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Spatial and temporal variability of biological indicators of soil quality in two forest catchments in Belgium

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ABSTRACT

Biological indicators, measurements based on the presence and activity of soil organisms, are increasingly being considered in assessments of forest soil quality. In addition to chemical indicators, such as soil organic carbon and pH, biological indicators can provide an early diagnosis of changes in soil quality and processes in response to environmental change and forest management actions. We investigated the spatial and temporal variability of selected bio-indicators in the forest floor of two catchments over three years. We further evaluated the sensitivity of these indicators to changes in the dominant tree species following reforestation and natural regeneration. Indicators of microbial abundance and activity (microbial biomass, potential respiration) and carbon maintenance costs (metabolic quotient) were higher under young spruce and mixed deciduous stands than pure stands of oak and beech. Our results indicate a greater microbial activity in autumn but a wider range of carbon substrate utilisation in spring. Assessment of seasonal differences in bio-indicator values is vital for the evaluation and planning of long-term studies and the development of reference values for forest soils in Belgium. Our results highlight the usefulness of these bio-indicators in identifying changes in soil quality, particularly in response to management activities, at small spatial scales.

1. Introduction

Soil biological indicators are increasingly being considered in assessments of forest soil quality in the context of sustainable forest management and to assess the impacts of forest management actions (Lemanceau et al., 2016; Stone et al., 2016). Biological indicators typically include soil fauna (earthworms, nematodes, collembola; Cluzeau et al., 2012; Pérès et al., 2011) and microorganisms (Raiesi and Beheshti, 2015), through measures of their abundance (biomass), activity (processes) and diversity (community structure). The importance of soil microorganisms in driving major biogeochemical processes (Delgado-Baquerizo et al., 2016; Schloter et al., 2003; van der Heijden et al., 2008) and their rapid growth under favourable conditions make them useful indicators of soil quality (Bauhus and Khanna, 1999; Kennedy and Stubbs, 2006), as they can provide early indications of changes in the functioning of soils (Kennedy and Stubbs, 2006; Schloter et al., 2003). In recent years, forest management practices have shifted from a reliance on single species stands to patches of different tree species or mixed stands (Bauhus et al., 2013). This increased heterogeneity of forest stands may increase the resilience of soil functioning at the landscape scale (van der Plas et al., 2016). Thus, for the sustainable management of forest soils it is essential to be able to identify changes in soil functioning at the scale of the forest patch.

Soil quality has been defined as the continued capacity of the soil to function as a vital living system, within ecosystem and land use boundaries (*sensu* Karlen et al., 1997). It depends on the physical, chemical and biological properties and processes in the soil as well as their interactions (Karlen et al., 2003), and its characterisation requires indicators that are sensitive to changes in vegetation and hydrology, and that are related to soil functions and processes (Allen et al., 2011). Traditionally in forestry, tree biomass and growth were used as the main indicators of soil quality (Schoenholtz et al., 2000). Soil physical properties, such as texture and bulk density, and chemical properties, such as total organic content, pH and nutrient levels, are also widely used (Zornoza et al., 2015). However, changes in these parameters over time are relatively slow (Kirschbaum, 2000) and thus they provide limited indication of short-term changes in soil quality. Bio-indicators, such as microbial biomass and activity, can be more easily related to soil processes than physical and chemical indicators because it is the direct activity of microorganisms that drives many soil functions and processes (Table 1).

The quality and quantity of plant residues in soil are key drivers of the biomass and activity of soil microbial communities, as heterotroph microorganisms rely on plant-derived carbon as their energy source

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Table 1
Description of each soil quality bio-indicator assessed in the study and the details on the ecosystem function to which it relates. The mean and 75% percentiles of each bio-indicator across both forest catchments is also included.

Bio-indicator	Description	Relation to ecosystem functioning	Mean (25 and 75 percentile)
Microbial biomass carbon (MBC, mg kg^{-1})	Weight of carbon in soil microorganisms	Decomposition, nutrient cycling, soil fertility, soil structure	3461.0 (2719.2, 4633.2)
Potential respiration (PR, μg g^{-1} h^{-1})	Soil CO_2 emissions from microbial respiration	Decomposition	5.3 (3.9, 7.3)
Microbial biomass nitrogen (MBN, mg kg^{-1})	Weight of nitrogen in soil microorganisms	Rates of biogeochemical cycling and indicator of soil fertility	542.7 (411.0, 734.2)
Net nitrogen mineralisation (Nmin, mg kg^{-1} d^{-1})	Net release of inorganic N from soil organic matter	Biogeochemical cycling, soil fertility	9.3 (6.4, 12.0)
Microbial quotient (qmic, MBC:Corg)	Soil microbial biomass carbon per unit of soil carbon content (Organic carbon availability/quality)	Decomposition	0.9 (0.7, 1.2)
Metabolic quotient (qCO_2, μg C-CO_2 mg^{-1} MBC h^{-1})	Soil respiration per unit of microbial biomass carbon	Indicator of the efficiency of carbon utilisation	1.5 (1.1, 1.9)
Metabolic potential (MP, %)	Percentage of 31 carbon substrates used by soil bacteria (Metabolic diversity of soil bacteria)	Decomposition	48.4 (37.9, 58.1)

(Zak et al., 2003). The quantity and biochemical composition of litter and root exudates can vary considerably between tree species (Binkley, 1996; Binkley and Giardina, 1998; Hättenschwiler et al., 2005; Scheibe et al., 2015), such that nutrient availability for microorganisms can be very different in forest stands of varying species composition and stand age (Brant et al., 2006; Carnol and Bazgir, 2013; Grayston et al., 1996; Wardle et al., 2004). Mixed species stands may provide a greater variety and a reduced temporal variability in nutrient availability for microorganisms (Hättenschwiler et al., 2005) thereby improving soil quality compared to single-species stands.

The use of biological indicators for assessing soil quality is relatively new, partly due to a better understanding of the functional role of soil organisms in soil processes (Stone et al., 2016). Their use has additional challenges to more conventional indicators because bio-indicators are more variable in space and time than physical and chemical indicators (Doran and Zeiss, 2000; Parkin, 1993). As such, their novelty and inherent variability means that currently there is a lack of base-line values for many indicators, especially in forest ecosystems, and there is a clear need to define reference values under different environmental conditions and locations (Cluzeau et al., 2012; Lemanceau et al., 2016; Pulleman et al., 2012). In addition, it is important to assess the temporal scales over which bio-indicators respond to changing environmental conditions in order to decide on sampling strategies and data interpretation (Thoms and Gleixner, 2013).

There is a wealth of studies looking at individual indicators in isolation, yet little is known on the relative abilities of different indicators to detect changes in soil quality on similar soil types over relatively short time frames with limited disturbances (Bending et al., 2004). Here we assess a range of bio-indicators (Table 1) measured over three years in two forest catchments, located on the same geological substrate. Although molecular indicators of biodiversity can provide important information on soil quality (Stone et al., 2016), here we focused on 'classical' indicators frequently used in monitoring networks and specifically selected for Wallonia because of their relevance, ease of use, limited cost and interpretability (Malchair et al., 2010; Ritz et al., 2009). Earthworms were not included, because of their limited presence in the acid sites studied. The objectives of this work were to (i) assess the spatial variability of the bio-indicators and to evaluate the sensitivity of selected bio-indicators to changes in the dominant tree species following reforestation and natural regeneration; (ii) assess the temporal variability of the bio-indicators; and (iii) provide data for the development of reference values for forest soils in Belgium.

