

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 Bangor DIVERSE 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.

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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 (Novara et al. 2014) 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; Sommer et al. 2016). 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 and 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 microbial communities (Fu et al. 2015). Thus, due to a variety of complex interacting factors, it is difficult to disentangle the direct effects of forest tree community composition from

the effect of soil properties on microbial community dynamics, especially under mature forests, where soil chemical properties may have already been changed. Further, it is difficult to distinguish between tree identity and forest tree community composition effects, because functional traits of single tree species can be masked or reduced in forest mixtures. Thus, only in experiments where both single species and mixtures of trees are studied simultaneously in the early afforestation stage can conclusions about the effect of tree identity and forest composition on the formation of soil microbial communities be made.

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

Materials and methods

Study site and soil sampling

Soils were obtained from the BangorDIVERSE forest experiment located at the Henfaes Research Centre, North Wales, UK (53°14'N, 4°01'W). Climate was characterized as hyperoceanic, with mean annual precipitation of 1034 mm and mean annual temperature of 11.5 °C (Campbell Scientific Ltd., Shepshed, UK). The site was set up in 2004 with a total area of 2.36 ha. Soils are classified as Eutric Fluvisols Cambisols (WRB 2006) (Fluventic Dystrachrept, USDA system) and have fine loamy texture (Smith et al. 2013). Each type of forests, namely: single species or two 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 m² 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 K₂O (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 100 g of fresh soil samples. The concentration of NH₄⁺ in soil solution was determined colorimetrically using the sodium-nitroprusside, while NO₃⁻ was determined colorimetrically using the VCl₃ (both procedures described in Mulvaney (1996)).

Phospholipid fatty acids analysis

Phospholipid fatty acids (PLFAs) were extracted from the soil samples according to Frostegard et al. (1991). Briefly, 4.5 g of fresh soil were placed into 50 ml centrifuge tubes, 25 µl of internal standard one added (1 µg µl⁻¹, 19:0 phospholipid) and lipids extracted twice (18 and 6 ml, respectively) by one phase mixture of chloroform, methanol and citric acid (0.15 M, pH 4.0) in the ratio 1:2:0.8 (v/v/v). Extracted lipids

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

were applied to the silica column and neutral-, glyco- and phospholipids were sequentially eluted from the column by chloroform (5 ml), acetone (20 ml) and methanol (20 ml), respectively. Collected phospholipids were saponified (0.3 M solution of BF_3 in methanol), obtained fatty acids were methylated (1 M solution of NaOH in methanol) and extracted in hexane. Finally, the samples were dried under a stream of N_2 and redissolved in toluene (185 μl) with addition of internal standard two (15 μl of 13:0 fatty acid methyl ester, 1 $\mu\text{g } \mu\text{l}^{-1}$).

The PLFAs were measured by GC-MS, having the following parameters: columns (15 m HP-1 methylpolysiloxane coupled with a 30 m HP-5 (5 % phenyl)-methylpolysiloxane column (both with an internal diameter of 0.25 mm and a film thickness of 0.25 μm)), He flow of 2 ml min^{-1} , and injection volume of 1 μl . The temperature program of GC-MS was set up to 80 °C and then ramped to 164 °C at 10 °C min^{-1} , then to 230 °C at 0.7 °C min^{-1} and finally to 300 °C at 10 °C min^{-1} . The quantity of PLFAs was calculated based on the 29 external standards (Gunina et al. 2014), which were prepared in 6 concentrations (Apostel et al. 2013). Final content of single PLFAs was presented as molar percentages (mol %) and total content was presented as nmol g^{-1} soil. Classification of PLFAs was done according to existing data on their presence in various groups of microorganisms: for Gram-negative (G-) bacteria the 16:1 ω 7c, cy17:0, 18:1 ω 7c, cy19:0 PLFAs were used (Leckie 2005; Lewandowski et al. 2015), for Gram-positive bacteria (G+) i15:0, a15:0, i16:0, i17:0 PLFAs were used (Leckie 2005; Lewandowski et al. 2015), for actinomycetes (Ac) 10Me16:0 and 10Me18:0 were used (Lewandowski et al. 2015; Leckie 2005), for fungi 18:2 ω 6 + 18:1 ω 9c were used and 16:1 ω 5c was assumed as arbuscular mycorrhiza (AM) fungi PLFA, but with caution due to its high possible input from G- bacterial biomass (Leckie 2005; Lewandowski et al. 2015).

