Structural and functional differences in the belowground compartment of healthy and declining beech trees

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ABSTRACT
Functional studies of tree decline have mainly focused on process inside the plant showing possible alterations of carbon transport, storage and hydraulic functions. However, the processes occurring at the plant-soil interface have been seldom investigated. Our objective is to examine carbon functional alterations in the belowground compartment in the case of a long term beech decline. Soil and nutrient content profiles were characterized under five healthy and five declining trees. Seasonal root growth and rhizodeposition were characterized using, ingrowth cores, and microbial biomass combined with soluble carbon organic content, respectively. Podzolisation associated with soil acidification and deficiencies in Ca, Mg and Mn were observed in the soil under declining trees, but not under healthy trees. Spring fine root growth was higher in declining trees than in healthy trees but there were only minor differences concerning rhizodeposition proxies. In our study, we showed that the tree health status is associated to a marked heterogeneity of soil characteristics. Podzolisation close to declining trees leads to a local mineral deficiency which probably stimulates their fine root growth but without altering their flux of carbon exudates.

1. Introduction
Tree decline is a complex phenomenon, in which several biotic and abiotic factors often interact with one another (Manion, 1981). Some of these factors, for example drought and/or pest-related damages, might be enhanced in temperate regions as a result of global warming (IPCC, 2014; Lindner et al., 2010), however its effect on the frequency and intensity of future tree decline and mortality events is still uncertain (Allen et al., 2010). To better predict the impact of global change on forests, it is therefore necessary to increase our understanding of the functional mechanisms underlying tree decline and mortality (Iréda and Badeau, 2008). Recent research on the short term or long term ecophysiological responses of trees to moderate or severe drought leading to decline, has shown that carbon (C) and/or water transport and/or storage could be altered during these events (Delaporte et al., 2016; McDowell, 2011; McDowell et al., 2008; Sala et al., 2010). To date, most of the studies have focused on processes occurring in the plant itself. Despite their potential importance in tree functioning (Högberg, 2011), processes occurring at the plant-soil interface have seldom been investigated.

Both internal and external factors influence root growth and the structure of the root system in trees. Tree age is an example of these internal factors: older trees spend more resources on maintenance than on tissue growth, allocating more resources into the production of fine absorbing roots and fewer into large structural roots (Pregitzer, 2002; West et al., 1999). However, (Zanetti et al., 2015) recently proved that root structure and growth are influenced more by soil and environmental factors than by genetic determinants. The availability of water and nutrients activates a series of genes/proteins at the root level, which inhibit or accentuate root growth (Gallais and Hirel, 2004; Giehl et al., 2014). For example, drought usually causes decreased root growth (Teskey and Hinckley, 1981).

The plant-soil interface commonly designated the rhizosphere, is the portion of soil under the influence of plant roots. These significantly modify water and nutrient availabilities, pH and microbial activity of the soil around them (Hinsinger et al., 2009) via rhizodeposition, i.e. the release of C by plant roots (Lynch and Whipps, 1990). Rhizodeposition strongly stimulates microbial activity in the vicinity of roots (Lynch and Whipps, 1990) and is a major component of the C balance of trees: it is estimated that between 40% and 70% of the assimilated C is transferred to the rhizosphere (Grayston et al., 1997). Plant roots interact with a wide array of microorganisms, and partially control the
processes of organic matter degradation/transformation and nutrient release (Taiz and Zeiger, 2013; Walker et al., 2003). Plants and microorganisms interact, exchanging molecule-signals which modify the behaviour and growth of the root system (Puga-Freitas and Blouin, 2015; Zhuang et al., 2013). However, soil microbial communities can also have negative effects on plant growth: either directly by acting as pathogens, or indirectly by competing with plants for nutrients (van der Heijden et al., 2008). Plants and rhizospheric micro-organisms interact tightly and depend highly on each other, so plants and soil can be considered as a continuum (Hogberg and Read, 2006).

The C balance of trees may be altered during decline (Anderegg et al., 2014; McDowell et al., 2008). More precisely, their photosynthetic C assimilation could be reduced (Anderegg et al., 2014) and/or C transport by phloem could be impaired (Sevanto et al., 2011). Girdling experiments have shown that interrupting C transport by phloem prevented rhizodeposition, and therefore limited the availability of resources to rhizospheric microorganisms (Hogberg et al., 2001). Moreover, when phloem transport is impaired by girdling, the stoichiometry of nutrients in the soil is altered: the inorganic N concentration increases (Hogberg et al., 2007; Weintraub et al., 2007), while organic C and N concentrations decrease (Dannenmann et al., 2009; Ekberg et al., 2007; Weintraub et al., 2007). Since rhizodeposition is a major source of labile C for soil microorganisms (Hogberg and Read, 2006), a reduction of the quantity and/or a modification of the quality of rhizodeposits may result in a decrease of total microbial biomass (Dannenmann et al., 2009; Hogberg and Hogberg, 2002), and of the diversity of the microbial community (Koranda et al., 2011; Schulze et al., 2005). Moreover, when rhizodeposition is altered by girdling, this can lead to decreased microbial activity in the soil (Hogberg and Read, 2006). The sensitivity of soil microbial communities to quantity and quality of C and N provided by tree roots is therefore highlighted by girdling experiments.

