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**1 Abstract**

2 Phosphorus (P) is an important nutrient for plant growth but its availability in soil is limited.  
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4 microorganisms system and reduce P availability. In this study we evaluated the rhizosphere effect  
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16 attributed to the release of labile organics through rhizodeposition. The availability of easy  
17 degradable compounds in the rhizosphere should foster the mineralization of the organic matter  
18 with a consequent increase of available P. Hence, we speculate that at high altitude the energy  
19 supplied by the plants through rhizodeposition to the rhizosphere heterotrophic microbial  
20 community is key for fuelling the rhizospheric processes and, in particular, P cycling.

1 **EFFECT OF BEECH (*FAGUS SYLVATICA* L.) RHIZOSPHERE ON PHOSPHOROUS**  
2 **AVAILABILITY IN SOILS AT DIFFERENT ALTITUDES (CENTRAL ITALY).**

3

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28 **Abstract**

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43 attributed to the release of labile organics through rhizodeposition. The availability of easy  
44 degradable compounds in the rhizosphere should foster the mineralization of the organic matter  
45 with a consequent increase of available P. Hence, we speculate that at high altitude the energy  
46 supplied by the plants through rhizodeposition to the rhizosphere heterotrophic microbial  
47 community is key for fuelling the rhizospheric processes and, in particular, P cycling.

48

49 **1. Introduction**

50 Phosphorus (P) is one of the most critical nutrients for the growth of plants and microorganisms,  
51 and is present in soil both in inorganic and organic forms. The inorganic P includes primary  
52 minerals (e.g., apatites, strengite) and secondary minerals such as calcium-, iron-, and aluminum-  
53 phosphates (Shen et al., 2011), while the organic P is mainly made up of phospholipids,

54 orthophosphate monoesters, nucleic acids, teichoic acids and phosphonates (Rubæk et al., 1999;  
55 Kruse et al., 2015). The content and distribution of these different P forms do not have a constant  
56 trend along the soil profile (Makarov et al., 2004; Chiu et al., 2005; Backnäs et al., 2012). In  
57 grassland and beech forest soils located on north-facing slope of southern Swiss Alps, Beck and  
58 Elsenbeer (1999) found that the variation with depth of the organic P concentration mainly depends  
59 on type and age of vegetation, and soil characteristics. Then, Chiu et al. (2005) studied grassland  
60 and coniferous forest soils and found that the inorganic orthophosphate concentration decreased, but  
61 orthophosphate monoesters increased with increasing soil depth. The generally low availability of P  
62 in soil is due to the scarce solubility of P-bearing compounds, both for inorganic and organic forms  
63 (Hinsinger, 2001). For example, in the soil solution P exists as orthophosphate anions and dissolved  
64 forms of organic P, with concentrations that range from 0.01 to 3.0 mg l<sup>-1</sup> (Frossard et al., 2000).  
65 However, plants are able to respond to P deficiency by root-exudation of organic acids that increase  
66 P availability in the soil close to the roots (Ström et al., 2002; Oburger et al., 2009; Zhao et al.,  
67 2010). Furthermore, the simple organic compounds comprising the plant exudates enhance the  
68 activity of the microorganisms that, in turn, favours P availability by *i*) soil acidification due to the  
69 CO<sub>2</sub> produced through respiration, and *ii*) releasing organic acids and phosphatase enzymes  
70 (Marschner et al., 2011). A great part of the complex interactions among P-bearing compounds,  
71 plant and microorganisms occurs in the small soil volume between fine roots and earthy material,  
72 the rhizosphere, where most of the chemical, biochemical and biological reactions take place (e.g.,  
73 Hinsinger et al., 2003; Richter et al., 2007; Lambers et al., 2009). Because of its high sensitivity to  
74 the environmental conditions (Turpault et al., 2007), the biogeochemical processes occurring in the  
75 rhizosphere can be strongly affected by climate. Indeed, climate (temperature, precipitation amount  
76 and pattern) is one of the key soil forming factors that inherently controls the soil profile  
77 development (Darwish and Zurayk, 1997; Fernández Sanjurjo et al., 2003; Riebe et al., 2004), soil  
78 microbial activity (Qiu et al., 2005; Devi and Yadava, 2006), soil organic matter dynamics (Jobbagy  
79 and Jackson, 2000; Brevik, 2013), and macro- and micro-nutrients cycles (Butler et al., 2012;

