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Response of soil microbial community to afforestation with pure and mixed species

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Abstract

Objectives

Afforestation changes soil chemical properties over several decades. In contrast, microbial community structure can be shifted within the first decade and so, the direct effects of tree species can be revealed. The aim of this study was to determine the alteration of soil microbial community composition 10 years after afforestation by trees with contrasting functional traits.

Methods

The study was conducted at the BangorDIVERSE temperate forest experiment. Soil samples were collected under single, two and three species mixtures of alder and birch, beech and oak - early and secondary successional species, respectively, and contiguous agricultural field. Soil was analysed for total carbon (C) and nitrogen (N) contents, and microbial community structure (phospholipid fatty acids (PLFAs) analysis).

Results and conclusions

The total PLFAs content (370-640 nmol g^{-1} soil) in forest plots increased for 30 to 110% compared to the agricultural soil (290 nmol g^{-1} soil). In contrast, soil C, N and C/N ratios were altered over 10 years much less - increased only up to 20% or even decreased (for beech forest).

Afforestation increased bacterial PLFAs by 20-120%, whereas it had stronger impact on the development of fungal communities (increased by 50-200%). These effects were proved for all forests, but were more pronounced under the monocultures compared to mixtures. This indicates that species identity has a stronger effect than species diversity. Principal component analysis of PLFAs revealed that under mono and three species mixtures similar microbial communities were formed. In contrast, gram-positive PLFAs and actinomycete PLFAs contributed mainly to differentiation of two species mixtures from other forests. Thus, at the early afforestation stage: i) soil biological properties are altered more than chemical, and ii) tree species identity affects more than species amount on both processes.

Keywords: woodland, plant microbial interactions, microbial biomarkers, land use change, forest composition, ammonium and nitrate, soil solution, tree identity.

Introduction

Forests in the European Union cover more than 180 million ha representing 41% of the total land area. In response to a range of European policies (e.g. EU Biodiversity Strategy, EU Forest Strategy) afforestation area has increased by 17 million ha in the last 25 years and this trend is expected to continue for the foreseeable future (EEA, 2015). Both pure and mixed species forests are used for afforestation of former arable and grassland soils, however, there is still a lack of information on the effects of various tree species on maximising soil function (e.g. enhancing carbon (C) and nitrogen (N) storage, promoting nutrient cycling and water storage), and especially on the changes in soil microbial communities. This fundamental knowledge would be useful to make informed management decisions to maximise both above and below-ground diversity and to promote sustainable landscape functioning.

Forest soil properties are altered by the processes of tree establishment, growth and mortality. Soil C and N stocks generally increase with forest age and achieve their maximum accumulation rates during the exponential tree growth phase (DeLuca and Boisvenue 2012), and gradually decline in late successional forest stages. Approximately 30-50 years after afforestation, soil C and N stocks begin to stabilize (Fu et al. 2015; Kalinina et al. 2011). The quality of leaf litter also changes with forest age (e.g. decrease in leaf nutrient content, increased in C/N and lignin/N ratios), which directly affects litter decomposition and soil nutrient supply (Trap et al. 2013). A well-known effect of afforestation is soil acidification (Berthrong et al. 2009) due to changes in soil base saturation, litter chemistry, rhizodeposition and absence of liming (Fu et al. 2015). The reported pH decrease for 27 year-old broadleaf forests was around 0.95 units (Fu et al. 2015), while it is estimated that between 80-100 years of forest development is required to obtain pH values close to those found in native forests (Ritter et al. 2003). Overall, this suggests that soil acidity and C and N stocks change very slowly during afforestation.

Concurrent with changes in soil chemistry, the biomass, quality composition and diversity of soil microbial communities can also be expected to shift following trees establishment (Grayston et

al. 1997b; Macdonald et al. 2009). Afforestation induce a rapid increase in microbial biomass with changes apparent within one year of tree planting (van der Wal et al. 2006). Afforestation typically stimulates the development of fungal communities (Jangid et al. 2011; Buckley and Schmidt 2003), whereas bacteria appear to be less sensitive to land use changes (Klein et al. 1995). In addition, the diversity and relative abundance of individual fungal and bacterial species have been shown to increase after afforestation. For example, Acidobacteria appeared to dominate under birch while Firmicutes and Proteobacteria were more dominant under young pine forests (Nazaries et al. 2015). Thus, microbial communities might serve as a primary indicator of ecosystems recovery as their changes occur more rapidly than for soil chemical properties.

Forests affect the composition of microbial communities not only directly (Fu et al. 2015), but also indirectly through changes in soil chemical and physical properties (Yannikos et al. 2014; Mann and Tolbert 2000) depending on the forest type, biodiversity, and land use history (Yannikos et al. 2014). The time range needed for microbial communities to evolve to those typical of native forests is estimated to be 30 - 50 years (Jangid et al. 2011; Buckley and Schmidt 2003; van der Wal et al. 2006) and is affected by the rate at which soil properties change (van der Wal et al. 2006). Generally, the composition of microbial communities formed under broadleaf forests is radically different from those formed under coniferous species (Li et al.; Cong et al. 2015). These differences can be ascribed mainly due to variations in leaf litter chemistry, changes in mycorrhizal communities and colonization. Comparison of soils formed under broadleaf forest has also revealed that tree species like beech promote development of microbial communities different from those developed under ash, lime and hornbeam forests, mainly due to low C/N ratio of beech litter, presence of microbial activity inhibitors in root exudates and more rapid decreases in soil pH (Scheibe et al. 2015). Composition of forest was also reported to affect microbial community structure, which was found for the beech grown in mono- and mixed forests (Thoms and Gleixner 2013). However, in addition to forest community composition, variations in functional traits of trees should be accounted for due to their strong potential effects on the formation and shaping of soil

109 microbial communities (Fu et al. 2015). Thus, due to a variety of complex interacting factors, it is
110 difficult to disentangle the direct effects of forest tree community composition from the effect of
111 soil properties on microbial community dynamics, especially under mature forests, where soil
112 chemical properties may have already been changed. Further, it is difficult to distinguish between
113 tree identity and forest tree community composition effects, because functional traits of single tree
114 species can be masked or reduced in forest mixtures. Thus, only in experiments where both single
115 species and mixtures of trees are studied simultaneously in the early afforestation stage can
116 conclusions about the effect of tree identity and forest composition on the formation of soil
117 microbial communities be made.

118 The objective of this study was to evaluate the effects of forest tree community composition
119 on soil microbial community structure at the early forest development stage (10 years after
120 afforestation). It was hypothesized that independent of forest type, i) microbial community structure
121 will change more strongly than soil physico-chemical properties and ii) fungal biomass will
122 increase faster than bacterial biomass; iii) monoculture forests will promote strong and more
123 specific changes in content of particular microbial groups, whereas in species mixtures these
124 responses will be dampened.