Statistical analysis

To compare the effect of forest development on soil chemical properties and on microbial biomarkers contents, changes of all parameters were calculated relatively to agricultural soil. Changes of the soil chemical properties (except pH) relatively to the agricultural soil have been calculated as:

$$\frac{Cp_f - Cp_{agr}}{Cp_{agr}}$$

where, Cp_f and Cp_{agr} are the values of chemical properties in the forest and agricultural soils, respectively. For pH absolute changes were calculated by subtracting pH of agricultural soil from pH of forest soils.

The increase of PLFAs of distinct groups relatively to agricultural plot was calculated as:

$$\frac{PLFA_f - PLFA_{agr}}{PLFA_{agr}}$$

where, $PLFA_f$ and $PLFA_{agr}$ are the contents of PLFAs of specific microbial groups in forest and agricultural soils (nmol g^{-1} soil), respectively. Data were checked for the normal distribution and homogeneity was tested by Levene's test. Calculated values were tested with one-way ANOVA and significant differences were obtained with Notched Box Plots.

Principal component analysis (PCA) of mol% of individual PLFAs was done to elucidate major variation pattern. The scores of the first two components from the PCA were used to separate the soils formed under various forests. Linear regression of PLFAs factor scores and soil properties (pH, total C and N, concentration of NH_4^+ and NO_3^-) was done to conclude about the correlation of PLFAs composition with environmental factors depending on the forest type. Statistical analyses were done in Statistica 12.0 and Microsoft Excel 2010.

Results

Afforestation effects on soil properties

Afforestation had weak effect on the C content: the maximal changes of soil C content was ca. 20 % relative to the agricultural soil (Fig. 1), and was maximal for the birch, alder + oak and birch + beech plots. However, C stocks in the upper 10 cm under pure oak, beech, two species mixtures with oak and three species mixtures were lower compared to the agricultural soil (Supplementary Table 2), mainly because of the low bulk density of the forest soils (it varied between 0.7–1 g cm⁻³ for forest soils and was 1.2 g cm⁻³ for the agricultural soil).

The effect of forest development on soil N content (Fig. 1) followed the same tendency as on C content, despite the contrasting N content of the various forest litters (Table 1). In general, changes of total N content in the forest soils were similar and ranged within ±15 %. The organic matter quality, characterized by C/N ratio, was the most strongly affected for the pure birch, birch + beech plots and alder + beech, where it had the highest increase relative to agricultural soil (Fig. 1).

10 years of afforestation decreased soil acidity by 1.0–1.2 units compared to the agricultural plot (Fig. 1).

The NO₃⁻ concentrations in soil solution decreased for the birch, beech and two forest mixtures with birch

compare to the agricultural soil (Fig. 1). In contrast, NH₄⁺ did not differ in the agricultural and forest soils (Fig. 1).

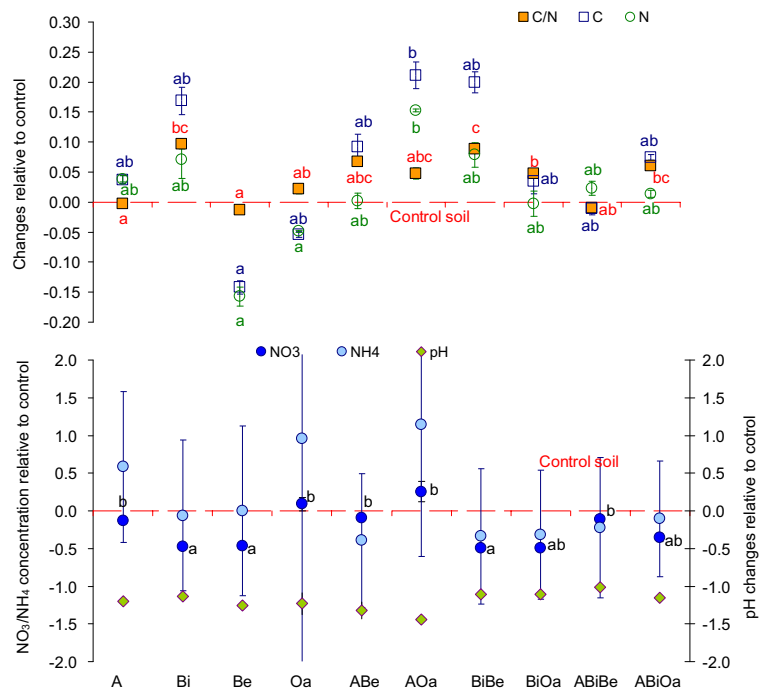
Afforestation effects on total PLFAs content