80 Vincent et al., 2014; Zhang et al., 2014). For example, in a phytotron experiment, Kumar et al.  
81 (2011) found that higher temperatures increased the amount of available P, but decreased organic P  
82 in the rhizosphere of wheat. Instead, as far as we are aware, *in vivo* studies on the P dynamics in the  
83 rhizosphere of forest species at different temperature have never been achieved, although they could  
84 help better understanding the resilience of forest ecosystems with respect to global (and more  
85 regionally localized) warming. The objective of this study was to provide novel field and laboratory  
86 information on this argument. To assess the influence of temperature on the rhizospheric P pool, we  
87 selected one of the main diffused forest species in Europe, i.e. the European beech (*Fagus sylvatica*  
88 L.). By using latitude and altitude as proxies for temperature change (Vincent et al., 2014), we  
89 contrasted the associated changes in European beech rhizosphere and bulk soil collected from the  
90 different horizons of forest soils at two altitudes (800 and 1000 m) on three mountains located  
91 within 1° of latitudinal gradient in central Italy. Specifically, we tested the hypotheses that: 1) soil  
92 organic C, total N, and organic and available P decrease with increasing latitude and altitude, and 2)  
93 the rhizosphere effect on P availability becomes more pronounced when potential nutrient  
94 limitations are more severe, as it happens with increasing latitude and altitude. The above two  
95 hypotheses were tested on rhizosphere and bulk soil from the three study areas through measuring  
96 of organic C, total N, organic and available P, and phosphatase activities; additionally, <sup>31</sup>P-NMR  
97 analyses were performed to assess the different forms of soil P.

98

## 99 **2. Materials and methods**

### 100 *2.1. Study sites*

101 As study areas, three calcareous massifs were selected on the Apennines chain (central Italy):  
102 Mount Terminillo (42°28' N, 12°59' E), Mount San Vicino (43°19' N, 13°03' E), and Mount Acuto  
103 (43°28' N, 12°41' E) (Figure S1 of the Supplementary Materials). For each area, two European  
104 beech (*Fagus sylvatica* L.) forests were chosen on the north-facing slopes at about 800 and 1000 m  
105 above sea level (a.s.l.). A description of the environmental conditions of each site is reported in

106 Table 1. Here it suffices to summarize that the three areas have a similar mean annual air  
107 temperature (MAAT) that varies from about 10°C at 800 m, to about 9°C at 1000 m a.s.l..  
108 Following the latitudinal transect, coldest and warmest months showed a contrasting trend as, going  
109 north, the mean of the coldest month (January) decreased of 1.6-2.0°C, while that of the warmest  
110 month (July) increased of 1.6-1.7°C. In all the areas, the mean temperature for both coldest and  
111 warmest months is lower in the soils at 1000 m than in those at 800 m a.s.l. of 0.6-1.0 and 1.0-  
112 1.1°C, respectively. The mean annual precipitation, similar at both altitudes, do not follow a  
113 latitudinal transect, and is the highest at Mount Acuto, the lowest at Mount San Vicino.  
114 All the forests were coppices in conversion, with the conversion that started from about 20 to about  
115 40 years ago. As indicated by the diameter at breast height (Table 1), the most recent conversions  
116 occurred at Mount Terminillo and Mount San Vicino, at 800 and 1000 m a.s.l., respectively; the  
117 oldest conversions started at Mount Terminillo for the coppice at 1000 m a.s.l., and at Mount Acuto  
118 for the woods at both altitudes. At all sites beech was the dominant tree, with dominances ranging  
119 from 80 to 100%. While the soil cover due to litter was always complete, the coverage due to  
120 understory ranged from 5 to 50%, with scarce to null signs of erosion.  
121 All the soils had developed from limestone rocks with small flintstone layers.

122

## 123 *2.2. Soil sampling*

124 During the winter 2014, at each altitudinal site two profiles were dug within a plot of about 100 m<sup>2</sup>,  
125 for a total of 12 profiles (3 latitudes x 2 altitudes x 2 profiles). The rationale of the winter sampling  
126 was that, in this season, root respiration and exudation, and root-associated microorganism activity  
127 are at their lowest intensity (Epron et al., 2001; Buée et al., 2005; Meinen et al., 2009; Ruehr and  
128 Buchmann, 2010; Calvaruso et al., 2014); hence, more reliable and stable information on the  
129 rhizosphere status can be obtained in winter rather than in more dynamic seasons like spring or  
130 summer. Each profile was opened at 50-60 cm from the stem of the biggest beeches found in the  
131 selected site. Approximately, the age of the trees ranged from about 40 years at Mount Terminillo

132 (800 m a.s.l.) to about 60-65 years at Mount Acuto. However, the age of the tree is of secondary  
133 importance as, for the protocol adopted to obtaining the rhizosphere samples (see below), we  
134 considered only the fine roots, which activity is little dependent on the age of adult plants as they  
135 are renewed every few year (Trumbore and Gaudinki, 2003; Agnelli et al., 2014).