125

126 **Materials and methods**

127 ***Study site and soil sampling***

128 Soils were obtained from the BangorDIVERSE forest experiment located at the Henfaes
129 Research Centre, North Wales, UK (53°14'N, 4°01'W). Climate was characterized as hyperoceanic,
130 with mean annual precipitation of 1034 mm and mean annual temperature of 11.5ºC (Campbell
131 Scientific Ltd, Shepshed, UK). The site was set up in 2004 with a total area of 2.36 ha. Soils are
132 classified as Eutric Fluvic Cambisols (WRB 2006) (Fluventic Dystrochrept, USDA system) and
133 have fine loamy texture (Smith A. et al. 2013). Each type of forests, namely: single species or two
134 and three species mixtures of European alder (*Alnus glutinosa* L.), Silver birch (*Betula pendula*

Roth), European beech (*Fagus sylvatica*, L.), and English oak (*Quercus robur* L.) were planted in four independent field replication, with a size replications were: 62, 121 and 196 m^2 for the single, two and three species forests, respectively. Forests were formed by tree species with contrasting functional traits: early primary and late successional stages species, N-fixing and non N-fixing, producing low and high litter quality. Monoculture species plots of alder, birch, beech and oak, two species mixtures of alder+beech, alder+oak, birch+beech, birch+oak, three species mixtures of alder+birch+beech, alder+birch+oak were used for the present experiment. The understory was formed mainly by grass, goose grass, nettle, bramble and dock. Only the plots taken for that study are mentioned, and for a full description of the experimental design see Ahmed et al. (2016). The main properties of the plant communities are presented in Table 1. Contiguous agricultural field (established before the BangorDIVERSE experiment), was chosen as a comparative soil due to its same historical land use and soil type. The latest cultivation species at the agricultural field was oilseed rape (*Brassica napus*) had been cultivated there following the addition of K_2O (20 kg ha^{-1}) and N (60 kg ha^{-1}). Soil samples were collected from the 0-10 cm depth from each field replication, and each sample was consisted of three independent soil cores. Each sample was divided into three parts: one was stored at 5 °C and used for extraction of soil solution, the second was dried at 105 °C and used for total C and N analysis (Supplementary Table 2), and the third was stored at -20 °C and used for phospholipid fatty acid (PLFA) analysis.

Analysis of soil quality indicators

Soil samples were dried at 105 °C and ball milled before C and N analysis by dry combustion (Elemental analyzer, Vario EL III, Jena, Germany). Soil C and N stocks were calculated based on the C and N contents and soil densities (it varied between 0.7-1 g cm^{-3} for forest soils and was 1.2 g cm^{-3} for the agricultural soil). Soil solution was obtained by the centrifugal drainage procedure described in Glanville et al. (2012) using 100g of fresh soil samples. The concentration of NH_4^+ in soil solution was determined colorimetrically using the sodium-

161 nitroprusside, while NO_3^- was determined colorimetrically using the VCl_3 (both procedures
162 described in Mulvaney (1996)).
163
164 ***Phospholipid fatty acids analysis***
165 Phospholipid fatty acids (PLFAs) were extracted from the soil samples according to
166 Frostegard (1991). Briefly, 4.5 g of fresh soil were placed into 50 ml centrifuge tubes, 25 µl of
167 internal standard one added (1 µg μl^{-1}, 19:0 phospholipid) and lipids extracted twice (18 and 6 ml,
168 respectively) by one phase mixture of chloroform, methanol and citric acid (0.15 M, pH 4.0) in the
169 ratio 1:2:0.8 (v/v/v). Extracted lipids were applied to the silica column and neutral-, glyco- and
170 phospholipids were sequentially eluted from the column by chloroform (5 ml), acetone (20 ml) and
171 methanol (20 ml), respectively. Collected phospholipids were saponified (0.3 M solution of BF_3 in
172 methanol), obtained fatty acids were methylated (1 M solution of NaOH in methanol) and extracted
173 in hexane. Finally, the samples were dried under a stream of N_2 and redissolved in toluene (185 µl)
174 with addition of internal standard two (15 µl of 13:0 fatty acid methyl ester, 1 µg μl^{-1}).
175 The PLFAs were measured by GC-MS, having the following parameters: columns (15 m
176 HP-1 methylpolysiloxane coupled with a 30 m HP-5 (5% phenyl)-methylpolysiloxane column (both
177 with an internal diameter of 0.25 mm and a film thickness of 0.25 µm)), He flow of 2 ml min^{-1}, and
178 injection volume of 1 µl. The temperature program of GC-MS was set up to 80 ºC and then ramped
179 to 164 ºC at 10 ºC min^{-1}, then to 230 ºC at 0.7 ºC min^{-1} and finally to 300 ºC at 10 ºC min^{-1}. The
180 quantity of PLFAs was calculated based on the 29 external standards (Gunina et al. 2014), which
181 were prepared in 6 concentrations (Apostel et al. 2013). Final content of single PLFAs was
182 presented as molar percentages (mol %) and total content was presented as nmol g^{-1} soil.
183 Classification of PLFAs was done according to existing data on their presence in various groups of
184 microorganisms: for Gram-negative (G-) bacteria the 16:1ω7c, cy17:0, 18:1ω7c, cy19:0 PLFAs
185 were used (Leckie 2005; Lewandowski et al. 2015), for Gram-positive bacteria (G+) i15:0, a15:0,
186 i16:0, i17:0 PLFAs were used (Leckie 2005; Lewandowski et al. 2015), for actinomycetes (Ac)

187 10Me16:0 and 10Me18:0 were used (Lewandowski et al. 2015; Leckie 2005), for fungi
188 18:2ω6+18:1ω9c were used and 16:1ω5c was assumed as arbuscular mycorrhiza (AM) fungi PLFA,
189 but with caution due to its high possible input from G- bacterial biomass (Leckie 2005;
190 Lewandowski et al. 2015).
191
192 ***Statistical analysis***
193 To compare the effect of forest development on soil chemical properties and on microbial
194 biomarkers contents, changes of all parameters were calculated relatively to agricultural soil.
195 Changes of the soil chemical properties (except pH) relatively to the agricultural soil have been
196 calculated as:
197 $$\frac{Cp_f - Cp_{agr}}{Cp_{agr}}$$
198 where, Cp_f and Cp_{agr} are the values of chemical properties in the forest and agricultural soils,
199 respectively. For pH absolute changes were calculated by subtracting pH of agricultural soil from
200 pH of forest soils.
201 The increase of PLFAs of distinct groups relatively to agricultural plot was calculated as:
202 $$\frac{PLFA_f - PLFA_{agr}}{PLFA_{agr}}$$
203 where, $PLFA_f$ and $PLFA_{agr}$ are the contents of PLFAs of specific microbial groups in forest and
204 agricultural soils (nmol g^{-1} soil), respectively. Data were checked for the normal distribution and
205 homogeneity was tested by Levene's test. Calculated values were tested with one-way ANOVA and
206 significant differences were obtained with Notched Box Plots.
207 Principal component analysis (PCA) of mol% of individual PLFAs was done to elucidate
208 major variation pattern. The scores of the first two components from the PCA were used to separate
209 the soils formed under various forests. Linear regression of PLFAs factor scores and soil properties
210 (pH, total C and N, concentration of NH_4^+ and NO_3^-) was done to conclude about the correlation of

211 PLFAs composition with environmental factors depending on the forest type. Statistical analyses
212 were done in Statistica 12.0 and Microsoft Excel 2010.
213
214 **Results**
215 ***Afforestation effects on soil properties***
216 Afforestation had weak effect on the C content: the maximal changes of soil C content was
217 ca. 20% relative to the agricultural soil (Fig. 1), and was maximal for the birch, alder+oak and
218 birch+beech plots. However, C stocks in the upper 10 cm under pure oak, beech, two species
219 mixtures with oak and three species mixtures were lower compared to the agricultural soil
220 (Supplementary Table 2), mainly because of the low bulk density of the forest soils (it varied
221 between 0.7-1 g cm^{-3} for forest soils and was 1.2 g cm^{-3} for the agricultural soil).
222 The effect of forest development on soil N content (Fig. 1) followed the same tendency as
223 on C content, despite the contrasting N content of the various forest litters (Table 1). In general,
224 changes of total N content in the forest soils were similar and ranged within ±15%. The organic
225 matter quality, characterized by C/N ratio, was the most strongly affected for the pure birch,
226 birch+beech plots and alder+beech, where it had the highest increase relative to agricultural soil
227 (Fig. 1).
228 10 years of afforestation decreased soil acidity by 1.0-1.2 units compared to the agricultural
229 plot (Fig. 1).
230 The NO_3^- concentrations in soil solution decreased for the birch, beech and two forest
231 mixtures with birch compare to the agricultural soil (Fig. 1). In contrast, NH_4^+ did not differ in the
232 agricultural and forest soils (Fig. 1).
233
234 ***Afforestation effects on total PLFAs content***
235 Maximal contents of total microbial PLFAs were observed for the oak, birch and alder forest
236 soils (Fig. 2). Total PLFA contents were higher for the oak, birch and alder monocultures forests

