee belemnite (PDB)

171   and air. The standard deviation of a measurement based on multiple analyses of the

172   standard was generally ≤0.1‰. Each sample was analyzed in three replicates. If the

173   standard deviation was >0.2‰, the measurement was repeated until the standard

174   deviation for all measurements fell to ≤0.2‰. The contribution ratios of

175   C4-plant-derived and C3-plant-derived carbon on the carbon of the sample (CR_{C4} and

176   CR_{C3}) were calculated as follows:

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178    $\delta^{13}C = \delta C_4 \times CR_{C4} + \delta C_3 \times (1 - CR_{C4})$,

179    $CR_{C3} = 100 - CR_{C4}$,

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181   where δC_4 and δC_3 are the $\delta^{13}C$ values of C4- and C3-plant-derived carbon, respectively

182   (Hiradate et al., 2004). In the present study, the δC_4 and δC_3 values were set to −13‰

183   and −27‰, respectively (Yoneyama et al., 2001). The C content derived from C3 and

184   C4 plants was then calculated for the size-separated HA fractions from the C content of

185   each HA fraction and CR_{C3} and CR_{C4}.

186   The ^{14}C contents of selected HA fractions were determined at the AMS facility

187   (NIES-TERRA) of the National Institute for Environmental Study (Uchida et al., 2004).

188   Sample graphitization was carried out according to the method of Uchida et al. (2005,

189   2008) as follows: Homogenized bulk sediment samples were combusted in sealed

190   quartz tubes (with CuO), and the resulting CO_2 was purified and graphitized by

191   reduction with H_2 in the presence of Fe powder as a catalyst.

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193   *2.5. Liquid-state ^{13}C NMR*

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195   Liquid-state ^{13}C NMR spectra were recorded with an NMR spectrometer (Avance

196   500, Bruker GmbH, Karlsruhe, Germany) using sample tubes with 5 mm in diameter.

197   Samples (ca. 30–50 mg) were dissolved in 0.4 ml of 0.5 mol l^{-1} NaOD in D$_2$O.

198   Chemical shifts were referenced to sodium 3-trimethylsilylpropionate-2,2,3,3-D$_4$ (TSP;

199   Euriso-top, Saint Aubin, France). To obtain quantitative conditions for integration of the

200   ^{13}C NMR spectra, ^{13}C signals were proton-decoupled using the following inverse-gated

201   decoupling technique parameters: spectrometer frequency = 125.76 MHz, pulse width =

202   45°, acquisition time = 0.839 s, and total repetition time = 2.5 s. To improve the

203   signal/noise ratio, line broadening of 50 Hz was used. Scans from 10,000 to 20,000

204   were accumulated. Resonance areas were calculated by electronic integration. Spectral

205   peaks were assigned according to the reports of Preston and Blackwell (1985), Schnitzer

206   and Preston (1986), Thorn et al. (1989), and Ricca and Severini (1993). To obtain

207   quantitative information, the spectra were divided into the following six regions

208   (Fujitake and Kawahigashi, 1999): alkyl C, 5–45 ppm; *O*-alkyl C, 45–110 ppm; aryl C,

209   110–145 ppm; *O*-aryl C, 145–165 ppm; carboxylic C, 165–190 ppm; and carbonyl C,

210   190–220 ppm. Aromaticity, as proposed by Watanabe and Fujitake (2008), was

211   expressed as the ratio of aryl C and *O*-aryl C to the total of alkyl C, *O*-alkyl C, aryl C,

212   and *O*-aryl C. Aliphaticity, as proposed by Golchin et al. (1995), was expressed as the

213   ratio of alkyl C to *O*-alky l C.

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215   **3. Results and discussion**

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3.1. Dynamics of isotopic ratios and their relationship with degree of darkness

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219   For all sites, the $\delta^{13}C$ value for each size-separated HA fraction increases with $\delta^{15}N$

220   value, although the gradient of the trend differs according to molecular size (Fig. 1).