136 The profiles were dug till the parent rock, and the soils were morphologically described according  
137 to Schoeneberger et al. (2012). As a whole, the soils at 800 m a.s.l. showed a *solum* made of the  
138 following sequence of horizons, with respective mean thicknesses (standard deviations in  
139 parentheses): O = 7.2 cm (2.4), A = 7.0 cm (3.2), AB = 7.0 cm (1.5), Bw1 = 13.2 cm (3.7), Bw2 =  
140 12.8 cm (4.7), Bw3 = 26.5 cm (8.9), Bw4 = 18.3 cm (4.2). The soils at 1000 m a.s.l. showed the  
141 following *solum*: O = 10.2 cm (6.2), A = 10.3 cm (3.2), AB = 14.2 cm (7.7), Bw1 = 19.8 cm (9.4),  
142 Bw2 = 10.0 cm (4.7). The mean thickness (excluding the O horizons) of the *solum* was 68.8 cm  
143 (36.3) at 800 m, and 49.2 cm (19.9) at 1000 m. The underneath C horizons are not part of the *solum*  
144 and were excluded from sampling. The litter was made by O horizons that were typical of the  
145 *amphimus* type of humus (Baize and Girard, 2008), which are present in soils with well-developed  
146 O horizons rich of pedofauna, and resting on A horizons with well-developed crumb structure.

147 Roughly, in the soils at 800 m the fine earth content ranged from 85% in the A horizon to 50% in  
148 the Bw3 and Bw4 horizons, while in those at 1000 m it went from 80% in the A horizon to 35-40%  
149 in the Bw1 and Bw2 horizons. All the soils had a *mesic* soil temperature regime, and an *udic* soil  
150 moisture regime, and were classified as Mollisols or Inceptisols (Table 1) according to the Soil  
151 Survey Staff (2014).

152 For each profile, a large amount of sample (at least 3 kg) from each mineral horizon forming the  
153 *solum* was collected and stored in a portable refrigerator for the transport to the laboratory.

154

### 155 2.3. Sample preparation

156 Within one week from the sampling, the beech rhizosphere of each sample was isolated by picking  
157 up the roots together with the adhering soil (Cocco et al., 2013; Massaccesi et al., 2015). The roots



158 with a diameter larger than 2 mm were discarded. After a light shaking to detach the weakly  
159 adhering soil particles, which were then added to the bulk soil (i.e., the soil not strictly adhering to  
160 the roots), the remaining soil material firmly adhering to the fine roots was considered as  
161 rhizosphere and was recovered by further shaking and gentle brushing. An aliquot of the field moist  
162 rhizosphere and bulk of each horizon was stored at 4°C for measuring the phosphatase activities,  
163 while the remaining material was air-dried and sieved through a 2 mm mesh to be used for the  
164 chemical and spectroscopic analyses.

165

#### 166 *2.4. Chemical analysis*

167 The soil pH was determined potentiometrically in water ( $\text{pH}_{\text{H}_2\text{O}}$ ) and in 1 M KCl solution ( $\text{pH}_{\text{KCl}}$ )  
168 (solid:liquid ratio of 1:2.5) after 30 minutes of stirring by a combined glass-calomel electrode. Total  
169 organic C content (TOC) was estimated by K-dichromate digestion, heating the suspension at 180  
170 °C for 30 minutes (Nelson and Sommers, 1996), and total N content was determined by a Carlo  
171 Erba EA1110 dry combustion analyzer (Carlo Erba Instruments, Italy). To measure the water  
172 extractable organic C (WEOC), 1 g of sample was placed into a plastic container, submerged with  
173 distilled water (solid:liquid ratio 1:10) and shaken overnight with an orbital shaker (140 rpm). The  
174 mixture was left to rest for a while, centrifuged at 1400 g for 10 minutes, and then filtered through  
175 Whatman 42 filter paper. The resulting solution was analyzed with a TOC-500A (Shimadzu, Japan)  
176 analyzer after the addition of a few drops of concentrated  $\text{H}_3\text{PO}_4$  to eliminate carbonates. The total P  
177 was determined by the ignition method, and the organic P was calculated by difference between the  
178 total and inorganic P content (Kuo, 1996). Available P was estimated according to Olsen et al.  
179 (1954).