Maximal contents of total microbial PLFAs were observed for the oak, birch and alder forest soils (Fig. 2). Total PLFA contents were higher for the oak, birch and alder monocultures forests compare to pure beech forest, whereas no differences were found between the two and three species mixtures. In the case of the two species mixtures where beech was present, total PLFA content increased relative to the beech monocultures, whereas, the opposite trends were observed for the pure oak forest and two species mixtures containing oak.

Afforestation effects on the content of specific microbial biomarkers

Afforestation increased fungal PLFAs content the most compared to other biomarkers, and were 50–200 % higher in the forest soils compared to the agricultural (Fig. 3). The maximal increase was found for the soils under birch, oak, alder and birch + beech. The two and three species forests increased their fungal biomarker content by 50–100 %.

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 (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)



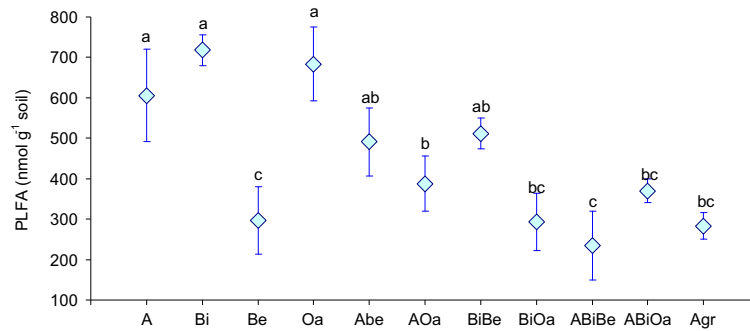


Fig. 2 Content of total PLFAs (nmol g^{-1} soil) in the different forest treatments and the agricultural soil. Data present mean \pm st error, $n = 4$. Letters above error bars present significant differences ($p < 0.05$) between the treatments. Forest treatments: A1 (alder), Bi

(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)

Bacterial biomarkers increased in forest soils (except beech, three species mixture with beech and birch + oak) by 20 to 110 % compared to the arable soil but without differences in the G+ and G- groups (Fig. 3). The content of G+ bacterial PLFAs were low for the monocultural beech forest, but increased for the two species

mixtures with beech. In contrast, the content of G+ PLFAs were higher for the monocultural oak forests, than for the birch + oak mixed forest.

Relative to the agricultural, the content of 16:1 ω 5 PLFAs (AM fungal or G- bacterial biomarker) increased by 30–120 % (Fig. 3) and the increase was higher under

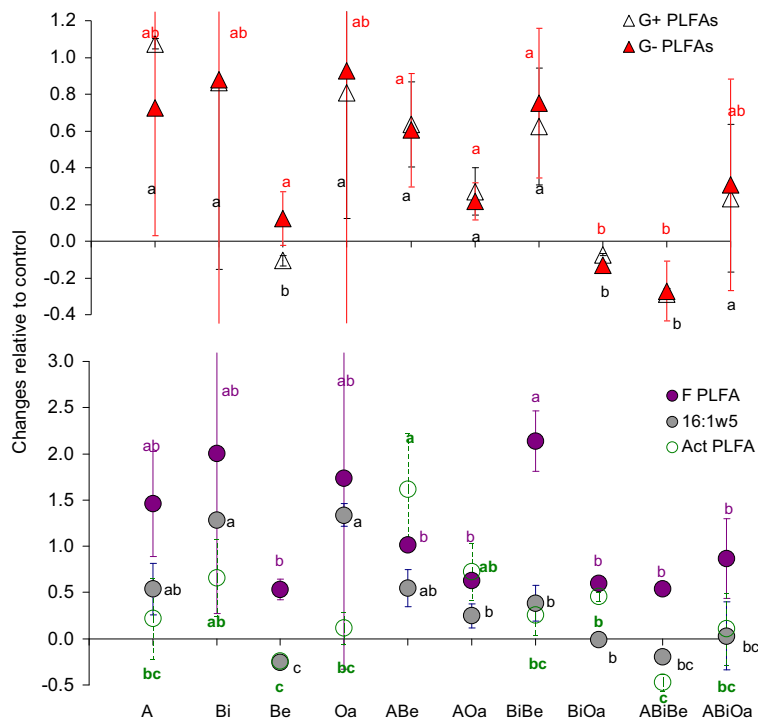


Fig. 3 Changes in the content (n mol g^{-1} soil) of specific microbial indicators PLFAs in the different forest treatments relative to the agricultural soils, presented as portion of changes. Data present mean \pm st. error, $n = 4$. Letters above error bars present significant differences ($p < 0.05$) between the plots for the each group separately. Top figure - red letters are for G- bacterial PLFAs, black