2. Materials and methods

2.1. Study sites

The study sites were located in two, *ca.* 80 ha, forested catchments (Waroneu, https://data.lter-europe.net/deims/site/LTER_EU_BE_17 and La Robinette, https://data.lter-europe.net/deims/site/LTER_EU_BE_27), in the state forest of Hertogenwald, Belgium (50°33′ N, 6°04′ E). The geological substratum of both catchments consists of quartzites, quartzo-phyllades and Revinian phyllads, covered with acidic brown soils. The structural B-horizon of the soils rests on a horizon with a silty texture called "fragipan", which impedes drainage. The soil is acidic, of type moder to dysmoder and presents a low base saturation. At Waroneu, in the early 1980s, forty percent of the area was covered with hardwoods (beech, *Fagus sylvatica* L., sessile oak, *Quercus petraea* (Matt.) Liebl., and birch, *Betula pendula* ROTH.) and 60% was covered with Norway spruce (*Picea abies* (L.) KARST.), planted in the 1930s. By 2011, the proportions had changed to 38% spruce, 45% deciduous and 17% open areas. Waroneu was limed in 1992 with 3 T/ha dolomite lime and 200 kg/ha potassium sulphate. La Robinette was initially covered with Norway spruce, but following windthrow in the 1990s, forest cover was significantly reduced and a mixed Sitka spruce (*Picea sitchensis* (Bong.) Carrière)-Norway spruce plot was established (hereafter "mixed spruce"). A further 22 ha were clear-cut in 1996, and in 1998 common alder (*Alnus glutinosa* (L.) GAERTN.), pedunculate oak (*Quercus robur* L.), silver birch (*Betula pendula* ROTH.), goat willow (*Salix caprea* L.), noble fir (*Abies procera* Rehd.) and rowan (*Sorbus aucuparia* L.) were planted in alternate rows, spaced by 2.5 m apart within four fenced plots of 2 ha. Alder, rowan, birch and oak were also planted within the catchment (with individual protection against deer damage). Spontaneous regeneration of Norway spruce resulted in thickets across the catchment, with trees in the same age class as the planted deciduous species (Carnol and Bazgir, 2013).

Six 30 by 30 m plots were established in each catchment representing the dominant tree species and drainage combinations (Table 2). The dominant tree height in each plot was defined as the mean height of the three trees with largest circumference at 1.30 m. Measurements of tree basal area and wood volume in each plot were performed according to the permanent inventory of forest resources in Wallonia (Alderweireld et al., 2015). Briefly, the circumference (C; 1.30 m) and the total tree height (VERTEX IV) were measured in circled plots (centred in the middle of the 30 × 30 m square) of 36 m, 18 m, and 9 m diameter for trees with $C > 120$ m, $70 < C < 119$, $20 < C < 69$, respectively. In mixed deciduous plots, all trees with $C > 20$ cm were measured. Wood volume was estimated following Vallet et al. (2006).

Table 2

Characteristics of the two forest catchments (Waroneu and La Robinette) and each sample plot. Open refers to unforested areas of the catchment. MAT: mean annual temperature; MAP: mean annual precipitation. The basal area ($m^2\,ha^{-1}$), wood volume ($m^3\,ha^{-1}$) and dominant tree height (m) were estimated in June 2016 for La Robinette and June 2013 for Waroneu.

Plot ID	Tree species	Planting date	Soil type	Drainage characteristic	Basal area ($m^2\,ha^{-1}$)	Wood volume ($m^3\,ha^{-1}$)	Dominant tree height (m)	Number of trees (ha^{-1})
Waroneu	*Altitude: 310–430 m; MAT: 7 °C; MAP: 1200 mm*							
W01	Adult spruce	1906	Hydromorphic	Poor	37.7	54.1	32.3	187
W02	Open		Hydromorphic	Poor				
W03	Oak	1885	Acid brown	Good	29.8	37.0	26.3	147
W04	Young spruce	1995	Hydromorphic	Poor	41.3	7.7	15.0	1258
W05	Adult spruce	1935	Acid brown	Good	40.6	57.8	30.7	285
W06	Beech	1860	Acid brown	Good	28.3	52.5	31.8	118
Plot ID	Tree species	Planting date	Soil Type	Drainage characteristic	Basal area ($m^2\,ha^{-1}$, 2016)	Wood volume ($m^3\,ha^{-1}$)	Dominant tree height (m)	Number of trees (ha^{-1})
Robinette	*Altitude: 470–530 m; MAT: 7 °C; MAP: 1300 mm*							
R07	Mixed deciduous[1]	1998	Hydromorphic	Poor	13.7	10.4	14	845
R08	Young spruce	1997–1998 (natural regeneration)	Hydromorphic	Poor	36.6	1.3	11.2	4401
R09	Adult spruce	1905	Acid brown	Good	52.4	76.4	32.2	236
R10	Mixed deciduous[1]	1998	Acid brown	Good	15.0	13.0	15.3	825
R11	Mixed spruce[2]	1993	Hydromorphic	Poor	67.9	13.7	15.5	1965
R12	Open		Hydromorphic	Poor				

[1] Common alder (*Alnus glutinosa* (L.) GAERTN.), European beech (*Fagus sylvatica* L.), Pedunculate oak (Quercus robur L.), Silver birch (*Betula pendula* ROTH.), Goat willow (*Salix caprea* L.), noble fir (*Abies procera* Rehd.) and Rowan (*Sorbus aucuparia* L.).

[2] Sitka spruce (*Picea sitchensis* (Bong.) Carr) and Norway spruce (*Picea abies* (L.) KARST.).

2.2. Soil sampling and physio-chemical analyses

Within each of the 12 plots, six forest floor samples (up to 10 cm depth) were taken with a 0.25 by 0.25 m template in randomly selected 10 by 10 m subplots. Sampling dates were adapted to local climatic conditions, to represent spring (10 June 2013, 23 May 2014, 11 May 2015) and autumn (10 January 2014, 25 November 2014, 10 December 2015). In particular, the 2013 spring sampling was delayed due to an exceptionally cold spring and late snow at the sampling site and the autumn sample in 2013 was performed in January 2014 due to technical problems and an exceptionally warm winter (Royal Meteorological Institute; https://www.meteo.be/). Autumns 2013–15 were all classified as ‘exceptionally warm’ and autumn samplings were performed before winter frost conditions. Fresh soil samples were sieved (4 mm) and stored at 4 °C until analyses. Water holding capacity (WHC, Haines-funnel system, Jenkinson and Powlson, 1976) and gravimetric water content (overnight drying at 105 °C) were estimated on one sample per plot, and all samples were adjusted to 60% water holding capacity before analyses.