The C flux from tree roots to soil micro-organisms can also be altered by environmental factors. For example, (Ruehr et al., 2009) showed that C transfer from beech trees to soil micro-organisms was significantly delayed under drought conditions. Moreover, it has been hypothesized that during a drought, adult beech trees could decrease the activity of free-living soil microorganisms by reducing rhizodeposition (Dannenmann et al., 2009). This decreased microbial activity could in turn affect the nutritional status of the tree, either positively by shifting the competitive balance for N in favour of the tree rather than of soil microorganisms (Dannenmann et al., 2009), or negatively by decreasing the microbial mineralisation (Kreuzwieser and Gessler, 2010), resulting in altered nutrient contents in trees (Sardans et al., 2008a, 2008b).

It is therefore possible that such impairment of the transfer of C to the roots and soil might lead to changes in rhizosphere microbial community during drought-induced tree decline, consequently affecting the nutritional status of trees. However, the relationship between tree decline and C flux to roots and soil is still very poorly documented. A study of unexplained Eucalyptus decline showed that diminished tree crown health was associated with a modification of soil functional diversity, which could indicate an alteration of the chemical composition of rhizodeposits in declining trees (Cai et al., 2010).

Since rhizodeposition cannot be assessed directly, we relied on several proxies to estimate quantity and quality of rhizodeposits. Root soluble organic carbon (Marchand, 2003), rhizospheric soluble organic carbon (Haynes and Francis, 1993), and microbial biomass (Haynes and Francis, 1993) were used as proxies of the quantity of rhizodeposits. Bacterial abundance in the rhizospheric soil was used as a proxy for both quantity and quality of rhizodeposits, by discriminating between copiotrophic and oligotrophic bacteria (Dennis et al., 2010). The functional diversity of soil bacteria was used as an indicator of the chemical diversity of rhizodeposits (Baudoin et al., 2002).

More than biological soil properties, physico-chemical properties and pedological characteristics of the soil could largely influence soil functioning. Indeed, soil properties are intimately linked with biotic processes through complex feedbacks controlling notably nutrient availability and fine tree health. For example, in the Fontainebleau forest, a scheme of functioning of Fagus sylvatica soil-tree system under Luvisol and Podzsol was proposed by Ponge et al. (1999). Tree height could be explained with the help of the following three variables: litter quality, soil-dwelling earthworms and access to lime. These authors showed that the calcium cycle and in particular its availability at the soil surface, related to the presence of active anecic species of earthworms, was positively correlated to trees dimensions. Soil properties may also influence the microbial community. For example, soil pH can affect the contribution made by different functional groups and the total size of the microbial community (Anderson and Domsch, 1993; Wardle, 1992).

In this study, we wondered whether the decline of mature beech trees observed at the crown level could be related to changes in the belowground compartment, and so we investigated three aspects of the belowground soil-plant continuum: soil profiles, root-related features (rooting depth, root mass and root growth); and rhizodeposition-related features (quantity and quality of rhizodeposits). More precisely, we sought answers to the following questions:

- Is tree decline associated with changes in soil quality (horizons, main chemical characteristics)?
- Are root features (mass and growth) altered by the health status of trees?
- Is the quantity and/or quality of rhizodeposits associated with tree decline?

2. Material and methods

2.1. Site description and sampling design

The study was carried out in the Fontainebleau state forest, France (48°22′N, 02°36′E, mean elevation 120 m a.s.l.), during 2013. This forest extends over 17,000 ha, 60 km southeast of Paris. The climate is temperate, with a mean annual temperature of 10.6 °C and a mean annual cumulative precipitation of 749 mm, well distributed throughout the year (averages for the period 1960–2010). The study site is a mature, even-aged, monospecific beech (Fagus sylvatica L.) stand with a surface of 5 ha, where in 2011 the mean tree age was 95 years and the dominant height 27 m. It has shown signs of decline since the late 1990s. Trees were growing on a rather shallow soil (mean depth 0.6 m), with a C:N ratio of 18.7 and an extractable soil moisture of 101 mm.

From the dominant storey, five trees with altered crowns (“declining”) and five trees with intact crowns (“healthy”) were chosen for the present survey. These trees are distributed throughout the plot described above. Crown loss was evaluated using the DEPEFEU (DEPErissement des FEUillus) protocol (Nagelis, 2010), which quantifies crown thinning on a scale from 0 (healthy tree) to 4 (dead tree). Selected healthy trees had large, dense crowns and abundant fine ramification (average DEPEFEU score: 0.9), while declining trees had reduced crown areas, very transparent crowns, only one or two main branches remaining and very limited fine ramification (average DEPEFEU score: 3.6). On average, declining trees had 70% less leaf area than healthy trees, as estimated with the DEPEFEU protocol. Mean diameters at breast height (45.9 ± 5 cm) and heights of the two groups (26.5 ± 4 m) were similar.