237 compare to pure beech forest, whereas no differences were found between the two and three species
238 mixtures. In the case of the two species mixtures where beech was present, total PLFA content
239 increased relative to the beech monocultures, whereas, the opposite trends were observed for the
240 pure oak forest and two species mixtures containing oak.
241
242 ***Afforestation effects on the content of specific microbial biomarkers***
243 Afforestation increased fungal PLFAs content the most compared to other biomarkers, and
244 were 50-200% higher in the forest soils compared to the agricultural (Fig. 3). The maximal increase
245 was found for the soils under birch, oak, alder and birch+beech. The two and three species forests
246 increased their fungal biomarker content by 50-100%.
247 Bacterial biomarkers increased in forest soils (except beech, three species mixture with
248 beech and birch+oak) by 20 to 110% compared to the arable soil but without differences in the G+
249 and G- groups (Fig. 3). The content of G+ bacterial PLFAs were low for the monocultural beech
250 forest, but increased for the two species mixtures with beech. In contrast, the content of G+ PLFAs
251 were higher for the monocultural oak forests, than for the birch+oak mixed forest.
252 Relative to the agricultural, the content of 16:1ω5 PLFAs (AM fungal or G- bacterial
253 biomarker) increased by 30-120% (Fig. 3) and the increase was higher under the birch and oak
254 treatments than for any other soils. Both beech alone and in three species mixtures forests
255 containing beech resulted in a decline of 16:1ω5 PLFAs relative to the agricultural soil. The content
256 of actinomycete PLFAs followed the same trend as 16:1ω5 PLFA, however, the highest increase
257 was found for the alder+beech plot.
258 PCA analysis of the PLFA data revealed that the first two PCA components explained 38
259 and 21% of the PLFA variation, respectively (Fig. 4). The first PCA component reflects differences
260 in soil pH (r^2=0.32; linear regression of scores for PC1 *vs.* soil pH) and was correlated with
261 saturated/monounsaturated ratio (r^2=0.45). The second PCA component was correlated with
262 fungal/bacterial ratio (r^2=0.69) and also can be explained by soil pH (r^2=0.73). Both PC1 and PC2

were correlated with the cyclo/precursor ratio (for PC1 r^2=0.38 and for PC2 r^2=0.40). Both ratios are presented in the Table 2.

According to the PCA results the agricultural soil was separated from the mono- and three species mixture forests along the PC1 and PC2 and only along PC2 from the two species mixtures forests. Bacterial biomarkers (18:1ω7, cy17:0, i15:0 and i17:0) contributed to the separation of forest soils from the agricultural plot along PC1, whereas fungal (18:2ω6,9 and 18:1ω9) and G- biomarkers (cy19:0) were responsible for the separation along PC2 (Fig. 4, top). The agricultural plot was different from the forests due to the high relative portion of i14:0, 16:1ω5 and 16:1ω7 PLFAs in total PLFAs content, which were 1.1-1.5 times higher in the agricultural relative to the forest soils (Supplementary table 1).

Single and three species mixtures forest soils were separated from the two species mixture forests along PC1 (Fig. 4, top). Based on the loading values (Fig. 4, bottom), Ac (10Me16:0 and 10Me18:0) and bacterial biomarkers (i16:0, i15:0, 18:1ω9) were the most important for separation the two species mixtures from single and three species mixtures forests. In contrast, mono- and three species mixtures were only weakly separated on PC 2, and no separation along PC1 was found.

Discussion

Afforestation effects on soil chemical properties

Afforestation typically results in an improvement in soil quality and an increase in total C and N content (Laganière et al. 2012; Kurganova et al. 2015; Paul et al. 2002). Soil C content increased by 20% (for some plots) in the top 10 cm when compared to the adjacent agricultural on which the forest was established (Fig. 1). Such small changes are related to: i) prolonged effects of former land use management on the total soil C content within the first 10 years after afforestation (Paul et al. 2002), ii) occurrence of opposing processes during afforestation: a) large inputs of tree litter which decomposes relatively slowly as the intrinsic microbial community is poorly adapted to

289 this new substrate, and at the same time b) intensive decomposition of the intrinsic agriculture-
290 derived SOC due to the increased activity and content of microbial biomass. As a result, C
291 mineralization can exceed accumulation in the surface soil layer during early afforestation.

292 Total soil N content in the forest soils were similar to the agricultural plot, except for pure
293 beech stand, where it decreased by 15% and alder+oak plot where N content increased by 15% (Fig.
294 1). N stocks were lower in all forest soils compared to the agricultural soil (Supplementary Table 2),
295 mainly because of decrease of soil density. Afforestation has a strong effect on N dynamics in soils
296 and induces changes in N mineralization, ammonification and nitrification rates (Li et al. 2014).
297 Moreover, young trees have a high demand for N, resulting in a redistribution from soils into tree
298 biomass (Uri et al. 2003). The dominating form of the N in soil solution in afforested soils was
299 nitrate, although this was lower than in the agricultural soil (Fig. 1, Supplementary Table 2). In
300 contrast, no strong effect of afforestation on NH_4^+ concentration was found. The decrease of NO_3^-
301 concentrations is common for forest soils is a consequence of lower pH, higher C input, absence of
302 fertilization and intensive uptake of N by plants, all of which reduce nitrification rates (Li et al.
303 2014).

304 In agreement with previous afforestation studies (Berthrong et al. 2009; Kalinina et al.
305 2011), a decrease in soil pH was observed in all forest plots. We ascribe this to, i) changes in the
306 amount of rhizodeposition, which is around 50% of total assimilated C belowground for trees *vs.*
307 10-40% for annual plants (Grayston et al. 1997a), ii) changes in root and ectomycorrhizal exudate
308 quality, which often contain a high variety and amount of organic acids (Grayston et al. 1997a), iii)
309 an increased uptake of cations by trees (Jobbágy and Jackson 2003), iv) shifts in litter quality, and
310 v) an absence of liming. We conclude therefore that while early afforestation does not promote
311 strong changes in some soil chemical properties (e.g. total C and N content, C/N ratio) it can
312 promote large changes in more dynamic soil quality indicators (e.g. pH and available N form).
313
314

315 ***Tree identity effects on total microbial PLFA***

316 Development of forests usually increases total PLFAs content (Jangid et al. 2011) and for
317 our study it was true mostly for the soils under the monoculture forests formed by alder, birch and
318 oak and also in two species forest mixtures with beech (Fig. 2). The total content of PLFAs was 2-3
319 times lower for the pure beech stands in comparison with the other broadleaf forest types (e.g.
320 hornbeam, lime, maple or ash) (Scheibe et al. 2015). This is a consequence of low pH and presence
321 of specific compounds in root exudates composition (Scheibe et al. 2015). The increase of PLFAs
322 content under the two species mixtures with beech is explained by presence of the pioneer species -
323 alder and birch, which are usually used to improve soil quality before planting the secondary forest
324 species such as beech (Frouz et al. 2015). Moreover, alder is an N-fixer, which can provide
325 additional N for microorganisms in soil under two species mixtures forests (Frouz et al. 2015;
326 Walker and Chapin 1986; Chapin et al. 1994). In contrast, mixtures containing both oak trees and
327 primary succession species did not stimulate an increase in microbial biomarkers content (Fig. 2).
328 The same effect was found for the three species mixtures because partly opposite effects of the tree
329 species (Fig. 2) compensating each other in mixtures. In conclusion, it appears that tree species
330 identity has a stronger effect than amount of species on the content of total PLFAs in the afforested
331 soils.