221   The $\delta^{13}C$: $\delta^{15}N$ ratios at each site are generally as follows: data for site G Fr. 1–4, site P

222   Fr. 1–5, and site Q Fr. 1–6 fall along the 1:3 line and data for site G Fr. 5–10, site P Fr.

223   6–10, and site Q Fr. 7–10 (except for Fr. 8) fall along the 1:1 line. Greater ^{13}C and ^{15}N

224   enrichment in SOM often reflects the degree of biodegradation and humification

225   (Marin-Spiotta et al., 2009). In addition, Connin et al. (2001) reported that the $\delta^{15}N$

226   value clearly increases relative to the $\delta^{13}C$ value with microbial degradation of litter that

227   is easily decomposed by microorganisms. Therefore, our $\delta^{13}C$: $\delta^{15}N$ ratio data suggest

228   that black HAs consist of two major components. Furthermore, within the same black

229   HA, components that generally fall along the 1:1 line may be more resistant to

230   microbial attack than those that fall along the 1:3 line.

231   The values of maximum peak molecular weight (Mp) and weight-averaged

232   molecular weight (Mw) for size-separated HA fractions clearly decrease with Fr.

233   number for all sites (Table 2). We therefore categorized Fr. 1–4 of site G, Fr. 1–5 of site

234   P, and Fr. 1–6 of site Q as higher-molecular-size fractions and Fr. 5–10 of site G, Fr.

235   6–10 of site P, and Fr. 7–10 of site Q as lower-molecular-size fractions based on the

236    $\delta^{13}C$: $\delta^{15}N$ ratio, Mp, and Mw within the same HA.

237   Table 3 shows the carbon content C derived from C3 and C4 plants for

238   size-separated HA fractions from each site. Fig. 2 also shows their relationship with the

239   degree of darkness (A_{600}/C values) for size-separated HA fractions from each site. The

240   estimated carbon content C derived from C4 plants in the lower-size fraction ranges are

241   as follows: site G 2.3–3.4 C g kg^{-1}, site P 2.2–2.6 C g kg^{-1}, and site Q 0.8–0.9 C g kg^{-1}

242   (Table 3). These values for the lower-size fractions of each HA correlate positively with

243   A_{600}/C values: r = 0.73 at site G, r = 0.77 at site P, and r = 0.91 at site Q (Fig. 2). In

244   contrast, the correlation coefficients of estimated carbon content C derived from C4

245   plants and A_{600}/C values in the higher-size fractions were much lower than those in the

246   lower-size fractions: r = 0.03 at site G, r = 0.01 at site P, and r = −0.75 at site Q (Fig.2).

247   A_{600}/C values for HAs are known to commonly correlate positively with aromatic C

248   content and C-layer plane size (Watanabe et al., 2005; Ikeya et al., 2011). Yamanoi

249   (1996) emphasized the importance of microparticles of charred pampas grassland (C4

250   plant) in the formation and accumulation of black HAs in Japanese volcanic ash soils.

251   Hiradate et al. (2004) also showed that the proportion of aromatic C in HAs correlates

252   significantly with the amount of C derived from the C4 plant of HAs from Japanese

253   volcanic ash soils. Our results therefore strongly suggest that the lower-size components

254   of black HAs have more stable charred C4-plant-derived C than do the higher-size

255   components within the same HAs. Furthermore, the mean values of carbon content C

256   derived from C4 plants of the lower-size components (site G 2.9 C g kg^{-1}, site P 2.4 C g

257   kg^{-1}, site Q 0.8 C g kg^{-1}) decrease much more during natural reforestation than do the

258   estimated C derived from C3 plants (site G 2.9 C g kg^{-1}, site P 2.2 C g kg^{-1}, site Q 2.1 C

259   g kg^{-1}), particularly from site P to site Q (Table 3).

260   In the long term (ca. 100 years), invasion of woody vegetation (C3 plants) into

261   grassland is generally thought to lead to an increase in the amount of litter input

262   (Jackson et al., 2002). This continuous addition of litter, particularly in deciduous

263   broad-leaved forests, probably compensates for the loss of C4-plant-derived C. In our

264   study, the A_{600}/C values for the different size fractions in each HA do not correlate well

265   positively with C3-plant-derived C, and C content appears to not change significantly

266   during reforestation, in contrast to the case for C4-plant-derived C (Fig. 2 and Table 3).

267   The δ^{13}C values across size fractions are also lower for site Q (from −23.2‰ to

268   −22.2‰) than for site G (from −20.9‰ to −19.1‰) and site P (from −20.2‰ to

269   −19.1‰) (Table 3), suggesting higher incorporation of products derived from newer C3

270   forests across all size fractions during long-term reforestation.