letters are for G+ PLFAs; bottom figure - violet letters are for fungal PLFAs, black are for 16:1 ω 5 PLFA and green are for actinomycetes PLFAs. Forest treatments: A1 (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)

the birch and oak treatments than for any other soils. Both beech alone and in three species mixtures forests containing beech resulted in a decline of 16:1 ω 5 PLFAs relative to the agricultural soil. The content of actinomycete PLFAs followed the same trend as 16:1 ω 5 PLFA, however, the highest increase was found for the alder + beech plot.

PCA analysis of the PLFA data revealed that the first two PCA components explained 38 and 21 % of the PLFA variation, respectively (Fig. 4). The first PCA component reflects differences in soil pH ($r^2 = 0.32$; linear regression of scores for PC1 vs. soil pH) and was correlated with saturated/monounsaturated ratio

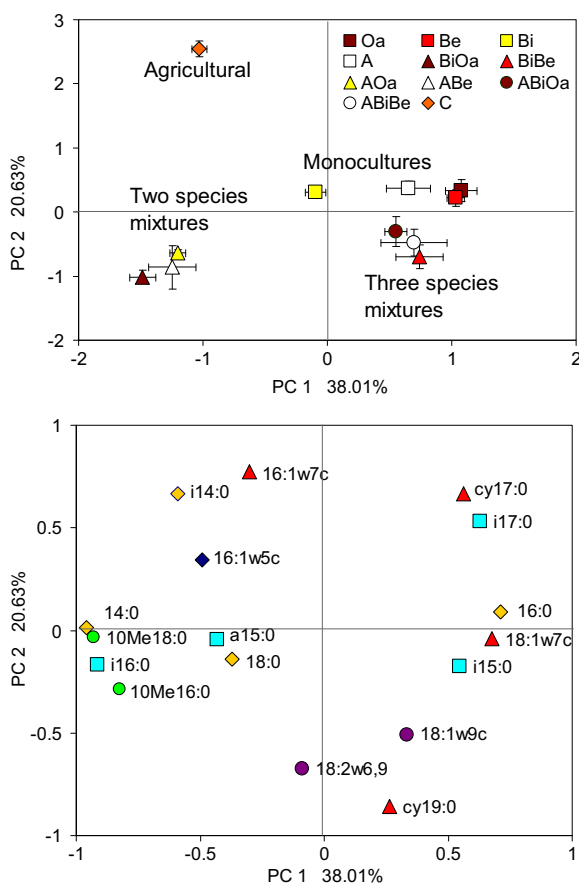


Fig. 4 Score plot of PCA presenting the separation of mono- and mixture species forests along the principal component PC1 and PC2 (top) and loading values for the PLFAs (bottom). Forest treatments: A1 (alder), Bi (birch), Be (beech), Oa (oak), ABe (alder + beech), AOa (alder + oak), BiBe (birch + beech), BiOa (birch + oak), ABiBe (alder + birch + beech), ABiOa (alder + birch + oak). Colors for the loading values of PLFAs indicate the following: red – Gram-negative bacterial, yellow – universal microbial biomarker, green – actinomycetes, blue – Gram-positive bacteria, violet – fungi

($r^2 = 0.45$). The second PCA component was correlated with 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 (18:1 ω 7, cy17:0, i15:0 and i17:0) contributed to the separation of forest soils from the agricultural plot along PC1, whereas fungal (18:2 ω 6,9 and 18:1 ω 9) 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, 16:1 ω 5 and 16:1 ω 7 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, 18:1 ω 9) 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 this new substrate, and at the same time b) intensive decomposition of the intrinsic