The soil profile at the foot of each tree was characterized. The pH of the soil solution was measured in the laboratory by mixing 5 g of air dried and sieved (< 2 mm) soil samples collected at depths of 0–10 cm, 10–20 cm and 20–30 cm with 25 mL of distilled water. Samples were then shaken during 45 min, and allowed to settle for an hour at room temperature. The pH of the resulting solution was then measured with a pH meter (P407, Consort, Belgium).
2.2. Root mass and growth

To assess root mass and growth, three ingrowth cores per tree were set up ca. 30 cm from the stem of each tree in January (2013/01/16). The three cores were evenly distributed around the stem, 10 cm in diameter, and 30 cm deep. They were divided in three 10 cm thick layers immediately after sampling. Then the segmented cores were placed in a cooler, and brought to the laboratory for treatment. Within three days from the sampling date, roots were separated from the soil by hand and washed in tap water to remove as much soil and debris as possible. Roots were then separated in three categories (Le Goff and Ottorini, 2001): coarse roots (more than 5 mm in diameter), medium roots (diameter between 2 mm and 5 mm), and fine roots (less than 2 mm in diameter). The soil was then sieved at 2 mm, and put back in place within a week of a sampling. The roots were freeze-dried during three days, then their dry weight was determined. In July (2013/07/10) and December (2013/12/17), this procedure was again applied to the same ingrowth cores. The roots contained by the cores on those two dates therefore grew during January–July and July–December periods.

2.3. Soil microbial activity measurements

In March (2013/03/22), June (2013/06/18) and November (2013/11/04), two superficial rhizospheric soil samples (between 0 and 10 cm deep) per tree were taken. Rhizospheric soil was defined as the soil that remained adherent to the roots when gently shaken. The two samples were taken at diametrically opposed locations, ca. 30 cm from the stem. The samples were brought to the laboratory in a cooler, and processed during the following week. Roots and rhizospheric soil were separated, and soil samples were sieved at 2 mm. The sieves were thoroughly rinsed with tap water, then cleaned with 90% ethanol between sample.

2.4. Microbial biomass and soluble organic C in soil and fine roots

To determine soil soluble organic C concentrations, rhizospheric soil samples were dried for 12 h at 37 °C then finely ground with a ball mill (MM301, Retsch, Germany). Five g of soil were extracted with 25 mL of distilled water for 18 h at 80 °C. To determine soluble organic C in roots, root samples were freeze dried for 3 days and ground to fine powder with a ball mill (MM301, Retsch, Germany). Half a g of root powder was extracted with 15 mL of distilled water at 60 °C. Microbial biomass was estimated by C and N content of microbial communities in fresh soil samples using the chloroform extraction-fumigation method (Vance et al., 1987). Briefly, two 7.5 g aliquots from each soil sample were first weighed. One was fumigated with chloroform at 25 °C, in the dark during 24 h, while the other was not. The organic C of both aliquots was extracted with a K₂SO₄ solution (0.5 M). The C content of the microbial biomass could not be measured in November due to technical difficulties.

The resulting solutions were filtered through Whatman GF/C glass microfiber filters (GE Healthcare, UK). Dissolved organic C and N were analysed in a Total Organic Carbon analyser (Shimadzu ON-LINE TOC- CSH, Tokyo, Japan). Results were expressed as g of organic C or N per g of dry soil.

2.5. Cultivable soil bacteria

Four g of fresh sieved rhizospheric soil were extracted with 40 mL of phosphate buffer (7.2 g Na₂, 2.8 g Na₂HPO₄, 0.4 g KH₂PO₄ L⁻¹ water, pH 7.2) during 10 min at room temperature, and shaken at 40 rpm. The resulting solution was centrifuged at 750 rpm during 10 min. Phosphate buffer was then added to the resulting supernatant to obtain 10⁻³ to 10⁻⁵ successive dilutions. A hundred μl of each of these dilutions were spread onto agar plates (Tryptic Soy Agar, Sigma-Aldrich, France) to estimate the number of cultivable bacteria, quantified as colony-forming units (CFU). Two replicates were prepared for each dilution. The agar plates were incubated in the dark at 27 °C. CFU were counted two days (fast-growing bacteria), and ten days (slow-growing bacteria) after inoculation. Results were expressed as log CFU per g dry soil.

2.6. Soil functional bacterial diversity

To estimate the functional diversity of the microbial community in the rhizosphere, catabolic profiles were established using Biolog Ecoplates™ (Biolog, USA). A hundred and fifty μl of the 10⁻³ dilution from the cultivable bacteria quantification were placed in each well of a Biolog Ecoplate™. The plates were incubated in the dark at 27 °C, and the colour development in the wells was determined every 24 h with a microplate reader (2960, Metertech, Taiwan) at 550 nm. Results were expressed as Average Well Colour Development (AWCD) and Shannon’s diversity index (H) (Cai et al., 2010; Gomez et al., 2006; Zak et al., 1994).