Loss on ignition (LOI) was measured as the relative weight loss from the oven-dry soil after overnight calcination at 550 °C. The total soil organic carbon content (Corg) was estimated as 58% of LOI (Allen, 1989). Soil pH, water-soluble C (WSC), hot water-extractable C (HWC) and exchangeable cations were analysed in the November 2014 samples only. Soil pH was measured in water and KCl (1:2 soil water ratio). WSC and HWC were assessed by the method of Ghani (Ghani et al., 2003; Haynes and Francis, 1993; Sparling et al., 1998). Fresh soil (5 g) was shaken (120 rpm) with distilled water (30 ml) for 30 min, centrifuged (3500 rpm, 15 min) and filtered (0.45 µm, Pall Corporation, MI, USA). The remaining wet soils were mixed with distilled water (30 ml) and placed in an oven for 16 h at 80 °C. After centrifugation and filtration, extracts were kept at 4 °C before analysis with a Total Organic Carbon analyser (Labtoc, Pollution and Process Monitoring Limited, UK). Exchangeable cations (Al^{3+}, Ca^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, $Zn^{2}+$) were extracted with 0.1 M $BaCl_2$ (1:5, w:v, Hendershot and Duquette, 1986). Chemical analysis of the filtered (filter 595 1/2, Whatman, Germany) and acidified $BaCl_2$ extracts (1% HNO_3 Suprapur) was performed using ICP-AESS (Varian, Australia).

2.2.1. Soil microbial parameters

2.2.1.1. Microbial biomass (C and N). Soil microbial biomass C and N (MBC and MBN, $mg\,kg^{-1}$) were determined by the chloroform fumigation extraction method (Brookes et al., 1985; Vance et al., 1987) followed by 0.5 M K_2SO_4 extraction of both fumigated and unfumigated soils. Fumigations were carried out for three days in vacuum desiccators with alcohol-free chloroform. After filtration (Whatman n° 42), the dissolved organic carbon in the fumigated and unfumigated extracts was measured with a Total Organic Carbon analyser (Labtoc, Pollution and Process Monitoring Limited, UK). Organic N in both extracts was measured with a continuous flow analyser equipped with a UV digestor (Autoanalyser3, BranLuebbe, Germany). MBC and MBN were calculated by dividing the difference of total extract between fumigated and unfumigated samples with a conversion factor of 0.45 for biomass C (Sparling and West, 1988) and 0.54 for biomass N (Brookes et al., 1985).

2.2.1.2. Net nitrogen mineralisation. Net N mineralisation (Nmin, $mg\,kg^{-1}\,d^{-1}$) was estimated as the difference in mineral N content of the soil during an aerobic laboratory incubation of 28 days, at 25 °C in the dark, following Hart et al. (1994). Inorganic nitrogen was extracted with 1 M KCl (1:5, w:v) following Allen (1989) and analysed colourimetrically using a continuous flow analyser (AutoAnalyser3, Bran-Luebbe, Germany). The net N mineralisation rate (Nmin) was calculated by dividing the net increase in inorganic N during the incubation period by the number of incubation days.

2.2.1.3. Potential respiration. Potential respiration (PR, $\mu g\,g^{-1}\,h^{-1}$) was measured as CO_2 accumulation in the headspace (250 ml) of an amber bottle (Supelco, USA) from 20 g of soil at 15 °C in the dark. Gas samples (4 ml) were taken at 0, 120, 150 and 180 min and analysed with an infrared absorption gas analyser (EGM-4, PPsystem, UK). Before taking the measurements, soil samples were kept at 15 °C for 12 h to equilibrate. PR production rates were estimated by the linear regression of CO_2-C against time.

2.2.1.4. Microbial and metabolic quotients. The microbial quotient (qmic, MBC:Corg) represents the availability of soil C and was calculated by dividing MBC by Corg. The metabolic quotient (qCO_2, $\mu g\, mg^{-1}\, h^{-1}$) represents the quantity of respired CO_2-C per unit of soil microbial biomass and was calculated by dividing the potential respiration (PR) by MBC (Anderson and Domsch, 1990).

2.2.1.5. Metabolic potential of soil bacteria. The metabolic potential (MP,%) of cultivable heterotrophic soil bacteria, also called community-level physiological profiling (CLPP), was measured using BIOLOG EcoPlates™, where each well contains one of 31 different carbon sources (Insam and Goberna, 2004; Rutgers et al., 2016). The percentage of the carbon sources used by the extracted bacteria, as indicated by the development of a purple colour due to the reduction of a tetrazolium dye present in the wells, was assessed for each soil sample. MP reflects the diversity of carbon utilisation pathways of those cells that are sufficiently active to utilise the specific carbon sources (Haack et al., 1995), and has been used as a proxy for the functional diversity of soil microbial communities (Chen et al., 2013; Preston-Mafham et al., 2002). The EcoPlates methodology allows a relatively quick and affordable assessment of the metabolic profile of the microbial community and has been found to be effective at distinguishing small changes in the environment (Winding et al., 2005). The rate of colour development in the wells is dependent on the inoculum cell density (Haack et al., 1995), therefore it is essential that metabolically active cells are inoculated and that the density is the same in all wells (Konopka et al., 1998). Prior to inoculation into the wells, bacterial density was assessed for one randomly chosen forest floor sample for each plot. One g of fresh soil was suspended in 9 ml sodium cholate (0.1%), vortexed for 1 min, and then serially diluted up to 10^{-4} in 0.85% NaCl. One hundred µl of the serial dilutions were plated on R2A agar and colonies were counted after 48 h at 20 °C. Fresh soil extracts were diluted to yield 1500 colony forming units (CFU) and inoculated (100 µl) into the EcoPlate wells. Colour formation was recorded visually after 72 h at 20 °C.

2.3. Statistical methods

We used linear mixed models (LMMs) to investigate the spatial and temporal variability in bio-indicators between the plots, using the *lme4* package in R (Bates et al., 2015). These models can account for hierarchical data and unbalanced designs, as we have in this case study (Bates et al., 2015). For all models, plot and sampling date were fitted as random effects to account for the non-independence of data points; i.e. multiple samples taken within plots and multiple samples taken over time. Catchment was included as a fixed effect because there were only two (La Robinette and Waroneu). All response variables, except MP, were log transformed to improve normality and homoscedasticity of errors. The dominant tree species (hereafter 'species'), soil drainage and season were fitted as categorical fixed effects. Species had seven levels (one for each species class), and soil drainage and season both had two levels (drainage: poor, good; and season: spring, autumn; Table 2).

To evaluate the importance of each explanatory variable for explaining the variation in bio-indictor values, linear mixed models were fitted to each bio-indicator separately in three steps: i) Null model (intercept only and random effects of plot and sampling date); ii) Full model (Null model plus main effects of catchment, species, season and drainage and the interaction of species with season); and iii) each combination of the main effects and the interaction term from the Full model. Model comparison, based on the Akaike Information Criterion for small sample sizes (AICc, Burnham and Anderson, 2002), was made in the pgirmess R package (function *selMod*; Giraudoux, 2017). The importance of an explanatory variable in explaining the variation in bio-indictor values was interpreted as those present in the most parsimonious model, i.e. the model with the smallest number of estimable parameters (K) within two AICc units (Δ_{AICc}) of the model with the lowest AICc. The variance of the random effect terms and estimates of fixed effect terms were obtained for the Null and Full models. The marginal coefficient of determination (R_m^2; variance explained by fixed effects) was calculated using *r.squaredGLMM* (MuMIn package, Bartoń, 2016).

In the initial model fitting process, we did not include any soil property predictors to prevent confounding effects, as they may be influenced by species and drainage. Instead, we added Corg, as a continuous fixed effect, to the Full model to evaluate the importance of total soil organic carbon content on bio-indicator values. The order of fitting predictors is such that variables added later in the modelling process are unlikely to influence those that had previously been added.