332

333 ***Afforestation effects on microbial community composition***

334 Afforestation increased the content of bacterial and fungal PLFAs, however, fungal
335 biomarkers increased 2 times higher than those for bacterial. Afforestation usually promotes
336 development of fungi (Yannikos et al. 2014; Macdonald et al. 2009; Carson et al. 2010) and induces
337 changes in fungal community composition (Carson et al. 2010). An increase in fungal biomarker
338 content after afforestation can be attributed to the both direct effects of the trees themselves and
339 indirect effects due to changes in the environment. Of the direct tree effects, fungal biomass is
340 stimulated by, i) a shift from easy decomposable crop residues to more recalcitrant leaf litter rich in

polyphenol/tannin compounds (Rousk and Baath 2007; Yannikos et al. 2014), and ii) development of plant species, which are strongly ectomycorrhizal such as birch, alder and oak (Baum et al. 2009). Of the indirect effects, i) termination of agricultural practice stimulates the development of fungi due to less physical disruption of hyphal networks (Helgason et al. 2009; Strickland and Rousk 2010), and ii) a decrease in soil pH suppresses bacterial growth and makes fungi more competitive in terms of substrate utilization (Swift et al. 1979; Zeller et al. 2001).

The 16:1ω5 PLFA can be used to estimate the content of AM fungal biomarkers (Thoms and Gleixner 2013; Madan et al. 2002) although we acknowledge that this may also be present in G- bacteria (Nichols et al. 1986). In contrast to fungal PLFAs, the content of the 16:1ω5 PLFA increased by 30 to 120% (for some cases) and even decreased (for beech and three species forest mixtures) (Fig. 3). This either can reflect i) the shift in fungal community from arbuscular mycorrhizal communities, inherent for agriculture and pasture soils, to ectomycorrhizal communities which dominate under forests (Macdonald et al. 2009) or ii) the changes in portion of microorganisms with rapid growth strategy in total microbial community (Priha et al. 1999).

Bacterial biomass was less affected by a shift away from an agricultural management regime than fungi. This is agreement with Klein et al. (1995) who suggested that abandonment of agricultural land and subsequent afforestation should not strongly affect that part of soil microbial community. However, based on our PLFA analysis, the amount of bacterial biomarkers increased with afforestation, which agrees with other findings (van der Wal et al. 2006). Also, there was a similar increase of G+ and G- biomarkers in the most forest plots (except three species mixtures with beech and birch+oak) (Fig. 3), which is in one line with data on similar portions of G+ and G- PLFAs found for the old growing oak and beech forests (Hackl et al. 2005). Increases in the G- bacterial biomarkers may be connected with the increasing the volume of rhizosphere in forest soils compare to agricultural (Thoms and Gleixner 2013), whereas increases in G+ biomarkers may occur due to intensive decomposition of C from previous land use.

366 The average increase of PLFAs associated with actinomycetes was 50-150% and was
367 detected only for pure birch stand and two species mixtures (the highest with presence of alder),
368 whereas for other plots they decreased or were similar to the agricultural soil (Fig. 3). Decrease in
369 actinomycete biomarker content is related to the increasing the content of fungal biomass which is
370 known to suppress the development of the actinomycete community (Lewandowski et al. 2015;
371 Boer et al. 2005). From this study we conclude that changes in the content of microbial biomarkers
372 following afforestation were greater compared to the major soil quality indicators. Afforestation
373 affected the development of fungal biomass to a greater degree than bacterial biomass. Shifts in the
374 content of particular biomarkers was found in all forest plots, suggesting that the amount of tree
375 species is not the main factor controlling soil microbial community changes. At the same time, the
376 relative increase in biomarker content was related to tree identity, revealing that individual tree
377 species promoted greater change relative to mixed-species forest. Further, no additive effects of
378 individual tree species were found.
379
380 ***Forest composition effects on soil microbial communities***
381 According to PCA analysis forest soil plots were different from the agricultural plot mainly
382 due to the fungal (18:2ω6,9 and 18:1ω9) PLFAs (Fig. 4, bottom). This is in accordance with general
383 increase of fungal biomarkers in forest soils (Fig. 3). Decrease of soil acidity contributed the most
384 to separation of forest and agricultural plots, which is frequently reported for forest soils (Scheibe et
385 al. 2015; van der Wal et al. 2006).
386 According to the PCA, one- and three species mixture forests were more similar in PLFA
387 composition than two species mixtures (Fig. 4, top). The most relevant groups in differentiation of
388 two species mixtures from monoculture and three species forests were 10Me16:0 and 10Me18:0,
389 common for actinomycetes (Zelles 1997) and branched PLFAs i16:0 and a16:0, common for G+
390 bacteria (Zelles 1997) (Fig. 4, bottom). The late successional tree species together with two early
391 primary successional species (three species mixture forests) stimulates development of microbial

communities similar to monoculture forests (Fig. 4, top). The most relevant PLFAs for separation of mono- and three species forests were fungal 18:1ω9 and cyclopropyl PLFAs cy17:0 and cy19:0 (Fig. 4, bottom).

Thus, the specific microbial community types were formed in the soils under the tested forest types already 10 years after planting. Similar microbial communities developed in soils under mono- and three species forest mixtures point on the absence of additive effect if two early primary successional species grow together. In contrast, simultaneous development of one early primary and one late successional tree species forms soil microbial communities with completely different composition.

Conclusions

Afforestation by one-, two- and three species mixtures with contrasting sets of functional traits, revealed the effects of trees identity and forest tree community composition on changes in soil chemistry and the structure of microbial communities. In support of our first hypothesis, total PLFA content increased more than 100% in forest soils compared to the agricultural, whereas changes in soil chemical properties (C and N contents, dissolved N forms) were altered to a lesser degree. Total PLFA contents for monocultural forests (except beech) were higher than for the mixtures, indicating that tree species identity has a stronger effect than number of species on the content of microbial biomarkers and no additive effects of increasing species number were observed.

The content of fungal biomarkers was changed by afforestation to much greater extent than for bacteria in agreement with our second hypothesis. Increase of particular biomarkers for all forests was independent of tree species amount, reflecting absence of additive effect of forest mixtures on the content of specific microbial biomarkers.

The PCA analysis revealed that two species mixtures were separated from one- and three species forests due to a higher abundance of actinomycetes and G+ bacterial biomarkers. In

contrast, microbial community composition for single species forests were similar to the three species mixtures, and could only be separated along PC2 due to a high abundance of G- bacterial biomarkers. Thus, development of forest monocultures, even formed by species having different functional traits promotes formation of similar microbial communities. In contrast, the simultaneous presence of early primary and late successional tree species stimulates the development of different community compositions, but this effect is dampened in mixtures of two early primary and late successional species.

Acknowledgements

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References

Ahmed IU, Smith AR, Jones DL, Godbold DL (2016) Tree species identity influences the vertical distribution of labile and recalcitrant carbon in a temperate deciduous forest soil. Special Section: Forests, Roots and Soil Carbon. Forest Ecology and Management 359:352–360. doi:10.1016/j.foreco.2015.07.018.

Apostel C, Dippold MA, Glaser B, Kuzyakov Y (2013) Biochemical pathways of amino acids in soil. Assessment by position-specific labeling and 13C-PLFA analysis. Soil Biology & Biochemistry 67:31–40.

Baum C, Toljander YK, Eckhardt K-U, Weih M (2009) The significance of host-fungus combinations in ectomycorrhizal symbioses for the chemical quality of willow foliage. Plant and Soil 323:213–224. doi:10.1007/s11104-009-9928-x.

Berthrong ST, Jobbaagy EG, Jackson RB (2009) A global meta-analysis of soil exchangeable cations, pH, carbon,and nitrogen with afforestation. Ecological Applications 19:2228–2241.

Boer WD, Folman LB, Summerbell RC, Boddy L (2005) Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiology Reviews 29:795–811. doi:10.1016/j.femsre.2004.11.005.

Buckley DH, Schmidt TM (2003) Diversity and dynamics of microbial communities in soils from agroecosystems. Environmental Microbiology 5:441–452.