2.7. Soil chemical analysis

The concentration of free Al, Ca, and some bases such as Mg and Mn was measured in four soil horizons: A, E1 or BP, E2 and BT. All A horizons were sampled at a depth between 5 and 10 cm; E1 or BP (under three trees, the top part of the E horizon (E1) was naturally substituted by a BP horizon) between 15 and 30 cm; E2 between 35 and 40 cm in all profiles excepted under healthy trees H1 and declining trees D109 and D110 (25–30 cm); BT between 50 and 55 cm, excepted under healthy trees H9 (80–85), H14 (75–80 cm), declining trees D109 (35–40 cm) and D110 (40–45 cm). Soil samples of each horizon were sieved at 2 mm, an aliquot were calcinated in a furnace at a temperature of 800 to 1200 °C. Thereafter, 200 mg of calcinated soil were weighted and placed in a Teflon beaker. Then, 3 mL of concentrated HNO₃ and 1 mL of concentrated HCl were added. The mixture was incubated during 30 min to room temperature, boiled and then evaporated to dryness. Once evaporated, 1.5 mL of concentrated HNO₃ was added and diluted in 50 mL deionised water. Mg, Mn, Ca and Al were then analysed by atomic absorption spectrophotometry (AAS 240 FS Varian, Agilent Technology, Australia).

2.8. Statistical analysis

The effect of health status was tested using Student’s t-tests. Potential differences between sampling dates were tested with pairwise paired Student’s t-tests. Wilcoxon tests were performed for distinguishing pH values and mineral elements (Al, Mg, Mn and Ca) concentrations in soil horizons under healthy or declining trees. All statistical analyses were performed with the R software package, version 3.1.3 (http://www.r-project.org/).

3. Results

3.1. Soil profiles, soil nutrient concentrations and pH

The soil profile at the foot of each tree is described in Table 1. The soil profiles of healthy trees all corresponded to typical Luvisol (Luvisol typique (Baize and Girard, 2009), haplic Luvisol (IUSS-Working-Group, 2015) or Hapludalf (Soil-Survey-Staff, 2014)), ca. 1 m deep. Soil profiles close to declining trees were more variable, ranging from shallow Luvisols (Trees 109 and 110, Table 1) to deeper Luvisols also characterized by eluvial E and argillic BT horizons but showing a spodic chocolate-coloured (Munsell hue 7.5YR, value 4, chroma 4 when moist) BP horizon (podzolic B horizon, characterized by illuvial accumulation of amorphous dispersible organic matter and aluminium-dominated sesquioxides coating soil particles and sometimes filling pores), above a lighter eluvial (E) horizon (Table 1). The podzolisation process was visible to the naked eye in the topsoil of three out of five declining trees (Table 1) and was undetectable under all the other investigated trees.
was also noticed that the OH horizon (Zanella et al., 2011a); equivalent to the Oa horizon in IUSS Working Group (2015) and Soil Survey Staff (2014) was absent in the topsoil of three out of five healthy trees (Table 1).

The soil nutrients content of the A horizon showed that the soil under healthy trees was richer (Table 2, P < 0.05) than the soil under declining trees. The significant higher Ca concentration in A, E2 and BT healthy soil horizons have to be underlined, with 70 times more Ca in the BT horizon of healthy trees than under declining trees (0.473 vs. 34.02 mg kg$^{-1}$, P < 0.001). Results also revealed that a process of podzolisation was occurring under declining but not under healthy trees. This process is localized at the foot of the trees and corresponds to podzolisation was occurring under declining but not under healthy trees (Table 1).

Concentration of soil nutrient (Mg manganese, Mn magnesium, Ca calcium, Al aluminium) in the different soil horizons under healthy and declining beech trees growing in the Fontainebleau state forest (France). Error bars represent standard errors. For each depth, significant differences (Student's t test) between healthy and declining trees are indicated by * (P < 0.05) and ** (P < 0.001).

### Table 2

<table>
<thead>
<tr>
<th>Horizon depth (cm)</th>
<th>Healthy</th>
<th>Declining</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.120 ± 0.04</td>
<td>0.278 ± 0.09</td>
</tr>
<tr>
<td>BP/E1</td>
<td>0.211 ± 0.11</td>
<td>0.271 ± 0.204</td>
</tr>
<tr>
<td>E2</td>
<td>0.105 ± 0.044</td>
<td>0.022 ± 0.09</td>
</tr>
<tr>
<td>Bt</td>
<td>0.107 ± 0.062</td>
<td>0.043 ± 0.09</td>
</tr>
</tbody>
</table>

In January, when ingrowth cores were set up, healthy and declining trees had a similar mass of fine and coarse roots per core, whatever the depth (Fig. 2). However, between 10 and 20 cm depth, declining trees had significantly fewer medium-sized roots than healthy trees (P < 0.05, Fig. 2b). In July and December, there were no medium and coarse roots both in healthy and declining trees: only fine roots had grown since the previous sampling. Between January and July, declining trees produced more root biomass than healthy trees at from 0 to 10 cm (P < 0.05, Fig. 2a) and 20 to 30 cm (P < 0.05, Fig. 2c). However, by December, healthy and declining trees show the same amounts of fine root, whatever the depth (Fig. 2).