271   The variation in Δ^{14}C value with molecular size shows a similar trend for all HAs:

272   first decreasing as molecular size increases from small to medium, then increasing as

273   molecular size increases from medium to large (Table 3). Based on our data for A_{600}/C,

274   δ^{13}C, and δ^{15}N values, we suggest that the more stable lower-size components in black

275   HAs may undergo different humification (stabilization) processes than do the less stable

276   higher-size components. As evidence, lower-size components showed that with

277   increasing molecular size, A_{600}/C and δ^{15}N values increase while the Δ^{14}C value

278   decreases (Figs. 1 and 2, Table 3). In addition, the Δ^{14}C values of size fractions in both

279   forest sites (sites P and Q) showed clearly higher values than those of site G (Table 3).

280   Δ^{14}C values for atmospheric CO_2 measured prior to atmospheric nuclear testing in the

281   1950s were ≤0‰; therefore, the measured Δ^{14}C values directly reflect the presence of

282   modern C introduced by nuclear weapon testing during the 1950s and 1960s (Nagao et

283   al., 2004). Thus, our results show that the amount of modern C in HAs increases with

284   reforestation even in the lower-size fractions, particularly in the earlier stages of

285   reforestation (from site G to P) (Table 3). The reasons for this are discussed along with

286   ^{13}C NMR spectroscopy data in the next section.

287

288   *3.2. Dynamics of black HAs during natural reforestation*

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290   ^{13}C NMR spectra and the C content of the functional groups of the size-separated

291   HA fractions are shown in Fig. 3 and listed in Table 4. All samples show essentially the

292   same peaks in the general chemical-shift regions, although peak magnitude varies. For

293   all HAs, molecular size correlates negatively with aryl C and positively with *O*-alkyl C

294   and alkyl C. For site G, total aryl C content is high (except for the largest-size fraction)

295   and *O*-alkyl C and alkyl C contents are relatively low (Table 4). In contrast, for sites P

296   and Q, total aryl C content decreases with increasing molecular size, whereas *O*-alkyl C

297   and alkyl C contents are more similar to those at site G (Table 4). These results strongly

298   support our abovementioned suggestion that for black HAs, lower-size components

299   undergo a different humification process than do higher-size components, and have

300   more polyaromatic structures derived from charred C4-plant materials; these moieties

301   may disappear during long-term natural reforestation.

302   Continuous input of fresh plant litter is well known to result in accumulation of

303   material that is highly resistant to biodegradation, such as resins, waxes, and other lipids

304   (Ziegler and Zech, 1989; Kogel-Knabner et al., 1992; Zech et al., 1992). In addition, the

305   trend of decreasing *O*-alkyl C and increasing alkyl C content (aliphaticity) with

306   decomposition has been reported for various soil types (Baldock et al., 1992; Golchin et

307   al., 1995). Furthermore, Willmann and Fakoussa (1997) demonstrated that HAs show

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308   increased aliphatic C (*O*-alkyl and alkyl C), together with decreased aryl C, after

309   incubation of the white rot fungal strain. Thus, the higher aliphatic chemical

310   characteristics of each size fraction in the present study (Table 4, Fig. 3) may be caused

311   by selective biodegradation of some HAs moieties and selective incorporation of

312   products derived from microbial synthesis and/or plant litter.

313   Our δ^{13}C data do not fully explain the higher incorporation of products derived from

314   newer C3 plants (forests and floor plants) across all size fractions during reforestation

315   from site G to P (Table 3). Alfredsson et al. (1998) reported that the decrease in soil C

316   with reforestation on temperate grassland may be attributed to changes in soil

317   macroflora and microflora associated with the root/rhizosphere systems in forest as

318   opposed to grassland. For example, ectomycorrhizal fungi associated with tree roots

319   have been found to increase the mineralization of organic matter in soil via production

320   of extracellular hydrolase enzymes (Marschner and Dell, 1994; George and Marschner,

321   1996). Overall, our measured C:N ratios are lower (Table 3) and aliphaticity is higher

322   (Table 4) for size fractions at site P than for those at site G, suggesting that microbial

323   activity is higher at site P than at site G.