Carson JK, Gleeson DB, Clipson N, Murphy DV (2010) Afforestation alters community structure of soil fungi. Fungal Biology 114:580–584. doi:10.1016/j.funbio.2010.04.008.

Chapin FS, Walker LR, Fastie CL, Sharman LC (1994) Mechanisms of Primary Succession Following Deglaciation at Glacier Bay, Alaska. Ecological Monographs 64:149–175.

Cong J, Yang Y, Liu X, Lu H, Liu X, Zhou J, Li D, Yin H, Ding J, Zhang Y (2015) Analyses of soil microbial community compositions and functional genes reveal potential consequences of natural forest succession. Scientific reports 5:10007. doi:10.1038/srep10007.

DeLuca TH, Boisvenue C (2012) Boreal forest soil carbon: distribution,function and modelling. Forestry doi:10.1093/forestry/cps003.

Frostegard A, Tunlid A, Baath E (1991) Microbial biomass measured as total lipid phosphate in soils of different organic content. Journal of Microbiological Methods 14:151–163. doi:10.1016/0167-7012(91)90018-l.

Frouz J, Voborilová V., Janoušová I., Kadochová Š, Matejícek L (2015) Spontaneous establishment of late successional tree species Englishoak (Quercus robur) and European beech (Fagus sylvatica) at reclaimed alder plantation and unreclaimed post mining sites. Ecological Engineering 77:1–8.

Fu X, Yang F, Wang J, Di Y, Dai X, Zhang X, H W (2015) Understory vegetation leads to changes in soil acidity and in microbial communities 27 years after reforestation. Science of the Total Environment 502:280–286.

Glanville H, Rousk J, Golyshin P, Jones DL (2012) Mineralization of low molecular weight carbon substrates in soil solution under laboratory and field conditions. Soil Biology & Biochemistry 48:88–95. doi:10.1016/j.soilbio.2012.01.015.

Grayston SJ, Vaughan D, Jones D (1997a) Rhizosphere carbon flow in trees, in comparison with annual plants. The importance of root exudation and its impact on microbial activity and nutrient availability. Applied Soil Ecology 5:29–56. doi:10.1016/s0929-1393(96)00126-6.

Grayston SJ, Vaughan D, Jones D (1997b) Rhizosphere carbon flow in trees, in comparison with annual plants. The importance of root exudation and its impact on microbial activity and nutrient availability. Applied Soil Ecology 5:29–56. doi:10.1016/s0929-1393(96)00126-6.

503 Gunina A, Dippold M, Glaser B, Kuzyakov Y (2014) Fate of low molecular weight organic
504 substances in an arable soil. From microbial uptake to utilisation and stabilisation. Soil Biology
505 & Biochemistry 77:304–313.

506 Hackl E, Pfeffer M, Donat C, Bachmann G, Zechmeister-Boltenstern S (2005) Composition of the
507 microbial communities in the mineral soil under different types of natural forest. Soil Biology &
508 Biochemistry 37:661–671.

509 Helgason BL, Walley FL, Germida JJ (2009) Fungal and bacterial abundance inlong-term no-till
510 and intensive-till soils of the Northern Great Plains. Soil Sci. Soc. Am. J. 73:120–127.

511 Jangid K, Williams MA, Franzluebbers AJ, Schmidt TM, Coleman DC, Whitman WB (2011) Land-
512 use history has a stronger impact on soil microbial communitycomposition than aboveground
513 vegetation and soil properties. Soil Biology & Biochemistry xxx:1–10.

514 Jobbágy EG, Jackson RB (2003) Patterns and mechanisms of soil acidification in the conversion of
515 grasslands to forests. Biogeochemistry 64:205–229. doi:10.1023/A:1024985629259.

516 Kalinina O, Krause SE, Goryachkin SV, Karavaeva NA, Lyuri DI, Giani L (2011) Self restoration
517 of post-agrogenic chernozems of Russia: soil development, carbon stocks, and dynamics of
518 carbon pools. Geoderma 162:196–206.

519 Klein DA, McLendon T, Paschke MW, Redente EE (1995) Saprophytic fungal-bacterial biomass
520 variations in successional communities of a semi-arid steppe ecosystem. Biology and Fertility of
521 Soils 19:253–256.

522 Kurganova I, Lopes De Gerenyu V, Kuzyakov Y (2015) Large-scale carbon sequestration in post-
523 agrogenic ecosystems in Russia and Kazakhstan. Catena 133:461–466.
524 doi:10.1016/j.catena.2015.06.002.

525 Laganière J, Paré D, Bergeron Y, Chen H (2012) The effect of boreal forest composition on soil
526 respiration is mediated throughvariations in soil temperature and C quality. Soil Biology &
527 Biochemistry 53:18–27.

528 Leckie SE (2005) Methods of microbial community profiling and their application to forest soils.
529 Forest Ecology and Management 220:88–106.

530 Lewandowski TE, Forrester JA, Mladenoff DJ, Stoffel JL, Gower ST, D'Amato AW, Balser TC
531 (2015) Soil microbial community response and recovery following group selection harvest.
532 Temporal patterns from an experimental harvest in a US northern hardwood forest. Forest
533 Ecology and Management 340:82–94.

534 Li H, Ye D, Wang X, Settles ML, Wang J, Hao Z, Zhou L, Dong P, Jiang Y, Ma Z Soil bacterial
535 communities of different natural forest types in Northeast China. Plant and Soil 383:203–216.
536 doi:10.1007/s11104-014-2165-y.

537 Li M, Zhou X, ZHANG Q, Cheng X (2014) Consequences of afforestation for soil nitrogen
538 dynamics in central China. Agriculture, Ecosystems and Environment 183:40–46.

539 Macdonald CA, Thomas N, Robinson L, Tate KR, Ross DJ, Dando J, Singh BK (2009)
540 Physiological, biochemical and molecular responses of the soil microbial community after
541 afforestation of pastures withPinus radiata. Soil Biology & Biochemistry 41:1642–1651.

542 Madan R, Pankhurst C, Hawke B, Smith S (2002) Use of fatty acids for identification of AM fungi
543 and estimation of the biomass of AM spores in soil. Soil Biology & Biochemistry 34:125–128.
544 doi:10.1016/s0038-0717(01)00151-1.

545 Mann L, Tolbert V (2000) Soil sustainability in renewable biomass plantings. Ambio 29:492–498.

546 Mulvaney RL (1996) Methods of Soil Analysis. Part 3 – Chemical Methods. Nitrogen – Inorganic
547 Forms. Soil Science Society of America, Inc., Wisconsin, USA.

548 Nazaries L, Tottey W, Robinson L, Khachane A, Al-Soudd WA, Sørensen S, Singh BK (2015)
549 Shifts in the microbial community structure explain the response of soil respiration to land-use
550 change but not to climate warming. Soil Biology & Biochemistry 89:123–134.

551 Nichols P, Stulp BK, Jones JG, White DC (1986) Comparison of fatty acid contentand DNA
552 homology of the filamentous gliding bacteria Vitreoscilla, Flexibacter,Filibacter. Archives of
553 Microbiology 146:1–6.

554 Paul KI, Polglase PJ, Nyakuengama JG, Khanna PK (2002) Change in soil carbon following
555 afforestation. Forest Ecology and Management 168:241–257. doi:10.1016/S0378-
556 1127(01)00740-X.

557 Priha O, Grayston SJ, Pennanen T, Smolander A (1999) Microbial activities related to C and N
558 cycling and microbial community structure in the rhizospheres ofPinus sylvestris, Picea
559 abiesandBetula pendulaseedlings in an organic and mineral soil. Fems Microbiology Ecology
560 30:187–199.

561 Ritter E, Vesterdal L, Gundersen P (2003) Changes in soil properties after afforestation of former
562 intensively managed soils with oak and Norway spruce. Plant and Soil 249:319–330.