The partial clear cutting of La Robinette catchment in 1996 and its reforestation/natural regeneration allowed an assessment of the differences in soil bio-indicator values following a change in the dominant tree species. In a separate analysis we assessed the changes in bio-indicator values from mature spruce stands to young spruce, mixed spruce and mixed deciduous stands. To evaluate a comprehensive change in soil quality by a combination of bio-indicators, we used a repeated-measure multifactor analysis of variance (MANOVA) to test whether the bio-indicator values varied between the plots in the La Robinette catchment. MANOVA tests whether there are statistically significant mean differences among groups in a linear combination of several dependent variables (Tabachnick and Fidell, 2007). An assumption of MANOVA is that the dependent variables are linearly independent. To meet this assumption, we only included Nmin, PR and qmic as a multivariate dependent variable. We excluded MP because it was only sampled on four occasions. Bio-indicator values were log transformed to fulfill the assumption of normality, as with the LMM analysis. Secondly, PCA was used to describe the similarities and differences in all bio-indicators between the plots.

3. Results

3.1. Bio-indicator ranges

The range of values of the indicators is given in Table 1. MBC, MBN and Nmin ranged from $3186\, mg\, kg^{-1}$, $486\, mg\, kg^{-1}$ and $5.3\, mg\, kg^{-1}\, d^{-1}$ in open stands in Waroneu, respectively, to $5329\, mg\, kg^{-1}$, $881\, mg\, kg^{-1}$ and $15.3\, mg\, kg^{-1}\, d^{-1}$ in under young spruce in La Robinette (Fig. S1). PR ranged from $4.3\, \mu g\, g^{-1} h^{-1}$ (beech, Waroneu) to $10.1\, \mu g\, g^{-1} h^{-1}$ (young spruce, La Robinette). Microbial quotient ranged from 0.6 under adult spruce to 1.4 under beech in Waroneu, whilst qCO_2 ranged from 1.1 under beech to 2.2 in open stands in Waroneu. Lastly, MP ranged from 30% under adult spruce in La Robinette to 55% and 54% under oak in Waroneu and young spruce in La Robinette, respectively. In general the bio-indicators were positively correlated (Table S1.1); MBC was positively correlated with Nmin and PR (Pearson $r > 0.59$, $p < 0.001$). MP and qCO_2 were relatively weakly correlated with the other indicators.

Soil pH_{H2O} across the 12 plots in November 2014 ranged from 3.6 (adult spruce, La Robinette) to 4.4 (young spruce, Waroneu) (Table 3). Soil pH_{KCL} was lowest in the adult spruce stands in both catchments (between 2.5 and 2.6) and highest in the beech stand in Waroneu (3.5). Exchangeable base cations were lowest under beech (Waroneu), adult spruce, mixed spruce and in the open area (La Robinette) and were highest under young spruce in both catchments. The beech plot at Waroneu had the lowest water-soluble C (WSC) and hot water-extractable C (HWC), whilst they were highest under young spruce at La Robinette. In the November 2014 samples, MBC and MBN were not correlated with soil pH, WSC, HWC, sum of exchangeable cations or exchangeable bases (Table S4). MP correlated negatively with soil pH (both H_20 and KCl) and qmic correlated negatively with soil pH KCl and exchangeable bases. Potential respiration, Nmin and qCO_2 all strongly positively correlated with WSC, HWC, sum of exchangeable cations and exchangeable bases (Table S4).

Table 3
Mean soil characteristics of the two forest catchments (Waroneu and La Robinette) in each sample plot in November 2014 (n = 6). WSC: water-soluble organic carbon, HWC: hot water extractable organic carbon. Different letters indicate significant differences between plots, across catchments (one-way ANOVA, $p < 0.05$).

Plot ID	Tree species	WSC ($mg\,g^{-1}$)	HWC ($mg\,g^{-1}$)	pH_{H20}	pH_{KCl}	Sum of exchangeable cations ($mmolc\,100\,g^{-1}$)	Exchangeable bases (Ca^{2+}, K^{+}, Mg^{2+}) ($mmolc\,100\,g^{-1}$)
Waroneu							
W01	Adult spruce	0.50 acd	15.7 be	3.7 ab	2.6 ab	15.0 abd	13.4 abe
W02	Open	0.57 ad	13.1 cdf	4.0 c	3.1 de	17.6 ab	11.7 ad
W03	Oak	0.50 acd	13.8 cdf	4.1 c	3.0 de	15.3 ad	9.0 adc
W04	Young spruce	0.76 ad	13.4 cdf	4.4 c	3.2 de	24.2 c	20.6 b
W05	Adult spruce	0.52 acd	15.1 ce	3.7 b	2.5 a	15.8 ad	13.7 abe
W06	Beech	0.25 c	9.7 f	4.2 c	3.5 c	13.4 bd	5.5 cd
La Robinette							
R07	Mixed deciduous[1]	0.53 acd	19.8 ab	3.9 bc	2.8 cd	19.5 ac	10.9 ad
R08	Young spruce	1.21 b	22.3 a	3.9 bc	2.7 bc	19.5 ac	17.3 b
R09	Adult spruce	0.60 ad	16.6 bc	3.6 a	2.6 ab	13.1 bd	7.1 cd
R10	Mixed deciduous	0.64 ad	14.7 cde	4.3 c	2.9 cd	15.2 abd	10.4 de
R11	Mixed spruce[2]	0.41 cd	10.4 df	3.8 b	2.7 bc	9.4 d	4.4 c
R12	Open	0.70 ad	12.7 cdf	4.3 c	3.1 de	11.2 d	5.2 c

[1] Common alder (*Alnus glutinosa* (L.) GAERTN.), European beech (*Fagus sylvatica* L.), Pedunculate oak (Quercus robur L.), Silver birch (*Betula pendula* ROTH.), Goat willow (*Salix caprea* L.), noble fir (*Abies procera* Rehd.) and Rowan (*Sorbus aucuparia* L.).

[2] Sitka spruce (*Picea sitchensis* (Bong.) Carr) and Norway spruce (*Picea abies* (L.) KARST.).

3.2. Spatial variability of bio-indicators across forest vegetation patches

There was considerable spatial variability in the bio-indicator values across the two catchments (Figs. 1 & S1). In general, the bio-indicators were well predicted; the marginal R^2 of the Full model (proportion of the total variation explained by the fixed effects) ranged from 0.14 for MBC and MBN to 0.34 for qmic (Table 4). Variance partitioning on the Null model (including the random effects for plot and sampling date) indicated important plot-level variation in all bio-indicator values (Table 4). Variation between the plots was largest for Nmin, qmic and qCO_2 (22.2%, 34.8% and 27% of the total variation, respectively) and smallest for microbial biomass (both C and N, 12.3% and 12.9%, respectively). Once species and drainage were included in the model (i.e. Full model; Table 4), plot-level variation in microbial biomass (both C and N) and qmic was close to zero. Some plot-level variation in PR, qCO_2 and MP remained after fitting the Full model.