180 Acid and alkaline mono-phosphatase activities were determined according to Tabatabai (1994).  
181 Briefly, in a 50-ml flask, 1 g of sample was added with 0.2 ml of toluene, 4 ml of modified  
182 universal buffer (at pH 6.5 and pH 11 for the acid and alkaline phosphatases, respectively), and 1 ml  
183 of 0.025 M *p*-nitrophenyl phosphate (*p*-NPP) solution. The mixture was incubated at 37 °C for 1 h

184 so to induce the transformation of *p*-NPP into *p*-nitrophenol (*p*-NP) via phosphatase. After  
185 incubation, 1 mL of 0.5 M CaCl<sub>2</sub> and 4 mL of 0.5 M NaOH were added to the samples, mixed for  
186 few seconds, and then filtered through a Whatman 42 filter paper. The color intensity of the filtrate  
187 was measured against a blank at 420 nm by a Lambda EZ 150 UV/VIS Spectrometer (Perkin Elmer,  
188 USA). The results are expressed as μg of *p*-NP produced by 1 g of soil per h of incubation (μg *p*-NP  
189 g<sup>-1</sup> h<sup>-1</sup>).

190 For the <sup>31</sup>P-NMR measurements, which were run only on the A horizons, the extracts were obtained  
191 by shaking 5 g of sample with 100 ml of a solution containing 0.25 M NaOH and 0.05 M EDTA for  
192 16 h at room temperature, in the dark. The mixture was centrifuged at 10,000 g for 30 min and the  
193 liquid phase, once separated from the precipitated, was freeze-dried. The freeze-dried NaOH-EDTA  
194 extracts were re-dissolved in 0.1 ml of 10 M NaOH solution and 0.5 ml of D<sub>2</sub>O, and transferred to a  
195 5-mm NMR tube for the analysis. The <sup>31</sup>P-NMR spectra were obtained using an Arance 600 MHz  
196 NMR spectrometer (Bruker, USA) operating at 243 MHz, with an acquisition time of 0.673 s, and a  
197 delay time of 0.5 s. For each sample 24,576 scans were run. Peak assignments were according to  
198 Turner et al (2003), and the intensities of signals were determined by integration.

199 The accuracy of the run soil analyses follows: pH in water, 0.14; pH in KCl, 0.11; TOC, 2.05 g kg<sup>-1</sup>;  
200 total N, 0.19 g kg<sup>-1</sup>; WEOC, 0.024 g kg<sup>-1</sup>; total P, 35 mg kg<sup>-1</sup>; organic P, 24 mg kg<sup>-1</sup>; available P, 2.5  
201 mg kg<sup>-1</sup>; acid and alkaline mono-phosphatase activity, 5 μg *p*-NP g<sup>-1</sup> h<sup>-1</sup>.

202

### 203 2.5. Statistical analyses

204 To test the effect of each variable (latitude, altitude, soil horizons, and soil fractions) on the soil  
205 properties we performed canonical redundancy analyses (RDA). The RDA model was tested for  
206 significance using 999 random permutations. The variations of soil properties as a function of  
207 latitude, altitude, soil horizons, and soil fractions were assessed by a Principal Component Analysis  
208 (PCA). For each property, all the data were standardized prior the RDA and PCA by subtracting the  
209 mean and dividing by the standard deviation.

210 The RDA indicated a lack of significant effect for latitude (ANOVA,  $F= 2.39$ ,  $P=0.057$ ), whereas  
211 the effects of altitude (ANOVA,  $F= 15.84$ ,  $P=0.001$ ), soil horizons (ANOVA,  $F=4.13$ ,  $P=0.001$ ) and  
212 soil fractions (ANOVA,  $F=62.86$ ,  $P=0.001$ ) on the soil properties were significant. Because of this,  
213 only these three latter significant variables were considered in further detail in our study, and the  
214 three latitudinal areas were therefore considered as replicates. Consequently, the analytical results  
215 obtained from the two samples collected for each horizon at each latitude and altitude were  
216 averaged, and these averages used as replicates so to have  $n=3$ . The data were checked for the  
217 normality of the distribution and the homogeneity of the variances by Shapiro-Wilk and Levene  
218 tests, respectively, and, if necessary, transformed by the Box and Cox (1964) procedure. To assess  
219 significant differences among altitudes, horizons and soil fractions (rhizosphere and bulk soil),  
220 three-way ANOVA was performed, and the comparison of means was assessed by Fisher's LSD  
221 post-hoc test ( $P<0.05$ ). Box plot diagrams were used to show the obtained data. The line inside each  
222 box represents the median. The bottom and top of the box are the first and third quartiles, while the  
223 upper and lower whiskers indicate the minimum and maximum values, respectively; the + sign  
224 within each box plot indicates the average.