563 Rousk J, Baath E (2007) Fungal biomass production and turnover in soil estimated using the
564 acetate-in-ergosterol technique. Soil Biology & Biochemistry 39:2173–2177.
565 doi:10.1016/j.soilbio.2007.03.023.

566 Scheibe A, Steffens C, Seven J, Jacob A, Hertel D, Leuschner C, Gleixner G (2015) Effects of tree
567 identity dominate over tree diversity on the soil microbial community structure. Soil Biology &
568 Biochemistry 81:219–227.

569 Smith A., Lukac M., Hood R., Healey J.R., Miglietta F., Godbold D.L. (2013) Elevated CO2
570 enrichment induces a differential biomass response in a mixed species temperate forest
571 plantation. New Phytologist 198:156–168.

572 Strickland MS, Rousk J (2010) Considering fungal. bacterial dominance in soils - Methods,
573 controls, and ecosystem implications. Soil Biology & Biochemistry 42:1385–1395.
574 doi:10.1016/j.soilbio.2010.05.007.

575 Swift MJ, Heal OW, Anderson JM (1979) Decomposition in Terrestrial Ecosystems. Oxford.

576 Thoms C, Gleixner G (2013) Seasonal differences in tree species' influence on soil microbial
577 communities. Soil Biology & Biochemistry 66:239–248.

578 Trap J, Hättenschwiler S, Gattin I, Aubert M (2013) Forest ageing: An unexpected driver of beech
579 leaf litter quality variability in European forests with strong consequences on soil processes.
580 Forest Ecology and Management 302:338–345.

581 Uri V, Lohmus K, Tullus H (2003) Annual net nitrogen mineralization in a grey alder (Alnus
582 incana(L.) moench) plantation on abandoned agricultural land. Forest Ecology and Management
583 184:167–176.

584 van der Wal A, van Veen JA, Smant W, Boschker H, Bloem J, Kardol P, van der Putten WH, Boer
585 W de (2006) Fungal biomass development in a chronosequence of land abandonment. Soil
586 Biology & Biochemistry 38:51–60.

587 Walker LR, Chapin FS (1986) Physiological controls over seedling growth in primary succession
588 on an Alaskan flood plain. Ecology 67:1508–1523.

589 Yannikos N, Leinweber P, Helgason BL, Baum C, Walley FL, van Rees K (2014) Impact of
590 Populus trees on the composition of organic matter and the soil microbial community in Orthic
591 Gray Luvisols in Saskatchewan (Canada). Soil Biology & Biochemistry 70:5–11.

Zeller V, Bardgett RD, Tappeiner U (2001) Site and management effects on soil microbial properties of subalpine meadows: a study of land abandonment along a north–south gradient in the European Alps. Soil Biology and Biochemistry 33:639–649. doi:10.1016/S0038-0717(00)00208-X.

Zelles L (1997) Phospholipid fatty acid profiles in selected members of soil microbial communities. Chemosphere 35:275–294. doi:10.1016/s0045-6535(97)00155-0.

Table captions

Table 1. Properties of the forest tree species.

Table 2. Ratios of saturated/monounsaturated (sat/mono) PLFAs (calculated as 14:0+15:0+16:0+17:0+18:0/16:1ω5+16:1ω7+18:1ω7+18:1ω9), cyclo/precursors (cy/prec) PLFAs (calculated as cy17:0+cy19:0/16:1ω7+18:1ω7) and fungal/bacterial (f/b) (calculated as 18:2ω6,9/ i15:0+a15:0+15:0+i16:0+16:1ω7+i17:0+a17:0+cy17:0+cy19:0+18:1ω7) for soils under the different forest treatments and the agricultural plots. Data present mean ± st. error, n = 4. Forest treatments: Al (alder), Bi (birch), Be (beech), Oa (oak), ABe (alder+beech), AOa (alder+oak), BiBe (birch+beech), BiOa (birch+oak), ABiBe (alder+birch+beech), ABiOa (alder+birch+oak). Agr - agricultural plot.

Figure captions

Fig 1. Changes of soil chemical properties in the various forest treatments relative to the agricultural soil (Agr). Data present mean±st. error, n=4. Letters above error bars present significant differences ($p < 0.05$) between the treatments for the each parameter separately. Red letters are for C/N ratios, blue letter are for C and green are for N. In case of pH no statistical differences between the forests were found, only differences between forest and agricultural soil was found. Forest treatments: Al

624 (alder), Bi (birch), Be (beech), Oa (oak), ABe (alder+beech), AOa (alder+oak), BiBe (birch+beech),
625 BiOa (birch+oak), ABiBe (alder+birch+beech), ABiOa (alder+birch+oak).
626
627 Fig 2. Content of total PLFAs (nmol g^{-1} soil) in the different forest treatments and the agricultural
628 soil. Data present mean±st error, *n*=4. Letters above error bars present significant differences ($p<$
629 0.05) between the treatments. Forest treatments: Al (alder), Bi (birch), Be (beech), Oa (oak), ABe
630 (alder+beech), AOa (alder+oak), BiBe (birch+beech), BiOa (birch+oak), ABiBe
631 (alder+birch+beech), ABiOa (alder+birch+oak).
632
633 Fig 3. Changes in the content (n mol g^{-1} soil) of specific microbial indicators PLFAs in the different
634 forest treatments relative to the agricultural soils, presented as portion of changes. Data present
635 mean±st. error, *n*=4. Letters above error bars present significant differences ($p< 0.05$) between the
636 plots for the each group separately. Top figure - red letters are for G- bacterial PLFAs, black letters
637 are for G+ PLFAs; bottom figure - violett letters are for fungal PLFAs, black are for 16:1w5 PLFA
638 and green are for actinomycetes PLFAs. Forest treatments: Al (alder), Bi (birch), Be (beech), Oa
639 (oak), ABe (alder+beech), AOa (alder+oak), BiBe (birch+beech), BiOa (birch+oak), ABiBe
640 (alder+birch+beech), ABiOa (alder+birch+oak).
641
642 Fig. 4. Score plot of PCA presenting the separation of mono- and mixture species forests along the
643 principal component PC1 and PC2 (top) and loading values for the PLFAs (bottom). Forest
644 treatments: Al (alder), Bi (birch), Be (beech), Oa (oak), ABe (alder+beech), AOa (alder+oak), BiBe
645 (birch+beech), BiOa (birch+oak), ABiBe (alder+birch+beech), ABiOa (alder+birch+oak). Colors
646 for the loading values of PLFAs indicate the following: red – Gram-negative bacterial, yellow –
647 universal microbial biomarker, green - actinomycetes, blue – Gram-positive bacteria, violet – fungi.
648
649
650

Table 1

Table 1. Properties of the forest tree species.

Plant species	English oak	European beech	Silver birch	European alder
Succession stage	Late	Late	Early primary	Early primary
Mycorrhization degree	High	High	High	Weak
Type of mycorrhization	Ecto	Ecto	Ecto	Ecto- and arbuscular
C/N ratio of plant litter	38.73	71.67	31.52	21.23

Table. 2

Table 2. Ratios of saturated/monounsaturated (sat/mono) PLFAs (calculated as 14:0+15:0+16:0+17:0+18:0/16:1ω5+16:1ω7+18:1ω7+18:1ω9), cyclo/precursors (cy/prec) PLFAs (calculated as cy17:0+cy19:0/16:1ω7+18:1ω7) and fungal/bacterial (f/b) (calculated as 18:2ω6,9/ i15:0+a15:0+15:0+i16:0+16:1ω7+i17:0+a17:0+cy17:0+cy19:0+18:1ω7) for soils under the different forest treatments and the grassland control plots. Data present mean ± st. error, *n* = 4. Forest treatments: Al (alder), Bi (birch), Be (beech), Oa (oak), ABe (alder+beech), AOa (alder+oak), BiBe (birch+beech), BiOa (birch+oak), ABiBe (alder+birch+beech), ABiOa (alder+birch+oak); Agr - agricultural plot.