324   This change in the microbial activity may reflect the difference in the chemical

325   properties and Δ^{14}C values for the size fractions at sites G and P. Kleber and Johnson

326   (2010) emphasized that the concentration of ^{14}C in an organic molecule is not at all

327   related to its chemical stability. They also stated that a decomposer organism is

328   indifferent to the ^{14}C content of an organic molecule. In our case, a clear difference in

329   the Δ^{14}C values of lower-size and higher-size fractions is not evident for all sites (Table

330   3). This agrees well with the report of Kleber and Johnson (2010). However, when the

331 vegetation cover changed drastically, the $\Delta^{14}C$ values as well as the chemical properties

332 of size fractions may be varied. For sites G and P, our data on the variation of

333 C4-plant-derived C as well as on the values of A_{600}/C values, $\delta^{13}C$, and $\delta^{15}N$ suggest

334 that the higher O-alkyl and alkyl C of size fractions from site P as compared with those

335 from site G may be caused mainly by selective biodegradation of HA moieties (old C)

336 except for the polyaromatic structures derived from charred C4-plant materials. For site

337 Q, the situation may differ; our data suggest a higher incorporation of products derived

338 from newer C3 plants (forest and floor plants) across all size fractions during

339 reforestation from site P to Q (Fig. 2 and Table 3). Hamer et al. (2004) reported that

340 mineralization of black carbon is stimulated by the addition of glucose. Indeed, the

341 O-alkyl C content (e.g., carbohydrate C) and aliphaticity is higher for size fractions

342 from site Q than for those from site G, even in lower-size fractions, as shown for site P

343 (Table 4). In addition, A_{600}/C values and C4-plant-derived C content for lower-size

344 fractions are clearly lower for site Q than for sites G and P (Fig. 2). Therefore, we

345 speculate that stimulating black HA biodegradation, even if the polyaromatic structures

346 are presumably derived from charred materials, may be achievable by continuous input

347 of new litter (deciduous leaves) during long-term reforestation, despite the fact that

348 biodegradation is slower for lower-size than for higher-size components.

349

350 **4. Conclusions**

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352   Our study strongly suggests that black HAs in Japanese volcanic ash soil consist of

353 two major types of components: one with a relatively lower molecular size and a

354 polyaromatic character (presumably derived from charred materials) and other with a

355 relatively higher molecular size and an aliphatic character. Lower-size components

356 exhibit slower rates of variation in their stable isotope ratios than do higher-size

357 components, strongly suggesting that stimulating the biodegradation of black HAs, even

358 of its lower-size components, can be achieved by continuous input of new litter such as

359 carbohydrate C moieties during long-term natural reforestation of Japanese pampas

360 grassland.

361

362 **Acknowledgments**

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370

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508 **Figure captions**

509

510 **Fig. 1.** Relationship between $\delta^{13}C$ and $\delta^{15}N$ values for size-separated HAs fractions

511 taken from each study site. The numbers indicate the fraction number.

512

513 **Fig. 2.** Carbon derived from C3- and C4-plant content and degree of darkness (A_{600}/C

514 values) for size-separated HAs fractions taken from each study site.

515

516 **Fig. 3.** ^{13}C NMR spectra of size-separated HAs fractions taken from each study site.

517 **Table 1**

518 Selected characteristics of soils used.

Sample	Depth (cm)	pH[a] (H$_2$O)	pH[a] (NaF)	T-C[a] (g kg^{-1} soil)	T-N[a] (g kg^{-1} soil)	Bulk density (g cm^{-1})	HA fraction[a] (gC kg^{-1} soil)
Site G	0-20	6.0	10.7	134	12.1	0.42	60.4
Site P	0-20	5.2	10.8	122	7.2	0.45	47.4
Site Q	0-20	5.3	11.3	88	4.6	0.51	30.2

519 [a] These data are from Iimura et al. (2010).

520 **Table 2**

521 Maximum peak molecular weight (Mp), weight-averaged molecular weight (Mw), and

522 polydispersity (Mw/Mn) of whole and size fractions of HAs estimated from calibration

523 curve of sodium polystyrene sulfonate (PSSNa).