225 The statistical analyses were performed using R software (R Core Team, 2014).

226

### 227 **3. Results**

#### 228 *3.1. PCA*

229 The PCA scoring plot (Figure 1) showed variations of the soil properties between rhizosphere and  
230 bulk and between the soils at 800 and 1000 m a.s.l., and identified the axes 1 and 2 that explained  
231 about 53 % and 24 % of the variation, respectively. All the soil parameters showed positive  
232 correlation with PC1 (Figure S2a of the Supplementary Materials), with TOC, alkaline and acid  
233 phosphatase activities, total N, and available and organic P contributing for more than 83 % to the  
234 variability (Table 2). While the  $pH_{H_2O}$  and  $pH_{KCl}$  only slightly affected PC1, they explained 57 % of  
235 the variability along PC2 axis. The scoring plot (Figure 1) showed that the soil properties were

236 affected mainly by altitude and soil fractions and to a lesser extent by soil horizons. Moreover, the  
237 PCA indicated that the differences between the rhizosphere and bulk were somewhat greater at  
238 1000 m than at 800 m a.s.l..

239

### 240 *3.2. Soil pH, organic C and total N*

241 In the soils at both altitudes,  $\text{pH}_{\text{H}_2\text{O}}$  and  $\text{pH}_{\text{KCl}}$  remained relatively constant throughout the profile,  
242 and no significant difference was observed between rhizosphere and bulk soil (Figure 2a, b).  
243 However, both  $\text{pH}_{\text{H}_2\text{O}}$  and  $\text{pH}_{\text{KCl}}$  from A to Bw2 horizons were higher at 1000 m than at 800 m  
244 altitude.

245 As expected, TOC content tended to decrease with depth throughout all the soils (Figure 3a). On a  
246 horizon by horizon comparison between the two altitudes, TOC content was greater at 1000 m than  
247 at 800 m. Then, TOC concentration was always higher in the rhizosphere than in the bulk soil of the  
248 same horizon. In contrast to TOC, WEOC concentration was similar at 800 and 1000 m a.s.l.  
249 (Figure 3b); however, whereas for the soils at 800 m no difference in WEOC content occurred  
250 between rhizosphere and bulk soil, at 1000 m the rhizosphere had consistently a greater WEOC  
251 content than the bulk soil.

252 The total N content decreased with depth in all the soils and, as for the TOC, on a horizon by  
253 horizon comparison it was higher at 1000 than at 800 m (Figure 3c). Rhizosphere and bulk showed  
254 similar total N contents at both altitudes, with the exceptions of the Bw1 horizon of the soils at 800  
255 m, where the rhizosphere displayed a greater total N content than the bulk soil.

256

### 257 *3.3. Total, organic and available phosphorus contents, and phosphatase activities*

258 The total and organic P content was much higher in the soils at 1000 m than in those at 800 m  
259 (Figure 4a, b). At both altitudes, rhizosphere and bulk soil generally did not show significant  
260 differences, with the exceptions of the Bw1 horizon for total and organic P, and Bw3 horizon for  
261 total P, always for the soils at 800 m. The available P content (Figure 4c) was also generally higher

262 in the soils at 1000 m than in those at 800 m; further, in the higher altitude soils the rhizosphere had  
263 a greater concentration of available P than the bulk soil in all the horizons, while in the soils at 800  
264 m this occurred in four over six horizons (A, AB, Bw2, and Bw3). The TOC:organic P ratio (Table  
265 S1 of the Supplementary Materials) showed a decreasing trend for both rhizosphere and bulk soil at  
266 800 m, but it was not significantly different between the two altitudes and between the fractions,  
267 with the exception of the Bw4 horizon at 800 m where the rhizosphere showed a greater value than  
268 that of the bulk soil.

269 In the soils at 800 m, alkaline and acid phosphatase activities (Figure 5a, b) decreased with  
270 increasing depth from the A to the Bw1 horizons (Bw2 for acid phosphatase), to remain rather  
271 constant more in depth. At 1000 m, both enzymatic activities decreased from the surface to the AB  
272 horizon, to remain rather constant in the horizon underneath. By contrasting horizon by horizon, the  
273 alkaline phosphatase activity (Figure 5a) was greater in the soils at 1000 m, where small differences  
274 between rhizosphere and bulk were observed only in the Bw1 horizon. The acid phosphatase  
275 activity (Figure 5b) was similar in the A and AB horizons at the two altitudes, and greater at 1000 m  
276 than at 800 m in the Bw1 and Bw2 horizons. The rhizosphere showed higher acid phosphatase  
277 activities than the bulk soil in the Bw1, Bw2 and Bw4 horizons at 800 m, and in the A and Bw1  
278 horizons at 1000 m.