Forest	A	Bi	Be	Oa	ABe	AOa	BiBe	BiOa	ABiBe	ABiOa	Agr
sat/mono	0.65±0.01	0.62±0.01	0.69±0.04	0.68±0.05	0.7±0.03	0.78±0.02	0.63±0.03	0.72±0.03	0.58±0.02	0.63±0.03	0.69±0.01
cy/prec	0.49±0.02	0.55±0.02	0.59±0.03	0.53±0.04	0.5±0.05	0.58±0.03	0.51±0.03	0.47±0.02	0.55±0.03	0.55±0.02	0.41±0.01
f/b	0.043±0.006	0.063±0.004	0.05±0.004	0.054±0.007	0.069±	0.062±0.003	0.076±0.013	0.074±0.004	0.064±0.01	0.055±0.009	0.033±0.001

Fig. 1

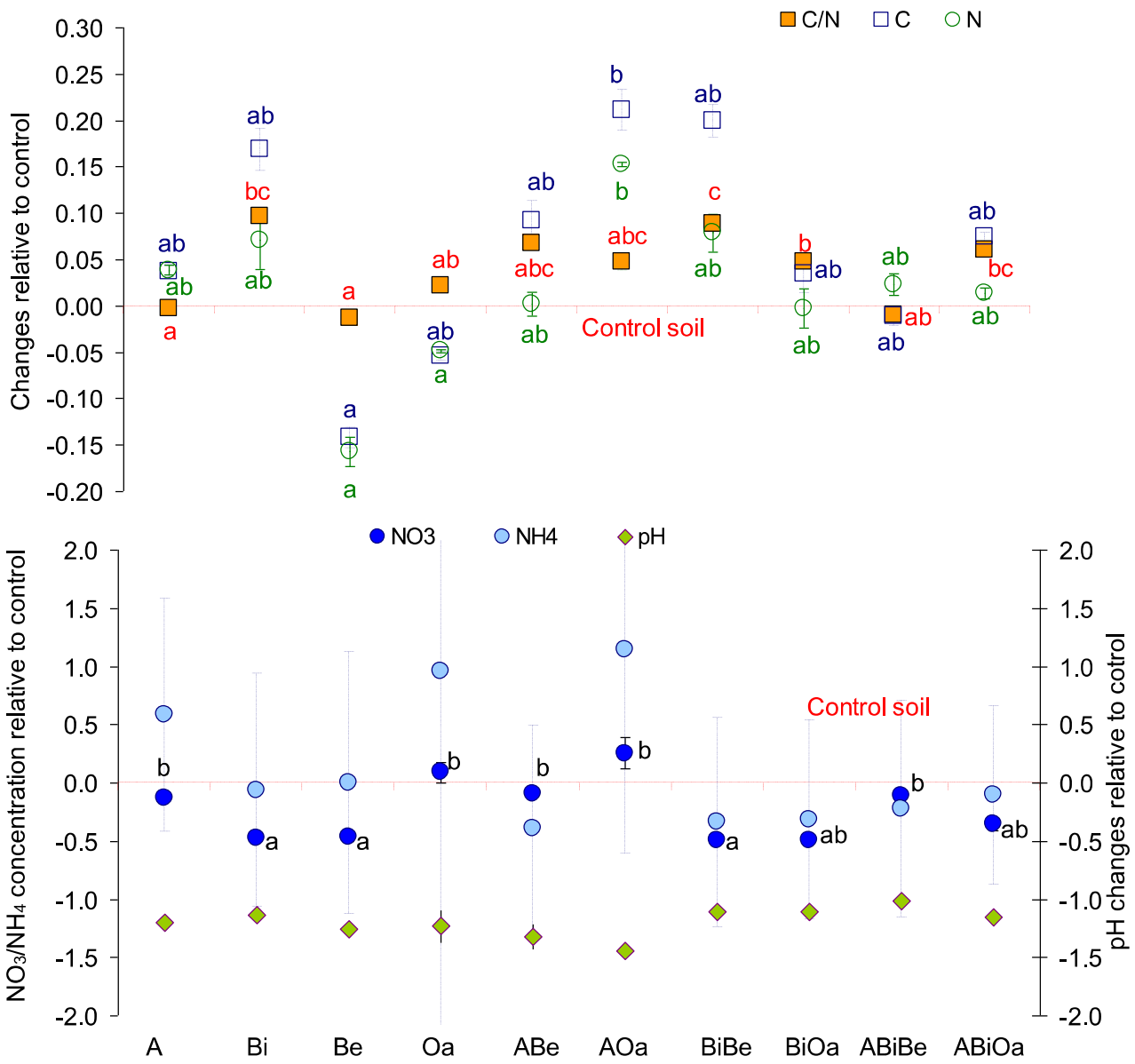


Fig. 2

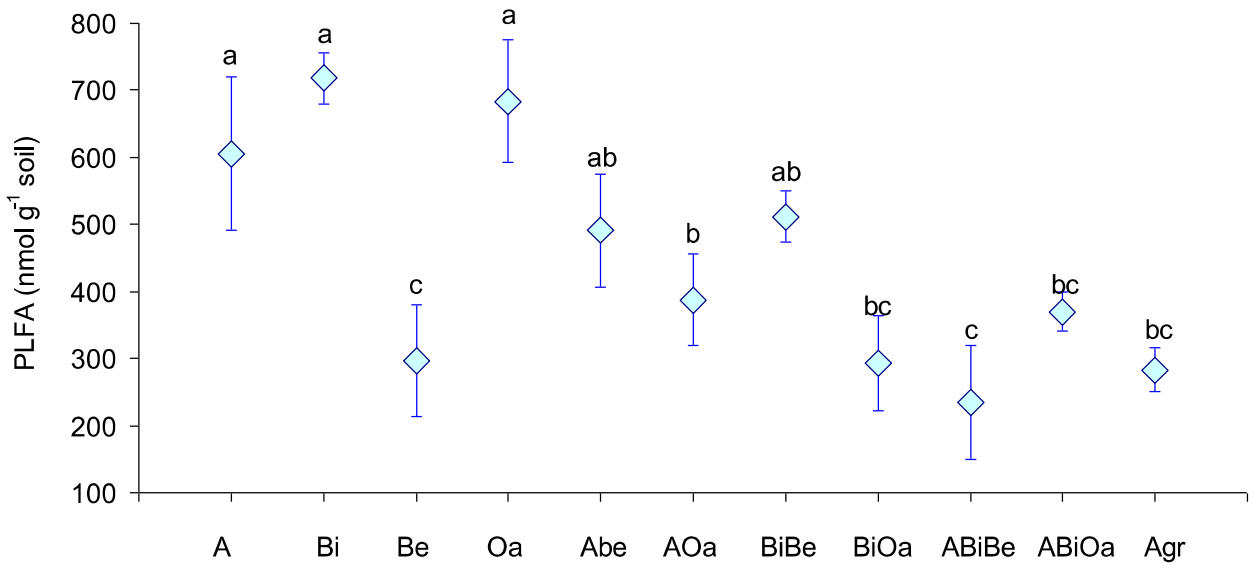

PLFA (nmol g-1 soil)
800
700
600
500
400
300
200
100
a
a
c
a
ab
b
ab
bc
c
bc
bc
A
Bi
Be
Oa
Abe
AOa
BiBe
BiOa
ABiBe
ABiOa
Agr