Species was in the most parsimonious model for all bio-indicators (Table S2), indicating that the dominant tree species was important in explaining differences between the plots for all bio-indicators (Fig. 1). Predicted MBC and MBN were particularly high under young spruce (4595 and 723 $mg\,kg^{-1}$, respectively) and oak (4534 and 726 $mg\,kg^{-1}$) and low in open stands (2894 and 454 $mg\,kg^{-1}$), compared to overall measured means of 3461 and 543 $mg\,kg^{-1}$ (Table 1), respectively. Potential respiration was also highest under young spruce (7.3 and 7.6 $\mu g\,g^{-1}h^{-1}$ in the two catchments but lowest under beech (3.8 $\mu g\,g^{-1}h^{-1}$ compared to a mean of 5.3, Table 1). Nmin was low in the open areas (5.14 and 3.85 $mg\,kg^{-1}\,d^{-1}$ in La Robinette and Waroneu, respectively, compared to a mean of 9.4, Fig. 1 and Table 1). Predicted qmic was high under oak and beech (1.3%, compared to an overall measured mean of 0.9%, Table 1) and low under adult spruce (0.6), whilst qCO_2 was low under oak and beech (1.1 $\mu g\,C\text{-}CO_2\,mg^{-1}\,MBC\,h^{-1}$, mean 1.5). Lastly, predicted MP was high in soil under young spruce (56%) and oak (55%) and low under adult spruce (35%, compared to a measured mean of 49% across all plots; Fig. 1 and Table 1).

Microbial biomass (both C and N), Nmin and qmic were higher in La Robinette catchment than in Waroneu (catchment was in the model with the lowest AICc and smallest number of estimated parameters for these indicators, Table S2), and there was some evidence that MP was lower in La Robinette catchment than in Waroneu. There were no differences between the catchments for PR and qCO_2 (Table S2).

We found limited support for the importance of soil drainage on bio-indicator values. Soil drainage was in the model with the lowest AICc for qmic (Table S2), which was higher in the better-drained soils (Table S3). Total organic soil carbon content (Corg) was generally very important in predicting bio-indicator values, as indicated by the large decrease in AICc in the model with Corg (Table 4). An increase in soil organic carbon was related to an increase in microbial biomass (both MBC and MBN), Nmin, PR and qCO_2 and a decrease in qmic. Only MP was insensitive to soil carbon levels (Table 4 & Fig. S2).

3.3. Changes in bio-indicators after reforestation and natural regeneration

Soil bio-indicator values (Nmin, PR and qmic) were significantly different between the plots in La Robinette catchment (MANOVA; Table 5). The change from mature to young spruce stands was correlated with an increase in the level of all three bio-indicators (Fig. S4). The first three axes of the PCA on all bio-indicator values in La Robinette plots explained 91% of the variation (Fig. 2 and Table S3). Microbial biomass (both C and N) and qmic loaded highest on the first PC (57%), PR and qCO_2 loaded on the second PC (23%), and Nmin and MP loaded on the third PC (11%). The young spruce and mixed deciduous plots clustered separately from the other plots in bio-indicator space (Fig. 2), but only in the autumn samples; the spring samples, across all the species had greater overlap between them. The autumn samples from the young spruce plots were characterised by higher levels of PR, Nmin and qCO_2 compared to the adult spruce plots, whilst the autumn samples from the mixed deciduous plots were characterised by higher levels of Nmin and qCO_2, compared to the adult spruce plots (Figs. 2 and S4).

3.4. Temporal or seasonal variability in bio-indicator values

Variance partitioning on the Null model indicated important temporal variation due to sampling date for all bio-indicators (Table 4). Temporal variability was greatest for microbial biomass (both C and N) and qmic and lowest for Nmin (34.4%, 33.4%, 27.8% and 5.4% of the total variation, respectively, Table 4). However, the variance component of sampling date was very similar between the Null and the Full model (which included season as fixed effect) for all bio-indicators, except MP (Table 4). This indicates that season explained very little of the variation between sampling dates for all bio-indicators except MP. For MP, the variation attributable to sampling date reduced from 21.8% in the Null model to 9.0% in the Full model. Species-specific seasonal differences were observed for PR, qCO_2, MP and weakly for Nmin (i.e. AICc support for the inclusion of species x season interaction term in the

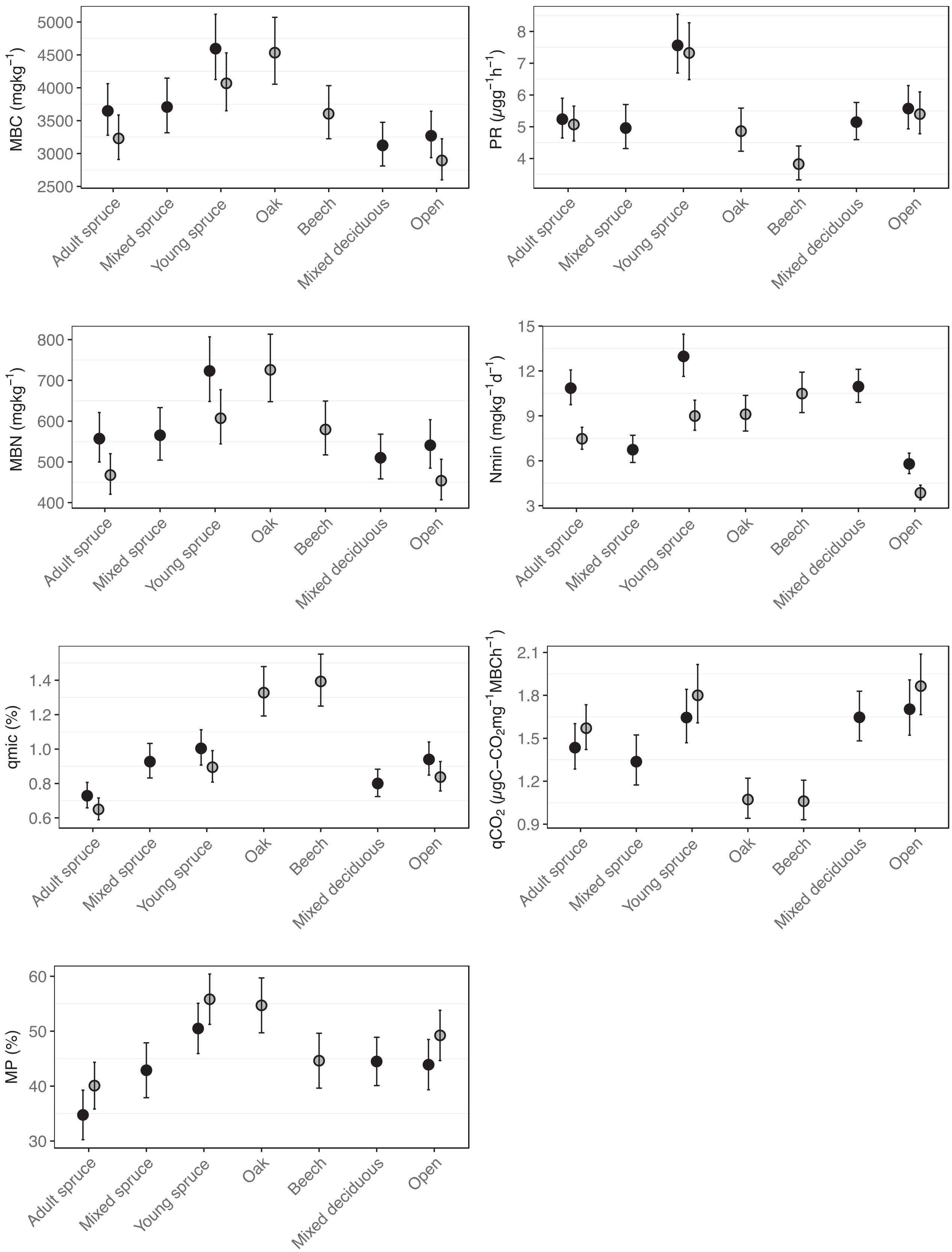

Fig. 1. Predicted spatial variability in soil bio-indicator values across the forest catchments. The model is bio-indicator ~ Species + Catchment + (1|Plot) + (1|Sample), where Plot is the Plot ID and Sample is the sampling time. Black filled circles indicate La Robinette catchment and grey circles indicate Waroneu catchment. The error bars indicate the standard error. MBC: microbial biomass C; PR: potential respiration; MBN: microbial biomass N; Nmin: net nitrogen mineralisation; qmic: microbial quotient; qCO$_2$: metabolic quotient; and MP: metabolic potential. Drainage and the interaction between species and season were not included in the prediction model for graphical visualisation purposes.