279

#### 280 3.4. <sup>31</sup>P-NMR spectroscopy

281 The <sup>31</sup>P-NMR spectra (Figure S3 of the Supplementary Materials) indicated that the orthophosphate  
282 monoesters (from 3 to 6 ppm of the chemical shift) were the dominating P forms of the spectral  
283 area, followed by inorganic orthophosphates (from 5.9 to 6.5 ppm), orthophosphate diesters (from -  
284 2 to 0 ppm) and pyrophosphates (from -3.5 to -5.5 ppm). The spectra showed similar patterns for  
285 rhizosphere and bulk soil. Between the two altitudes, the orthophosphate diesters were more  
286 abundant at 1000 m than at 800 m for both rhizosphere and bulk soil (Figure 6).

287

288 **4. Discussion**

289 *4.1 Altitude and rhizosphere effect on pH, C and N*

290 The PCA performed on all the measured soil properties showed a marked difference between  
291 rhizosphere and bulk of the studied soils, indicating that the beech roots induce a rhizosphere effect.  
292 Similar results were reported by several other authors. For example, Wang et al. (2001) studied the  
293 soil solution chemistry of beech and Norway spruce and found lower pH and nutrient  
294 concentrations in the rhizosphere than in the bulk soil solutions. Esperschütz et al. (2009), studying  
295 carbon fluxes from beech trees into the rhizosphere found that during the growing season the  
296 rhizosphere had a higher amount of dissolved organic matter than the bulk soil. Calvaruso et al.  
297 (2011) found that the rhizosphere of several tree species including beech was enriched in organic C,  
298 N, Ca, Mg and K with respect to the bulk soil, and the extent of the rhizosphere effect depended on  
299 the tree species. Finally, Cesarz et al. (2013) accomplished a rhizotron experiment and found that  
300 beech induced a strong rhizosphere effect mostly because of roots exudates and associated  
301 microbial community. Further, the PCA scoring plot (Figure 1) highlighted a clear distinction  
302 between the soils at 800 m and those at 1000 m, confirming the effect of altitude on soil properties,  
303 as commonly was reported by many authors (e.g., Tsui et al., 2004; Seibert et al., 2007; Cioci et al.,  
304 2008).

305 Even though the pH was the parameter that most strongly differentiated rhizosphere and bulk on the  
306 basis of PCA2, contrasting the two fractions for each horizon, no difference occurred for both  
307  $\text{pH}_{\text{H}_2\text{O}}$  and  $\text{pH}_{\text{KCl}}$ . The weak rhizosphere effect on soil pH was ascribed, at least partly, to the  
308 sampling period as the reduced winter root activity probably lessened the release of  $\text{H}^+$  at the soil-  
309 root interface in response to the lowered uptake of cations (Hinsinger et al., 2003). However,  
310 Calvaruso et al. (2014), in a study conducted on soil solutions of an acidic beech forest soil,  
311 measured lower pH values in the rhizosphere than in the bulk soil during winter, the reverse in  
312 spring, and similar pH values in the two fractions in fall. In our case, the scarcity of pH differences  
313 between rhizosphere and bulk could be also ascribed to the nature of the parent material, whose

314 carbonate content makes the rhizosphere little susceptible to acidification as *i*) the carbonate  
315 dissolution neutralizes the activity of the released protons (Agnelli et al., 2016), and *ii*) the rate of  
316 chemical weathering and decarbonation in mountain soils is minor than at lower altitudes (Riebe et  
317 al., 2004). However, these observations support the higher pH values that we found for both  
318 rhizosphere and bulk of the soils at the higher elevation.

319 The TOC and total N content followed a similar decreasing trend with depth for both rhizosphere  
320 and bulk soil at the two altitudes. This result was expected for soils with well-developed A horizons  
321 characterized by a strong crumb structure whose cement is mostly made of organics. According to  
322 several authors (e.g., Lemenih and Itanna, 2004; Dai and Huang, 2006; Follett et al., 2012), the  
323 higher content of TOC and total N at 1000 than at 800 m was related to climatic conditions and,  
324 among those, mostly to the temperature, which drops with increasing altitude; in contrast, the  
325 precipitation generally do not follow such a clear altitudinal trend (Körner, 2007; Griffiths et al.,  
326 2009). The accumulation of higher amounts of organic matter at 1000 m may have been fostered by  
327 the combination of the different effects caused by the lower temperature on the plant biomass  
328 production and on the activity of the soil microbial community. Although the plant biomass  
329 production decreases with increasing altitude (Zianis and Mencuccini, 2005), the higher amounts of  
330 TOC happens because of a lower microbial activity, which is due to colder soil temperatures  
331 occurring at higher elevations (Blume et al., 2002; Xu et al., 2014). In our study areas, a larger TOC  
332 content was found in the rhizosphere than in the bulk in the soils at both altitudes, as it has generally  
333 been observed in many different environments (e.g., Turpault et al., 2007; Zhao et al., 2010). As at  
334 1000 m the rhizosphere had a larger WEOC content than the respective bulk soil in all the horizons,  
335 whereas this did not occur at 800 m, it suggested that beech was able to induce a stronger  
336 rhizosphere effect at the higher altitude. The enrichment of WEOC in the rhizosphere is mainly  
337 attributed to rhizodeposition processes (Chiang et al., 2006; Tuason and Arocena, 2009), which  
338 supply most of the energetic substrates for the rhizosphere microbial community (Koranda et al.,  
339 2011; Cesarz et al., 2013). According to Kuzyakov (2002, 2010), the availability of easily