HAs	Apparent molecular weight (kDa)[a]		Polydispersity[b]
	Mw	Mp	(Mw/Mn)
Site G			
Whole	5.35	2.79	3.10
Fr.1	28.9	20.4	15.3
Fr.2	14.3	13.1	3.70
Fr.3	12.4	11.0	2.00
Fr.4	9.11	8.41	2.38
Fr.5	7.74	7.18	2.01
Fr.6	6.35	5.80	1.74
Fr.7	5.49	4.87	1.66
Fr.8	4.61	3.85	1.59
Fr.9	3.73	3.09	1.56
Fr.10	2.39	2.03	1.61
Site P			
Whole	8.00	3.32	9.50
Fr.1	88.4	58.1	22.5
Fr.2	36.8	26.7	16.4
Fr.3	22.1	17.5	4.04
Fr.4	18.0	14.4	3.33
Fr.5	14.8	11.9	2.27
Fr.6	11.7	9.66	2.64
Fr.7	9.76	8.03	2.43
Fr.8	8.46	6.82	2.24
Fr.9	6.23	5.07	2.43
Fr.10	3.42	2.66	2.42
Site Q			
Whole	12.9	3.61	5.55
Fr.1	58.8	34.6	4.88
Fr.2	20.4	17.3	1.96
Fr.3	13.2	11.8	2.68
Fr.4	9.72	8.85	3.14
Fr.5	7.99	7.15	2.75
Fr.6	6.74	6.00	2.42
Fr.7	5.70	4.91	2.22
Fr.8	4.65	4.02	2.05
Fr.9	3.76	3.09	1.87
Fr.10	2.38	1.98	1.89

524 [a] Except for site P, data are from Asakawa et al. (2011), which are matching of site G

525   and Q to SGG and SGM HA, respectively.

526   [b] Polydispersity is the ratio of the weight-averaged (Mw) to number-averaged

527   molecular weight (Mn).

528 **Table 3**

529 C and N contents, isotopic ratios (δ^{13}C, δ^{15}N and Δ^{14}C values), and C3- and

530 C4-plant-derived C contents of whole and size-separated HAs fractions.

Sample	C (g kg^{-1})	N (g kg^{-1})	C/N	δ^{13}C (‰)	C3-C[a] (g kg^{-1})	C4-C[b] (g kg^{-1})	δ^{15}N (‰)	Δ^{14}C (‰)
Site G								
whole	60.4	3.46	17.5	−19.6	28.4	32.0	4.41	−212
Fr.1	6.2	0.34	18.2	−19.1	2.7	3.6	5.19	−156
Fr.2	6.4	0.42	15.4	−19.1	2.8	3.6	4.50	
Fr.3	6.2	0.31	19.8	−19.4	2.8	3.3	3.89	−228
Fr.4	6.9	0.34	20.5	−19.4	3.2	3.7	3.75	
Fr.5	5.5	0.30	18.5	−19.6	2.6	2.9	4.72	−222
Fr.6	6.4	0.35	18.3	−19.6	3.0	3.4	4.50	
Fr.7	5.7	0.34	17.0	−19.7	2.7	3.0	4.39	−225
Fr.8	5.9	0.38	15.5	−19.9	2.9	3.0	4.38	
Fr.9	6.0	0.31	19.4	−20.2	3.1	2.9	4.01	−199
Fr.10	5.2	0.39	13.4	−20.9	2.9	2.3	4.00	
Site P								
whole	47.4	3.52	13.5	−19.6	22.3	25.1	4.45	24
Fr.1	4.9	0.32	15.1	−19.1	2.1	2.8	4.83	22
Fr.2	4.9	0.38	12.9	−19.2	2.2	2.8	4.95	
Fr.3	4.9	0.36	13.7	−19.3	2.2	2.7	4.66	19
Fr.4	4.9	0.35	13.9	−19.5	2.3	2.7	4.39	
Fr.5	4.5	0.33	13.6	−19.4	2.1	2.4	4.39	5
Fr.6	4.8	0.52	9.1	−19.5	2.2	2.6	4.56	
Fr.7	4.5	0.32	14.1	−19.6	2.1	2.4	4.64	
Fr.8	4.7	0.32	14.5	−19.6	2.2	2.5	4.57	
Fr.9	4.8	0.33	14.3	−19.7	2.3	2.5	4.53	27
Fr.10	4.5	0.27	16.7	−20.2	2.3	2.2	4.13	
Site Q								
whole	30.2	2.08	14.5	−22.6	20.5	9.7	5.18	−16
Fr.1	3.1	0.22	14.4	−22.2	2.0	1.1	5.72	10
Fr.2	3.1	0.23	13.6	−22.5	2.1	1.0	5.40	
Fr.3	3.1	0.22	13.6	−22.5	2.1	1.0	4.95	−15
Fr.4	3.0	0.22	13.9	−22.5	2.1	1.0	4.84	
Fr.5	3.0	0.23	12.8	−22.6	2.0	1.0	4.52	−80
Fr.6	3.0	0.24	12.5	−22.7	2.0	0.9	4.12	
Fr.7	3.0	0.20	15.2	−22.9	2.1	0.9	4.63	−35
Fr.8	3.1	0.20	15.5	−23.0	2.2	0.9	4.07	
Fr.9	3.0	0.17	17.4	−23.1	2.1	0.8	4.49	−7
Fr.10	2.9	0.16	18.6	−23.2	2.2	0.8	4.40	