Table 4
Comparison of the Null, Full and Carbon models, based on AICc for each bio-indicator. Models were fitted using maximum likelihood estimation. The Null model (intercept-only) had random errors for plot and sampling date (Sample). The Full (Species × Season + Drainage + Catchment) and Carbon (Full + Corg) models had additional predictors based on the Null model. The marginal R_m^2 (proportion of variation explained by the fixed effects) and the percentage variance explained by the random effects (Variance) are given for each model.

Microbial biomass C (MBC)					Microbial biomass N (MBN)			
Model	AICc	R_m^2	Variance (%)		AICc	Rm^2	Variance (%)	
			Plot	Sample			Plot	Sample
Null	251.2		12.3	35.8	282.8		12.9	34.4
Full	242.5	0.14	0	34.4	276.9	0.14	0	33.4
Carbon	97.2	0.31	0.2	29.5	156.8	0.28	0	32.0
Net nitrogen mineralisation (Nmin)					Potential respiration (PR)			
Model	AICc	R_m^2	Variance (%)		AICc	R_m^2	Variance (%)	
			Plot	Sample			Plot	Sample
Null	670.1		22.2	6.3	485.7		16.3	17.1
Full	659.1	0.26	0.01	5.4	483.2	0.16	3.6	16.5
Carbon	565.4	0.40	1.6	6.0	249.1	0.49	2.2	9.8
Microbial quotient (qmic)					Metabolic quotient (qCO_2)			
Model	AICc	R_m^2	Variance (%)		AICc	R_m^2	Variance (%)	
			Plot	Sample			Plot	Sample
Null	124.0		34.0	30.0	202.6		27.0	21.2
Full	106.6	0.34	0.4	27.8	191.3	0.25	6.4	19.2
Carbon	84.2	0.36	0	29.5	146.0	0.31	4.9	18.8
Metabolic potential (MP)								
Model	AICc	R_m^2	Variance (%)					
			Plot	Sample				
Null	2335.5		14.2	21.8				
Full	2332.0	0.29	1.7	9.0				
Carbon	2333.5	0.29	2.0	9.3				

Table 5
Repeated-measure multivariate analysis of variance (MANOVA). Net nitrogen mineralisation (Nmin), potential respiration (PR) and microbial quotient (qmic) were used as the multivariate response.

	df	Pillai	Approx. F	Num df	Den df	Pr(> F)
Season	1	0.196	0.163	3	2	0.913
Plot	5	0.653	11.132	15	600	< 0.0001
Plot × Season	5	0.163	2.318	15	600	0.003

Full model, Fig. 3 & Table S2). For Nmin, PR and qCO_2 the difference between the seasons was greatest under young spruce, where the values of all three bio-indicators were higher in the autumn than the spring (Fig. 3). Nmin was also predicted to be higher in autumn than in spring in mixed deciduous stands. MP was higher in spring than in autumn under all species, except young spruce and open stands (Fig. 3). We found no support in the model for seasonal differences in MBC, MBN or qmic, even though they had the greatest temporal variability (Table S2). In addition, we found no seasonal differences in Corg between the plots (Fig. S3).

4. Discussion

Growing interest in the use of soil biological indicators related to microbial biomass and activity to detect changes in soil quality has emerged from the recognition of the functional importance of microorganisms for many ecosystem services. We detected changes in microbial biomass, activity and metabolic potential within two forest catchments. Our results highlight the usefulness of these bio-indicators in identifying changes in soil functioning quality, particularly in response to management activities, at small spatial scales.

4.1. Spatial variability and changes in bio-indicators after reforestation and natural regeneration

We found tree species-specific variation for all bio-indicators within the catchments. Under oak and beech, high values of microbial quotient and low values of metabolic quotient indicated both good carbon availability and good carbon use efficiency by the microorganisms (Wardle and Ghani, 1995), and thus low carbon maintenance demands (Cotrufo et al., 2013; Xu et al., 2014). Both species were located on well-drained acid brown soils in the Waroneu catchment. Higher microbial biomass and microbial quotient may be related to better carbon availability in the well-drained soils, as drainage was included in the best model. However, this was not the case for the metabolic quotient, indicating that drainage is unlikely to explain the good carbon use efficiency under oak and beech. Comparatively, we found higher microbial biomass and activity (potential respiration) under oak, but a higher metabolic potential under beech. A possible interpretation of this is a lower soil quality under beech than oak. This is supported by lower water-soluble C and hot water-extractable C in the November 2014 samples under beech than oak and lower litter quality and higher forest floor C/N ratio under individual beech trees than oak trees in La Robinette catchment (Carnol and Bazgir, 2013; Haynes, 2005). The lower