340 degradable compounds (and their consumption by the microflora) triggers a further mineralization  
341 of stable organic matter through the so-called “priming effect”. As a consequence, the rhizosphere  
342 priming effect induced by root exudation boosts the organic matter cycling and the microbially-  
343 mediated release of nutrients (Kaiser et al., 2011). This process may play a key role in the  
344 rhizosphere of the soils at higher altitude, where a scarcer soil development and a generally lowered  
345 microbial activity due to climatic constraints limited nutrients availability. The allocation of plant  
346 resources in the rhizosphere through rhizodeposition can therefore be seen as a strategy of the plants  
347 to overcome ecosystem (nutrient availability) restrictions (Boddy et al., 2008; Massaccesi et al.,  
348 2015). The fact that at 800 m the WEOC/TOC ratios for both rhizosphere and bulk were higher than  
349 those at 1000 m (data not shown), indicated a more active organic matter cycling occurring at the  
350 lower altitude, where the microbial activity has less limitations because of a milder temperature  
351 (Pietikäinen et al., 2005; Creamer et al., 2015).

352

#### 353 *4.2. Altitude and rhizosphere effect on P availability and related enzymatic activities*

354 In agreement with many previous observations on forest soils, the total P content was mostly made  
355 up of organic P, which showed mean contents that are commonly reported for mountain soils (e.g.,  
356 Makarov et al., 2004; Talkner et al., 2009). According to Turner et al. (2002) and Stutter et al.  
357 (2015), the greater concentration of organic P in the soils at 1000 m was attributed to the higher  
358 abundance of organic matter in these soils. The dependence of the organic P concentration on soil  
359 organic matter content was also confirmed by the TOC:organic P ratio, which showed no difference  
360 between the two altitudes and between rhizosphere and bulk soil, although this ratio could be also  
361 affected by the amount of available P (Makarov et al., 2004) and P plant uptake (Saikh et al., 1998).  
362 As the organic P is the main source of available P in soil (Turner et al., 2014), the larger  
363 concentration of available P for both rhizosphere and bulk in the soils at 1000 m was ascribed to a  
364 greater alkaline phosphatase activity all throughout these soils, which was probably induced by the  
365 larger WEOC and TOC contents (Lemanowicz and Krzyzaniak, 2015; Stutter et al., 2015), and the



366 higher pH of these soils, all factors able to promote the alkaline phosphatase activities (Nannipieri  
367 et al., 2011). For the Bw1 and Bw2 horizons, also the acid phosphatase activity was higher in the  
368 soils at 1000 m, but in this case the fostering factors were probably only the high contents of  
369 WEOC and TOC. The question on whom, between plants roots and rhizosphere microbial  
370 community, was the main responsible for the different production of phosphatase in the soils at 800  
371 and 1000 m remains open. Previous studies found that the acid and alkaline phosphatase activities  
372 are higher in the rhizosphere than in the bulk soil (Marschner et al., 2005; Zhao et al., 2007; Shi et  
373 al., 2011) because of rhizodepositions, which fuels the microbial activity and enhances the  
374 production of extracellular enzymes in the rhizosphere (Brzostek et al., 2013). However, also the  
375 plant roots produce enzymes (Nannipieri et al., 2011; Rejsek et al., 2012). To this regard, in a  
376 mesocosm experiment with young *Fagus sylvatica* L., Hofmann et al. (2016) found that plant  
377 phosphatases contributed lesser than microbial ones to the total phosphatase activity in P-rich soil.  
378 In our case, we hypothesized that the greater alkaline phosphatase activities found in the  
379 rhizosphere of the soils at 1000 m was due to the release from both beech roots and a specifically  
380 adapted microbial biomass. In the soils at 1000 m, the larger phosphatase activities and the higher  
381 availability of easily degradable organics (WEOC) probably counterbalanced the minor microbial  
382 activity caused by the lower temperature.