531 [a] Carbon content C derived from C3-plants.

532 [b] Carbon content C derived from C4-plants.

533

534 **Table 4**

535 Composition of C functional groups, aromaticity and aliphaticity of size-separated HAs

536 estimated from ^{13} C NMR.

HAs	% of carbon species (δ, ppm)						aromaticity	aliphaticity
	carbonyl 220-190	carboxyl 190-165	*O*-aryl 165-145	aryl 145-110	*O*-alkyl 110-45	alkyl 45-5		
Site G								
Fr.1	1.8	18.4	3.0	23.6	34.6	18.5	0.33	0.54
Fr.2[a]								
Fr.3	2.0	16.6	9.0	37.8	22.8	11.8	0.57	0.52
Fr.4	3.0	19.9	7.7	43.3	15.8	10.3	0.66	0.66
Fr.5	3.4	18.5	10.0	38.5	19.6	9.91	0.62	0.50
Fr.6	1.2	17.8	8.4	33.5	25.2	13.8	0.52	0.55
Fr.7	3.8	17.0	10.4	39.6	18.3	10.9	0.63	0.60
Fr.8	2.5	15.9	6.8	44.9	17.9	11.9	0.63	0.67
Fr.9	3.6	17.9	7.5	47.7	14.0	9.36	0.70	0.67
Fr.10	0.9	15.5	7.4	45.8	18.5	12.0	0.64	0.65
Site P								
Fr.1	5.1	13.8	4.4	11.5	41.3	24.0	0.20	0.58
Fr.2	4.4	12.9	6.9	18.2	34.7	22.8	0.30	0.66
Fr.3	5.3	17.3	8.8	21.8	28.8	18.0	0.40	0.62
Fr.4	7.1	17.2	7.6	18.3	29.9	19.9	0.34	0.66
Fr.5	4.0	17.0	8.4	21.3	29.3	20.0	0.38	0.68
Fr.6	6.1	16.6	7.8	23.6	27.7	18.3	0.41	0.66
Fr.7	5.1	15.3	6.2	24.5	29.6	19.3	0.39	0.65
Fr.8	5.8	15.0	7.3	20.0	29.9	22.1	0.34	0.74
Fr.9	4.1	17.9	6.8	29.9	23.8	17.4	0.47	0.73
Fr.10	5.4	17.2	7.2	33.0	21.2	15.9	0.52	0.75
Site Q								
Fr.1	6.0	19.3	7.0	22.8	25.4	19.4	0.40	0.76
Fr.2	4.2	18.1	4.8	20.6	31.2	21.1	0.33	0.68
Fr.3	3.0	17.7	6.2	26.3	27.1	19.6	0.41	0.72
Fr.4	3.7	19.8	7.9	29.9	22.4	16.2	0.50	0.72
Fr.5	2.1	18.2	6.0	25.8	26.4	21.5	0.40	0.81
Fr.6	3.0	18.9	6.7	31.3	23.0	17.1	0.49	0.74
Fr.7	4.0	19.1	6.7	34.0	20.6	15.6	0.53	0.76
Fr.8	2.8	19.3	5.8	32.9	23.0	16.2	0.50	0.70
Fr.9	2.8	18.6	7.9	34.2	22.4	14.1	0.54	0.63
Fr.10	3.5	20.7	7.6	35.8	18.9	13.6	0.57	0.72

537 [a] Not determined.