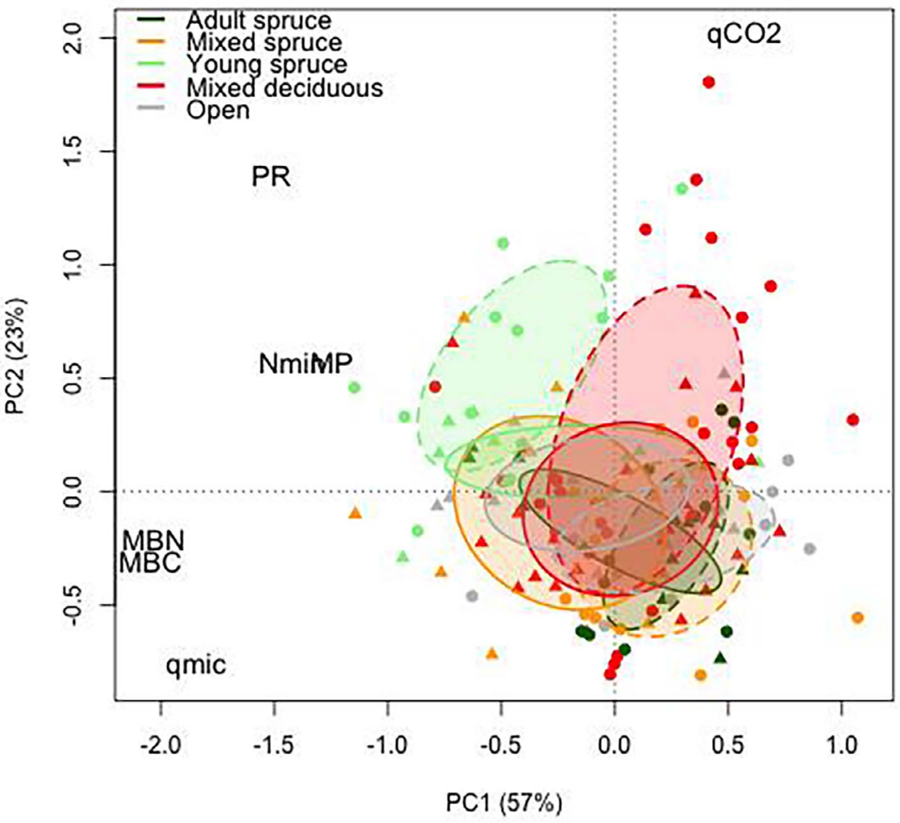

Fig. 2. PCA biplot of bio-indicator values in the La Robinette catchment plots. The ellipsoids indicate the standard error of bio-indicator values under each species and season. Solid lines and triangles indicate spring samples and dashed lines and circles indicate autumn samples. PC1 explained 57% of the variation in bio-indicator values and PC2 23%. MBC: microbial biomass carbon; MBN: microbial biomass nitrogen; PR: potential respiration; Nmin: net N mineralisation; qCO_2: microbial quotient; qmic: metabolic quotient; and MP: metabolic potential.

carbon availability under beech may enable a wider range of carbon substrate utilisation due to less competition by opportunistic species (Fontaine et al., 2003). The lack of ground vegetation in the beech plots may also explain some of the differences due to differences in root exudation (Grayston et al., 1996).

In contrast, under young spruce, microbial biomass, potential respiration and metabolic quotient were high and the microbial quotient was low, indicating poor carbon quality and less efficient microbial utilisation of carbon, and thus high carbon maintenance costs (Xu et al., 2014). Bacterial metabolic potential was particularly high in the young spruce stands, which suggests that a greater range of carbon substrates were being utilised but at a lower efficiency than in the beech and oak stands. Low carbon quality and high maintenance can be explained by the high C/N ratio of spruce litter (Carnol and Bazgir, 2013; Vesterdal et al., 2008) and thus lower soil quality under spruce. There is some indication that nitrogen availability may have been higher under young than adult spruce; microbial biomass N and N mineralisation were both higher in the young spruce stands. This may be due to higher root exudation in the dense young spruce stands, as it has been shown that root exudation stimulates nitrogen transformation (Yin et al., 2013). A higher N availability may in part explain the higher metabolic potential and microbial activity (potential respiration rates) under young than adult spruce trees, although evidence that N availability can influence decomposition rates is equivocal (Prescott, 1995). Under adult spruce, all the bio-indicators were low, except N mineralisation, which strongly suggests a low soil quality.

Interestingly, bio-indicators values were very similar between the open areas and mixed deciduous stands (apart from for N mineralisation). In particular, the microbial quotient was low and the metabolic quotient high. This suggests a low soil carbon quality and high maintenance costs, possibly due to the low carbon inputs in the young stands. Higher N mineralisation in the mixed stands indicates a better soil quality than the open stands, most likely to due a higher litter quality. Overall, our results support recent forestry management practices that advocate mosaics of different tree species in the forest to maintain resistance and resilience of ecosystem functioning at a landscape level (Brang et al., 2014).

Our findings demonstrate significant impacts of reforestation and natural regeneration on indicators of soil quality in La Robinette catchment. It is well acknowledged that a change in the system induces some stress on the microorganisms, as they are not initially adapted to the new substrates (Shade et al., 2012). However the clear cutting of the catchment dates back almost 20 years and it is unlikely that the microorganisms are still under stress, thus any differences more likely reflect changes in dominant tree species. Changes in bio-indicators were most pronounced with the development of young spruce and mixed deciduous stands. The change from adult to young spruce was associated with an increase in microbial biomass (both C and N), greater potential respiration and higher metabolic quotient values. This suggests a greater substrate availability and microbial activity but at higher maintenance costs for the microorganisms. Similarly, the change to mixed deciduous stands was associated with an increase in N mineralisation and higher metabolic quotient values, suggesting higher decomposition rates and nutrient availability for the microorganisms but also higher maintenance costs. Several species, often referred to as ‘soil improving species’ (Augusto et al., 2002; Raulund-Rasmussen and Vejre, 1995), were present in the mixed species plots. For example, the presence of birch in forests stands has previously been found to improve soil quality by increasing microbial biomass and activity (Schua et al., 2015) and stimulating non-symbiotic microbial nitrogen fixation (Nohrstedt, 1988). Similarly, in the mixed plots of our study, soil pH was found to be higher under individual birch and rowan trees than the other species (Carnol and Bazgir, 2013).

4.2. Temporal variability in bio-indicator values

We found considerable variability in all bio-indicators across the sampling dates, most likely due to variation in climatic conditions