### Table 3

<table>
<thead>
<tr>
<th>Soil horizon</th>
<th>Mg mg kg$^{-1}$</th>
<th>Mn mg kg$^{-1}$</th>
<th>Ca mg kg$^{-1}$</th>
<th>Al mg kg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declining</td>
<td>Healthy</td>
<td>Declining</td>
<td>Healthy</td>
<td>Declining</td>
</tr>
<tr>
<td>A</td>
<td>0.120 ± 0.04 a</td>
<td>0.150 ± 0.109 a</td>
<td>0.521 ± 0.23 a</td>
<td>0.590 ± 0.21 a</td>
</tr>
<tr>
<td>BP/E1</td>
<td>0.211 ± 0.11 b</td>
<td>0.271 ± 0.17 a</td>
<td>0.204 ± 0.169 a</td>
<td>0.312 ± 0.219 a</td>
</tr>
<tr>
<td>E2</td>
<td>0.105 ± 0.044 a</td>
<td>0.022 ± 0.09 c</td>
<td>0.093 ± 0.01 a</td>
<td>0.089 ± 0.04 a</td>
</tr>
<tr>
<td>Bt</td>
<td>0.107 ± 0.062 a</td>
<td>0.043 ± 0.09 a</td>
<td>0.093 ± 0.01 a</td>
<td>0.089 ± 0.04 a</td>
</tr>
</tbody>
</table>

Concentrations of soluble organic carbon in roots were similar in healthy and declining trees (Table 3). They increased significantly between March and June (P < 0.001). They were not significantly different between March and June (P < 0.001) (Table 3).
3.4. Carbon in the microbial biomass

Similar amounts of C and N were contained in the rhizospheric microbial biomass of healthy and declining trees, at all sampling dates (Table 3). The C:N ratio of the microbial biomass was similar in healthy and declining trees in March and June (Table 3), however C and N content and C:N ratio of the microbial biomass varied significantly between these two sampling dates. The C content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$). The C:N content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$). The C:N content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$). The C:N content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$). The C:N content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$). The C:N content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$). The C:N content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$). The C:N content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$). The C:N content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$). The C:N content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$). The C:N content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$). The C:N content of the microbial biomass increased significantly between March and June ($P < 0.05$), and then decreased between June and November ($P < 0.001$).

3.5. Cultivable bacteria counts

In March and June, healthy and declining trees had similar numbers of fast-growing cultivable bacteria in their rhizospheric soil (Fig. 3a). However, by November, declining trees had significantly fewer fast-growing cultivable bacteria in their rhizospheric soil than healthy trees.
(P < 0.05, Fig. 3a). Healthy trees had similar numbers of fast-growing cultivable bacteria in their rhizospheric soils in March and June, and these had increased by November. The numbers of fast-growing cultivable bacteria in the rhizospheric soils under declining trees were similar at all sampling dates (Fig. 3a). Rhizospheric soils under declining trees had fewer slow-growing bacteria than under healthy trees in March (P < 0.01). However, soils under trees of both health statuses had increased by November. The numbers of fast-growing bacteria in the rhizospheric soils under declining trees were statistically similar at all sampling dates (P ≥ 0.05), with a thick (> 2 cm) OH horizon present under all the trees used in our experiment except three healthy trees. The absence of the organic horizon in these 3 healthy trees might be explained by the presence of active endogeic earthworms directly transforming OL and OF at the A horizon of a Dysmull humus form (Delhaye and Ponge, 1993; Ponge et al., 1999, 2005). Additionally, beech litter (C:N = 40) promotes podzolisation on this substrate by producing massive amounts of soluble organic acid compounds that alter the structure of clay and form organo-metallic complexes with iron and aluminium (Robin et al., 2011b) which originate from iron-magnesium clay minerals altered by the acid soil solution. Organo-metallic complexes migrate downwards (significant increase in Al concentration with depth under declining trees, Table 2), colouring the subjacent clearer E horizon of a preexistent Luvisol. The process starts with the formation of a Dysmoder humus form (Zanella et al., 2011b), with a thick (> 2 cm) OH horizon present under all the trees used in our experiment except three healthy trees. The absence of this organic horizon in these 3 healthy trees might be explained by the presence of active endogeic earthworms directly transforming OL and OF at the A horizon of a Dysmull humus form (Delhaye and Ponge, 1993; Ponge et al., 1999).