Fig. 3

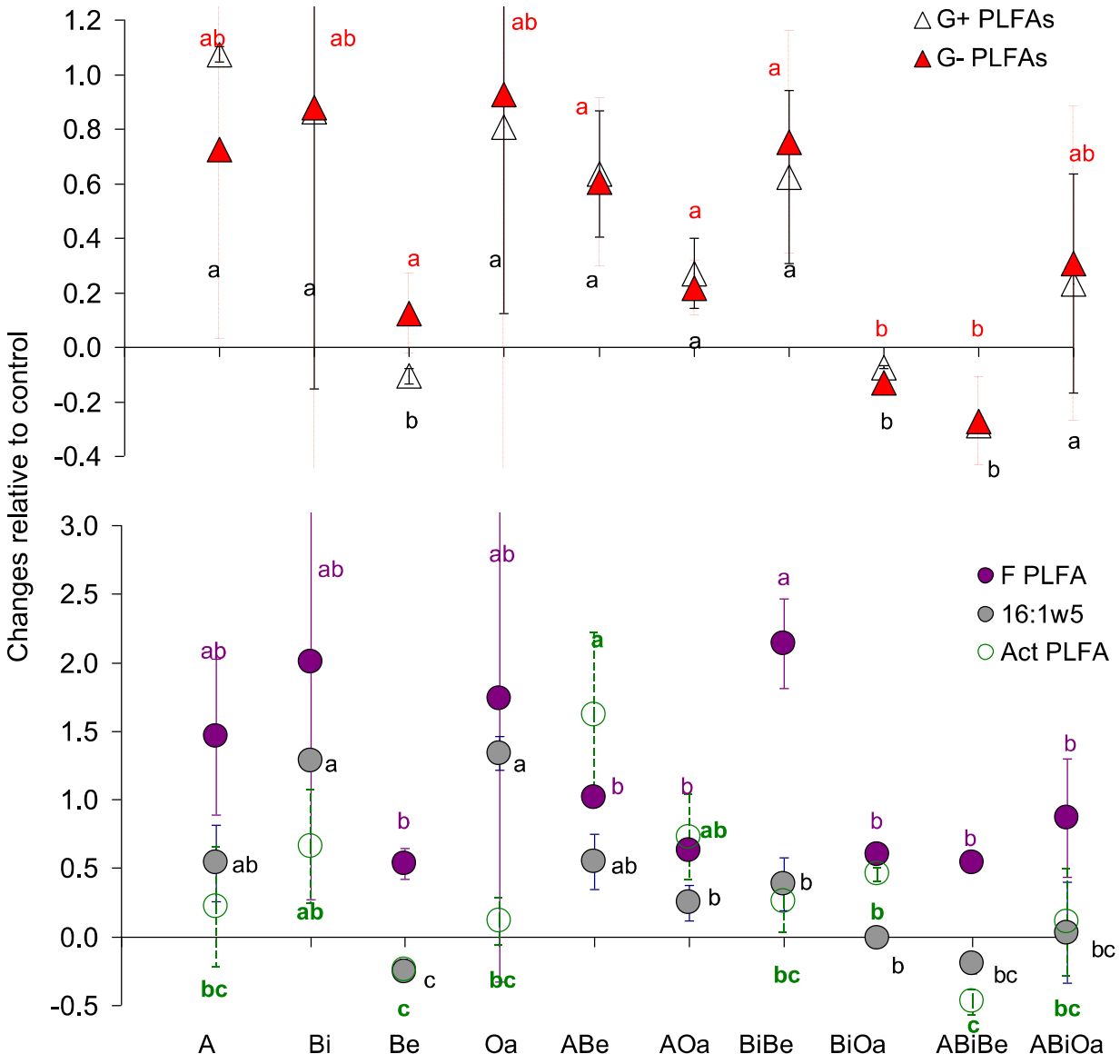
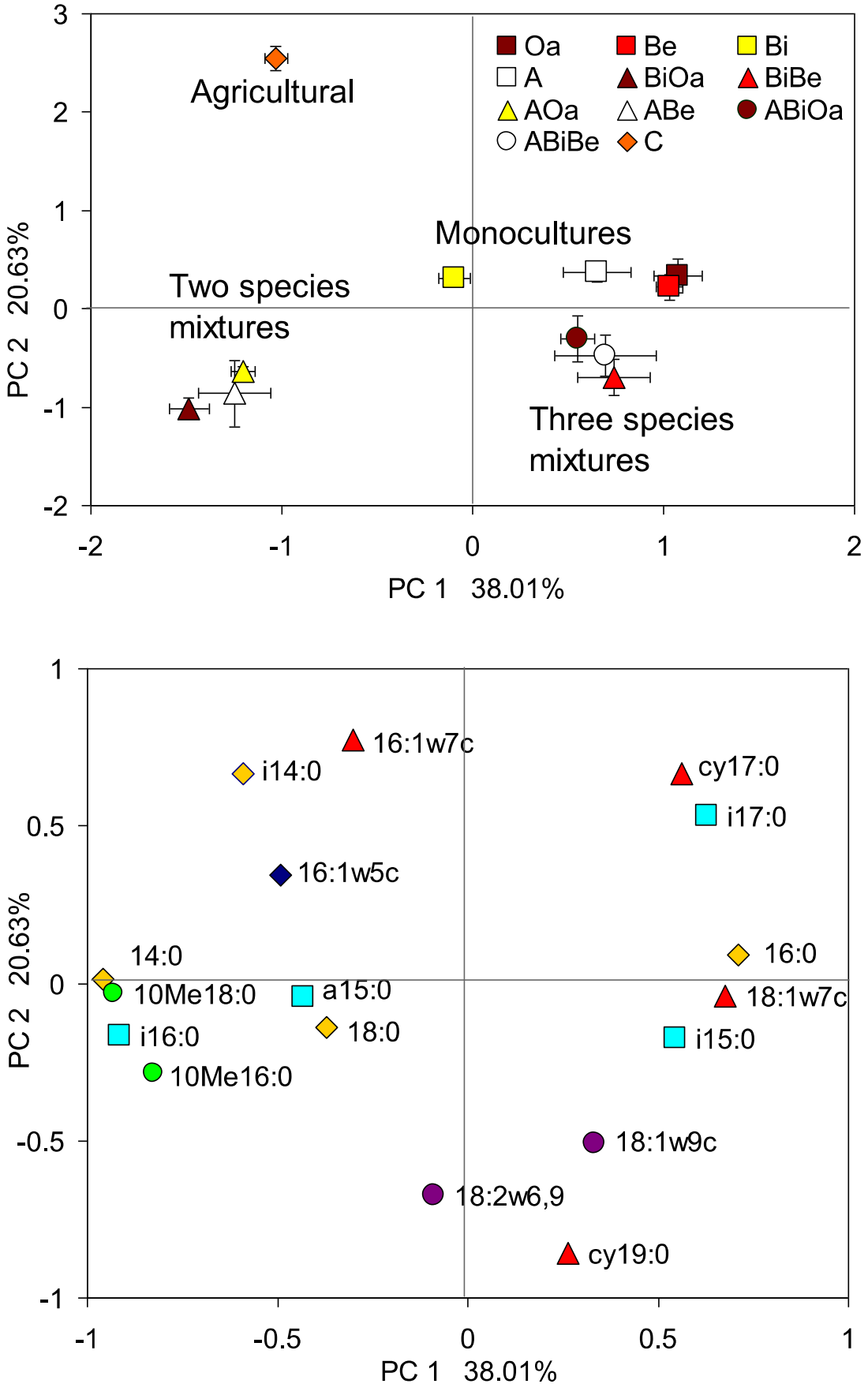

Changes relative to control
G+ PLFAs
G- PLFAs
F PLFA
16:1w5
Act PLFA
A
Bi
Be
Oa
ABe
AOa
BiBe
BiOa
ABiBe
ABiOa

Fig. 4


Oa
Be
Bi
A
BiOa
BiBe
AOa
ABe
ABiOa
ABiBe
C
Agricultural
Monocultures
Two species mixtures
Three species mixtures
PC 2 20.63%
PC 1 38.01%
16:1w7c
i14:0
cy17:0
i17:0
16:1w5c
14:0
16:0
10Me18:0
a15:0
18:1w7c
i16:0
18:0
i15:0
10Me16:0
18:1w9c
18:2w6,9
cy19:0

Electronic supplementary material