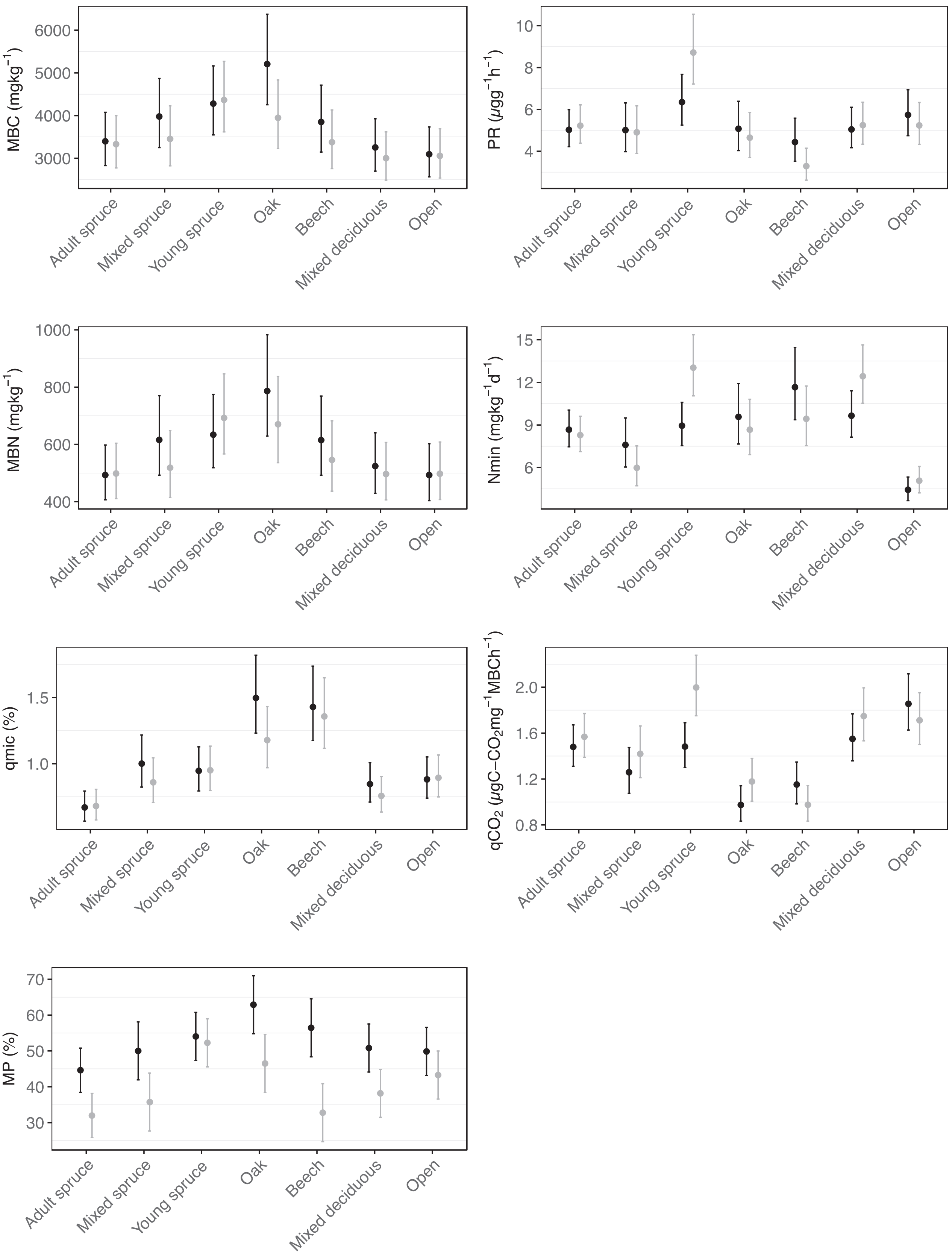

Fig. 3. Predicted seasonal variability in soil bio-indicator values across the forest catchments. The model is bio-indicator ~ Species + Season + Species: Season + (1|Plot) + (1|Sample), where Plot is the Plot ID and Sample is the sampling time. Black circles indicate spring samples and grey circles indicate autumn samples. The error bars indicate the standard error. MBC: microbial biomass C; PR: potential respiration; MBN: microbial biomass N; Nmin: net nitrogen mineralisation; qmic: microbial quotient; qCO_2: metabolic quotient; and MP: metabolic potential. Drainage and catchment were not included in the prediction model for graphical visualisation purposes.

within and between the years. Temporal variability was highest for microbial biomass (both C and N) and microbial quotient, but there were no clear differences between spring and autumn sampling. The absence of clear seasonality could be due to a decrease in inter-seasonal meteorological differences due to climate change, with globally earlier spring and delayed autumn conditions (Menzel et al., 2006), and, in particular, later frosts and an increase in frost-free periods in Belgium (Ministry for Social Affairs, 2009). On a global scale, contrasting temporal dynamics of microbial biomass have been reported (reviewed in Wardle 1998), with lower temporal variability in N rich forest soils (Wardle, 1998). Soil moisture (Serna-Chavez et al., 2013) and soil organic carbon content (Zak et al., 2003) have also been put forward to explain soil microbial biomass across a large range of sites. In our sites, temporal variability in soil microbial biomass is most likely due to differences in soil moisture content, as well as belowground C allocation and root exudation patterns between sampling dates (Bardgett et al., 2005).

Soils sampled in spring consistently had a greater metabolic potential of soil bacteria than autumn samples under all species except young spruce, where there was no difference. A possible explanation could be that high root exudation in the spring could lead to a greater range of substrates available and therefore a greater microbial metabolic potential (Bardgett et al., 2005). However, under young spruce, root exudation is likely to be high during the whole year, which may explain the lack of seasonal differences in metabolic potential under young spruce. In contrast, potential respiration, N mineralisation and metabolic quotient were higher in autumn than spring under young spruce, which suggests a greater microbial activity later in the year. Similarly, N mineralisation was higher in autumn in the mixed deciduous plots. Importantly, for all of the bio-indicators weak or no seasonal differences were predicted in the open stands, which implies that the observed seasonal differences are most likely due to differences in the quality and quantity of plant residues from of the dominant tree species. In summary, our results indicate that spring sampling is more effective to identify differences in the range of carbon substrates used (i.e. metabolic potential), whilst autumn sampling is more effective for identifying differences in microbial activities.

4.3. Establishment of reference values

The microbial biomass values (both C and N) presented here are broadly within the range of comparable forest stands (Bauhus and Khanna, 1999). The N mineralisation values reported here are slightly higher, and potential respiration, lower than similar studies (Welke and Hope, 2005). Microbial and metabolic quotient values are within the ranges described in comparable studies (Anderson, 2003; Anderson and Domsch, 1993; Bauhus et al., 1998; Moscatelli et al., 2005). Nevertheless, in contrast to ecotoxicological and chemical indicators, where clear threshold values can be established (Clements and Rohr, 2009; Smolders et al., 2009), the interpretation of biological indicators of soil quality has greater uncertainties associated with them and is subject to interpretation. We found differences in bio-indicator values between plots situated in a restricted geographical area, on the same geological substrate with relatively homogenous soil type. This poses the question of scale for establishing reference values. Should they be, for instance, by soil type, geographical region or dominant species? Broad ranges can be defined at a large scale but then it may be impossible to discriminate changes, while narrow ranges at a local scale may be too detailed and unfeasible for many monitoring networks due to the work effort involved. Whereas the establishment of reference ranges based on the dominant tree species is likely to be impractical in relation to sampling intensity and applicability, our findings suggest that bio-indicators are useful for the detection of process-based changes following management. They are thus useful for the inference of ecosystem functioning and may help in the establishment of management plans according to prioritised soil ecosystem services. Temporal variability in bio-indicators is challenging to avoid and requires careful consideration of sampling times for specific bio-indicators in monitoring programs and the inclusion of temporal variability in reference values.

The interpretation of differences in bio-indicators is challenging because higher values do not necessarily mean better soil quality. For example, higher respiration can be interpreted as higher soil microbial activity and thus better quality, but the increased release of greenhouse gas CO_2 to the atmosphere is undesirable with regards to the global carbon balance. Furthermore, higher CO_2 emissions could reflect higher maintenance cost of the microorganisms and thus lower soil quality, illustrating the usefulness of ecophysiological quotients such as the microbial and metabolic quotients (Anderson and Domsch, 2010). Also, the relationship between some indicators and ecosystem functioning is not clearly established. For example, although microbial biomass is widely used as indicator of soil quality (Zornoza et al., 2015), others question the usefulness of microbial biomass for inferring process rates (Rousk, 2016; Wang et al., 2003). It is also now recognised that relationships between ecosystem services need to be considered in order to provide multiple services in sustainably managed landscapes (Bennett et al., 2009). These considerations highlight the need to interpreted bio-indicators with regards to the desired soil ecosystem service (i.e. nutrient cycling or carbon sequestration) and that a combination of indicators is needed to infer changes in processes, such as the carbon cycle (i.e. microbial biomass, microbial quotient and metabolic quotient) and the N cycle (N mineralisation and microbial biomass N).

5. Conclusions

Using a standard set of conventional bio-indicators, we detected differences in soil quality in forest patches of different tree species in two 80 ha catchments. In particular, we found a change in soil quality in the transition from mature spruce to young spruce and mixed deciduous stands, indicating higher microbial activity and nutrient cycling, but also higher maintenance costs under these stands. Temporal variability was highest for microbial biomass but we only found clear seasonal trends for bacterial metabolic potential. Our results suggest greater overall microbial activity in autumn but that a wider range of carbon substrates were metabolised in spring, which has important implications for the planning of long-term studies. The interpretation of results concerning bio-indicators remains challenging, as higher values do not necessarily imply better soil quality. The use of eco-physiological quotients in combination with other indicators, such as microbial biomass (both C and N) and N mineralisation, is needed to infer changes in the functioning of the forest ecosystem.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2018.02.020.

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