A recent process of podzolisation can explain the trend observed in the first 20 cm of soil under declining trees, concerning A and BP

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Date</th>
<th>Healthy trees</th>
<th>Declining trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil soluble organic C</td>
<td>March</td>
<td>3.14 ± 0.81 a</td>
<td>3.72 ± 0.53 a</td>
</tr>
<tr>
<td>(mg g dry soil⁻¹)</td>
<td>June</td>
<td>2.39 ± 0.12 b</td>
<td>2.66 ± 0.07 b</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>1.93 ± 0.29 b</td>
<td>2.00 ± 0.19 b</td>
</tr>
<tr>
<td>Root soluble organic C</td>
<td>March</td>
<td>2.08 ± 0.37 a</td>
<td>1.59 ± 0.10 a</td>
</tr>
<tr>
<td>(mg g dry matter⁻¹)</td>
<td>June</td>
<td>6.70 ± 1.59 b</td>
<td>8.01 ± 1.05 b</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>7.87 ± 0.87 b</td>
<td>7.19 ± 1.28 b</td>
</tr>
<tr>
<td>Microbial biomass C</td>
<td>March</td>
<td>1.51 ± 0.21 a</td>
<td>1.12 ± 0.19 a</td>
</tr>
<tr>
<td>(mg C g dry soil⁻¹)</td>
<td>June</td>
<td>2.64 ± 0.52 b</td>
<td>2.12 ± 0.31 b</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Microbial biomass N</td>
<td>March</td>
<td>0.08 ± 0.01 a</td>
<td>0.07 ± 0.01 a</td>
</tr>
<tr>
<td>(mg N g dry soil⁻¹)</td>
<td>June</td>
<td>0.11 ± 0.01 b</td>
<td>0.09 ± 0.01 b</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>0.05 ± 0.01 a</td>
<td>0.06 ± 0.01 a</td>
</tr>
<tr>
<td>C:N ratio of the microbial biomass</td>
<td>March</td>
<td>18.87 ± 4.33 a</td>
<td>16.00 ± 1.99 a</td>
</tr>
<tr>
<td></td>
<td>June</td>
<td>24.00 ± 0.83 b</td>
<td>23.55 ± 1.28 b</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

### 3.6. Functional bacterial diversity

Rhizospheric bacterial communities under declining trees tended to have a lower AWCD than those under healthy trees in March and June, and a higher AWCD in November, though these differences were not statistically significant. Nevertheless, the AWCD of rhizospheric bacterial communities under healthy and declining trees displayed contrast-ing temporal dynamics (Fig. 4a). Under healthy trees, AWCD decreased consistently between March and November, whereas under declining trees, it decreased between March and June, then increased between June and November (Fig. 4a). Shannon’s diversity index (H’) was not affected by tree health status on any date (Fig. 4b). It showed small, but significant, variation during the growing season: it decreased between March and June, then increased between June and November (P < 0.0001, Fig. 4b). Rhizospheric bacterial communities under healthy and declining trees mostly used the same carbon sources, but at different intensities (Table 3). Carbon sources used by bacterial communities under trees of only one of the two health statuses were usually some of the least used (Table 4).
horizons. This recent process is superposed to an older one, invisible by the naked eye and detected by the measure of Al concentration done in the lower part of the profile, between 30 cm (lower part of E horizon: E2) and 60 cm (until BT horizon). In Fontainebleau sands, a similar superposition of podzolisation processes has been related to the historical natural succession of beech and oak forests (Robin, 1979; Robin et al., 1983).

E and BT horizons are thicker under healthy than under declining trees (Fig. 5). Some previous studies reported that the closer the limestone layer to the soil surface was, the weaker the process of podzolisation (Delhaye and Ponge, 1993; Ponge et al., 1999; Robin, 1990) was. In addition, Ponge et al. (1999) ascertained a significant negative correlation between the depth of the limestone layer and the calcium content of beech litter: the depth of the limestone layer controls the availability of calcium for the trees. On the reverse, in our plots, we found that the deeper the limestone table was, the healthier the trees were and the richer in Ca was the litter. It could be explained by the fact that the limestone layer observed under declining trees was nearer to the soil surface but less accessible, because located below a massive BT horizon forming an obstacle for roots (Gregory, 2006; Maeght et al., 2013). Consequently, it has impeded tree root access to the underlying limestone layer. Such a physical obstacle could be a cause of calcium deficiency in declining trees which grow on a soil with a less accessible calcium source even if the limestone layer is nearer to the surface (Fig. 5). The possible role of calcium in their decline seems to be confirmed by the observation of faster increases in soil pH under

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**Table 4**

Rankings of carbon sources used by healthy and declining beech trees growing in the Fontainebleau state forest (France) in March, June and November. A carbon source is considered to be used significantly when its average colour development is above 0.25. Green indicates carbon sources for which healthy and declining trees share the same rank. Yellow indicates carbon sources that are used by both healthy and declining trees, but at different ranks. Red indicates carbon sources used by only one of the two health statuses. Different letters represent different C sources. A list of the carbon sources corresponding to each letter is available Supplementary data (Table A1) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