383 The mineralization of organic P compounds by hydrolysis of mononucleotides, sugar phosphates,  
384 phosphoproteins and inositol-phosphates via phosphatases is the process responsible for the release  
385 of inorganic orthophosphates, which are part of the available P and can be taken up by living  
386 organisms (Turner and Haygarth, 2005; Nannipieri et al., 2011). However, no substantial difference  
387 in phosphatase activities was found between rhizosphere and bulk soil at both altitudes. Because of  
388 this, the larger available P content in the rhizosphere of all the horizons of the soils at 1000 m was  
389 attributed to an intense P cycling that, in the soil close to the roots, was triggered by the exudation  
390 of labile organic compounds (Ström et al., 2002; Palomo et al., 2006); these would have promoted  
391 the microbial activity and the consequent release of P and other nutrients through the organic matter

392 mineralization (Kuzyakov, 2010). Further, the higher content of available P in the rhizosphere  
393 compared to the bulk soil may be also favoured by the P uptake, which induces desorption of P  
394 from mineral surfaces (Gerke, 2015). However, a P solubilisation due to root release of protons  
395 following nutrient uptake and of organic acids cannot be excluded. This latter explanation would be  
396 valid even if no pH difference was detected between rhizosphere and bulk soil because of the  
397 buffering action of the calcareous parent material, and the complex spatial and temporal pattern of  
398 micro-niches occurring in the rhizosphere (Richter et al., 2007; Faget et al., 2013).

399 The hypothesis of a more intense P cycling occurring in the soil close to the roots was not supported  
400 by the results of the  $^{31}\text{P}$  NMR analysis, as no significantly different proportions of P forms between  
401 rhizosphere and bulk soil were detected. This absence of differences between the fractions may be  
402 partly attributed to the soil variability (above and belowground) occurring even at the same altitude.

403 The most represented form of the P pools was that made of orthophosphate monoesters, either at  
404 800 and 1000 m. This fact was rather expected as inositol-phosphates (which are the main  
405 component of the P monoesters) are strongly stabilized in soil by abiotic reactions with minerals,  
406 which thereby hinder their biological degradation and favour their accumulation in soil (Turner et  
407 al., 2002; Giaveno et al., 2010). The only difference between the sites showed by the NMR spectra  
408 was the larger proportion of orthophosphate diesters in the soils at 1000 m. As orthophosphate  
409 diesters are considered indicators of microbial P cycling (Stutter et al., 2015), their greater amount  
410 at 1000 m than at 800 m supports the occurrence of a general stronger P turnover in the soils at the  
411 higher altitude. This is consistent with the concept that high-altitude ecosystems, due to more  
412 pronounced nutrient limitations when compared to lower altitude ones, are more dependent on  
413 mineralization of soil organic matter by microbial community (Parfitt et al., 2005). Indeed, when  
414 the amount of available P in the soil is limited, P is largely immobilized in organic forms  
415 (Bünemann et al., 2012).

416

## 417 **5. Conclusions**

418 In this work we evaluated the rhizosphere effect of beech in forest soils of central Italy, at two  
419 altitudes (800 and 1000 m) and along 1° of latitudinal gradient. While the small latitudinal gradient  
420 did not affect the rhizosphere and bulk soil properties, significant changes occurred between the  
421 soils at the two altitudes, and a marked rhizosphere effect was detected in those at 1000 m. These  
422 differences were observed in spite of the spatial and morphological heterogeneity of the forest soils,  
423 which possibly affected the extent of the rhizosphere effect at the different altitudes. However, the  
424 fact that we tested our hypotheses in natural soils, where above and belowground heterogeneities  
425 are independent variables, may be considered as an added value to our research, and indicates that  
426 sampling designs able to control the main climatic and physiographic variables may allow obtaining  
427 significant results studying *in vivo* rhizosphere.

428 The clear rhizosphere effect that was found at the higher altitude and it was expressed by a greater  
429 TOC, WEOC and available P concentrations, was attributed to rhizodepositions, which represent  
430 the main source of energetic substrates for the rhizospheric microbial community. The greater  
431 availability of easily degradable compounds (WEOC) in the rhizosphere should boost the  
432 mineralization of organic matter, which in turn may favour the mineralization of the organic P  
433 forms and increase the amount of available P (Figure 7). Therefore, we speculated that at high  
434 altitude the energy supplied by the plants through rhizodeposition to the rhizosphere heterotrophic  
435 microbial community is key for fuelling the rhizospheric processes and, in particular, P cycling.

436 Our results suggested that an increase of the air temperature of about 1°C, which is expected  
437 globally for the year 2050 (IPCC, 2013), and that is equivalent to the temperature shift between our  
438 study sites at 800 and 1000 m a.s.l., might cause a shortage of available P in the high altitude beech  
439 forest soils.

440

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444

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