Table 2 Ratios of saturated/monounsaturated (sat/mono) PLFAs (calculated as 14:0 + 15:0 + 16:0 + 17:0 + 18:0/16:1 ω 5 + 16:1 ω 7 + 18:1 ω 7 + 18:1 ω 9), cyclo/precursors (cy/prec) PLFAs (calculated as cy17:0 + cy19:0/16:1 ω 7 + 18:1 ω 7) and fungal/bacterial (f/b) (calculated as 18:2 ω 6,9/11:5:0 + a15:0 + 15:0 + i16:0 + 16:1 ω 7 + i17:0 + a17:0 + cy17:0 + cy19:0 + 18:1 ω 7) for soils under the different forest treatments and the agricultural soil. Data present mean \pm 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 \pm 0.01	0.62 \pm 0.01	0.69 \pm 0.04	0.68 \pm 0.05	0.7 \pm 0.03	0.78 \pm 0.02	0.63 \pm 0.03	0.72 \pm 0.03	0.58 \pm 0.02	0.63 \pm 0.03	0.69 \pm 0.01
cy/prec	0.49 \pm 0.02	0.55 \pm 0.02	0.59 \pm 0.03	0.53 \pm 0.04	0.5 \pm 0.05	0.58 \pm 0.03	0.51 \pm 0.03	0.47 \pm 0.02	0.55 \pm 0.03	0.55 \pm 0.02	0.41 \pm 0.01
f/b	0.043 \pm 0.006	0.063 \pm 0.004	0.05 \pm 0.004	0.054 \pm 0.007	0.069 \pm 0.000	0.062 \pm 0.003	0.076 \pm 0.013	0.074 \pm 0.004	0.064 \pm 0.01	0.055 \pm 0.009	0.033 \pm 0.001

agriculture-derived SOC due to the increased activity and content of microbial biomass. As a result, C mineralization can exceed accumulation in the surface soil layer during early afforestation.

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

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

Tree identity effects on total microbial PLFA

Development of forests usually increases total PLFAs content (Jangid et al. 2011) and for our study it was true mostly for the soils under the monoculture forests formed by alder, birch and oak and also in two species forest mixtures with beech (Fig. 2). The total content of PLFAs

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

Afforestation effects on microbial community composition

Afforestation increased the content of bacterial and fungal PLFAs, however, fungal biomarkers increased 2 times higher than those for bacterial. Afforestation usually promotes development of fungi (Yannikos et al. 2014; Macdonald et al. 2009; Carson et al. 2010) and induces changes in fungal community composition (Carson et al. 2010). An increase in fungal biomarker content after afforestation can be attributed to the both direct effects of the trees themselves and indirect effects due to changes in the environment. Of the direct tree effects, fungal biomass is 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 16:1 ω 5 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 16:1 ω 5 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.

The average increase of PLFAs associated with actinomycetes was 50–150 % and was detected only for pure birch stand and two species mixtures (the highest with presence of alder), whereas for other plots they decreased or were similar to the agricultural soil (Fig. 3). Decrease in actinomycete biomarker content is related to the increasing the content of fungal biomass which is known to suppress the development of the actinomycete community (Lewandowski et al. 2015; Boer et al. 2005). From this study we conclude that changes in the content of microbial biomarkers following afforestation were greater compared to the major soil quality indicators. Afforestation affected the development of fungal biomass to a greater degree than bacterial biomass. Shifts in the content of particular biomarkers

was found in all forest plots, suggesting that the amount of tree species is not the main factor controlling soil microbial community changes. At the same time, the relative increase in biomarker content was related to tree identity, revealing that individual tree species promoted greater change relative to mixed-species forest. Further, no additive effects of individual tree species were found.

Forest composition effects on soil microbial communities

According to PCA analysis forest soil plots were different from the agricultural plot mainly due to the fungal (18:2 ω 6,9 and 18:1 ω 9) PLFAs (Fig. 4, bottom). This is in accordance with general increase of fungal biomarkers in forest soils (Fig. 3). Decrease of soil acidity contributed the most to separation of forest and agricultural plots, which is frequently reported for forest soils (Scheibe et al. 2015; van der Wal et al. 2006).

According to the PCA, one- and three species mixture forests were more similar in PLFA composition than two species mixtures (Fig. 4, top). The most relevant groups in differentiation of two species mixtures from monoculture and three species forests were 10Me16:0 and 10Me18:0, common for actinomycetes (Zelles 1997) and branched PLFAs i16:0 and a16:0, common for G+ bacteria (Zelles 1997) (Fig. 4, bottom). The late successional tree species together with two early 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 18:1 ω 9 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.

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