<table>
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<tr>
<th>Ranking</th>
<th>March Healthy trees</th>
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<th>June Healthy trees</th>
<th>Declining trees</th>
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Fig. 4. Indicators of the functional bacterial diversity in the rhizospheric soils of healthy (black) and declining (grey) beech trees growing in the Fontainebleau state forest (France) in March, June and November: Average Well Colour Development (AWCD, a) and Shannon’s diversity index (H, b). Error bars represent standard errors. For each date, results of the Student’s t-test between healthy and declining trees are indicated by ns. (P > 0.05).
healthy than declining trees. Furthermore, Ponge et al. (1999) showed that the densities of soil-dwelling endogeic earthworms, known for their high calcium requirement (Piearce, 1972), were negatively correlated with the depth of the limestone layer. These worms can contribute to the preservation of a Dysmull humus form (no OH horizon under three of the five healthy trees) instead of a Dysmoder (presence of a thick OH horizon under all declining trees), thereby slowing or arresting podzolisation (Fig. 5). Calcium deficiency could therefore have a direct and indirect (through a feedback loop induced by the calcium content of the litter) role in the observed decline. A study on sugar maple emphasize the importance of Ca and Al to health and highlight the vulnerability of sugar maple stands to declines in growth and vigor following continued anthropogenic Ca depletion (Bal et al., 2015; Schaberg et al., 2011). In our case, the Ca role could be confirmed by measuring the calcium content in beech leaves and fresh litter fall.

The soil under healthy trees is richer in Al but also in nutrients than the soil under declining trees (Table 2). The difference in base content between the two soils reaches a maximum in BT horizons where we measured 2–3 times more Mg, Mn and 60 times more Ca under healthy compared to declining trees. This abundance of nutrients and the high quantity of calcium neutralizes the toxicity of Al, reversing the process illustrated by (Godbold et al., 1988) for coniferous trees (even a little concentration of Al reduces Ca uptake by spruce roots).

4.2. Root densities and growth

The majority of the fine root mass was found in the upper 10 cm of soil, which is coherent with previous observations (Mainiero and Kazda, 2006). These superficial roots have been shown to be particularly important to drought response. Indeed, they enable rapid water transport when the soil is wet, but disconnect the tree from the driest soil patches when drought-induced embolism develops (Alder et al., 1996; Martínez-Vilalta et al., 2002).

Initial fine root densities were not significantly affected by tree decline (Fig. 2), a finding coherent with results obtained on beech, which showed that the size of the fine root system is only weakly influenced by soil fertility and acidity (Leuschner et al., 2004). Therefore, even if soil profiles under healthy and declining trees differed (Table 1), this probably had no effect on fine root densities. However, declining trees had no medium-sized roots between 10 and 20 cm below the surface in January (Fig. 2b). This absence could be related to the presence of a BP horizon at these depths under some of the declining trees (Table 1). It can therefore be hypothesized that adverse soil properties at depths of 10 to 20 cm hindered the exploration of this layer by roots of declining trees.

During the first half of the year, declining trees produced more fine roots than healthy trees in 0–10 and 20–30 cm layers (Fig. 2). Trees living on nutrient poor soils can in some cases increase the production of fine roots (Dréanou, 2006). An increased root production in the organic layer has also been observed in Picea abies, where the production of fine roots in acidic soils was twice that in less acidic soils (Jentschke et al., 2001). However, the root growth of Picea abies decreased at a deeper level in the profile (Jentschke et al., 2001), in contrast to our observations. In beech, stronger root growth has been associated with more acidic topsoil pH (Leuschner and Hertel, 2003). This enhanced root growth in acidic and nutrient poor sites can be
interpreted as a compensatory response to low nutrient availability in soils with low biological activity (Vogt et al., 1995). This relation may explain the stronger fine root growth we observed in declining trees, as topsoil pH and nutrient availability were lower under declining than under healthy trees. In addition, it has been suggested that fine root production and turnover are sensitive indicators of changing soil environment (Leuschner and Hertel, 2003). The enhanced growth of fine roots in declining trees during the first part of the growing season could therefore reflect differences in soil properties (for example nutrient availability) under healthy and declining trees, along with the transition from Luvisols to Podzols, and the corresponding change of the humus system from Mull to Moder.

### 4.3. Rhizodeposition and bacterial biodiversity

Due to the complex nature of the soil environment, the quantity and quality of rhizodeposition cannot be assessed directly. Therefore, various proxies were used to study these variables. Root and soil soluble C contents and C content of microbial biomass are all proxies of the quantity of rhizodeposits (Haynes and Francis, 1993; Lynch and Whipp, 1990; Marchand, 2003; Vance et al., 1987). The health status of trees did not significantly alter these proxies, so the quantity of rhizodeposits was probably similar in healthy and declining trees. Since rhizodeposition is a major source of substrates for rhizosphere microorganisms (Lynch and Whipp, 1990), indicators of microbial abundance can be used as proxies for the quantity of rhizodeposits. In addition to the C content of the microbial biomass presented above, the abundance of cultivable bacteria can therefore be used as an indicator not only of the quantity, but also of the quality of rhizodeposits (Dennis et al., 2010).

In November, rhizospheric soils under declining trees had fewer fast-growing bacteria than under healthy trees (Fig. 3), a result that can be linked with the observations made by (Carletti et al., 2009) that acid soil and harsh climatic conditions lead to less diverse, more specialized and more stable soil bacterial communities than favourable environmental conditions, whereas an environment favourable to microbial life leads to more diverse, more uniform and very active universal communities. Although the Shannon index showed similar functional diversities under healthy and declining trees, the less favourable topsoil conditions under declining trees (more acidic, and probably with a lower nutrient content associated with podzolisation) could have led to the development of more specialized and more slower-growing bacterial communities than in the topsoil of healthy trees at the end of the leafy season (Fig. 5).

In March, soils under declining tree had fewer slow-growing bacteria than those under healthy trees (Fig. 3). This observation suggests slight differences in the quantity and/or composition of rhizodeposits and their temporal dynamics during periods of low C transfer by the trees to the soil (Epron et al., 2011). The composition of rhizodeposits has been investigated through catabolic profiles of the microbial community (Baudoin et al., 2002; Grayston et al., 1997). The overall catabolic activity (as indicated by AWCD) of the bacterial community of rhizospheric soil tended to be lower under declining than healthy trees in March and June, yet higher in November. This resulted in contrasting patterns of seasonal evolution in healthy and declining trees (Fig. 4a). Variation in the catabolic activity of rhizospheric soil under healthy and declining trees could be related to different environmental conditions. Indeed, the thinner canopies of declining trees allow higher irradiance levels to reach the ground, which could lead to a greater variability of soil temperature and/or water availability in the vicinity of declining trees, contributing to decreased biological activity in their rhizosphere. In contrast to observations in Eucalyptus (Cai et al., 2010), this difference in microbial activity was not associated with differing Shannon’s diversity indexes (Fig. 4b), and the bacterial communities in the rhizosphere of healthy and declining trees used mainly the same substrates (Table 3). The functional diversity of rhizospheric bacterial communities was therefore not related to tree decline, and the composition of rhizodeposits of healthy and declining trees was probably mostly similar.

### 4.4. Decline and plant-soil relationships

The main findings of this study are summarized in Fig. 5. Overall, the aforementioned proxies did not indicate any clear differences in the quality and quantity of rhizodeposits under healthy and declining trees. Seasonal variations of the quantity and quality of rhizodeposition are stronger than the variations induced by the health status of trees. The prevalence of the seasonal effect on rhizodeposition over alterations of internal C fluxes of the tree has already been reported for beech (Rasche et al., 2011). Indeed, in a girdling experiment, impaired phloem transport caused less variation in the phylogenetic composition of archael and bacterial communities than the seasonal variation of rhizodeposition (Rasche et al., 2011). In contrast, the C flux to roots (greater growth of fine roots) increased in declining trees during the first half of the year (Figs. 2 and 5). However, root growth and rhizodeposition were estimated locally, so this study may not have detected differing spatial patterns of root growth and/or rhizodeposition between healthy and declining trees. The response of soil microbial community structure (bacteria, archaea, fungi, notably mycorrhiza) to those C fluxes should be investigated more precisely. Any shift in this community structure could alter plant nutrition and indirectly the health status.

Many environmental factors influence rhizodeposition, both qualitatively and quantitatively. Of these, some nutrient deficiencies of the tree (particularly those of iron and phosphorus) are known to increase rhizodeposition (Hirsch et al., 2013; Ryan et al., 2001). The increased root growth of declining trees is correlated with a lower nutrient availability in the rhizospheric soil of declining trees. This is particularly relevant for Al concentration to explain the podzolisation process observed under declining trees (Tables 1, 2 and Fig. 5). Indeed, the formation of a BT horizon closer to the soil surface under declining than under healthy trees could isolate the roots of declining trees from the underlying limestone layer, and therefore induce a calcium deficiency as revealed by soil Ca analysis (Table 2, Fig. 5). This deficiency, in turn, exacerbates podzolization by creating an unfavourable environment for endogenic earthworms, and therefore contributing to the formation of a dysmoder humus form under declining trees (Fig. 5).

### 5. Conclusions

Our study shows that the interactions between tree, climate and soil conditions leading to the decline can be complex. In addition to the role of water and C supply, mineral nutrition should also be taken into account when trying to understand the functional mechanisms underlying tree decline. Mineral nutrition might however not be a decisive triggering factor, but rather a predisposing and/or contributing factor (sensu Manion, 1981), interacting with other environmental conditions such as drought (Becker and Levy, 1988).

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Appendix A  Supplementary data

Supplementary data associated with this article can be found in the online version, at 10.1016/j.apsol.2017.05.004 .

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