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1 **Immediate and carry-over effects of increased soil frost on soil respiration**
2 **and microbial activity in a spruce forest**

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20 **Key words:** Snow exclusion, soil enzyme, microbial biomass, nitrogen availability, soil
21 aggregate, fine root

22 **Running title:** Frost effect on soil carbon cycling

23 **Abstract**

24 Increased soil frost associated with winter climate change could have immediate and carry-over
25 effects on biological processes in high-altitude forest soils, but the nature of these processes
26 remain poorly understood. We conducted a snow-exclusion experiment to investigate the
27 immediate and cross-seasonal effects of increased soil frost on soil CO₂ efflux and biological
28 activity in a subalpine spruce forest on the eastern Tibetan Plateau, China. The increased frost
29 reduced soil CO₂ efflux by ~15 and ~19% in the winters of 2015/2016 and 2016/2017,
30 respectively. Increased frost tended to decrease soil basal respiration, the amount of microbial
31 phospholipid fatty acids and the activities of enzymes involved in soil carbon cycling during
32 the winters. Winter soil nitrogen availabilities were higher in the snow-exclusion treatment than
33 in the control plots. However, these effects did not carry over to the following growing season.
34 Our results suggest that increased frost reduces winter soil respiration by direct environmental
35 effects (e.g. soil temperature) and indirect biological processes (e.g. microbial biomass and
36 activity), whereas increased frost did not have any cross-seasonal effects. These findings
37 underscore the ecological importance of seasonal snowpack and microbe-associated carbon
38 processes in subalpine forests where winter snowfall is decreasing substantially.

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45 **1. Introduction**

46 Seasonal snow cover is a major control of biogeochemical cycling in cold environments
47 (Jusselme et al., 2016). Many snowy areas at high latitudes and altitudes have experienced
48 substantial climate change in recent decades, and this trend is predicted to continue in this
49 century (IPCC, 2013). Climate-induced changes are particularly rapid in high latitude and
50 alpine ecosystems, where rising temperatures have profound effects on winter conditions, such
51 as snowfall, soil frost and extreme climatic events (Liu et al., 2012; IPCC, 2013). Winter
52 precipitation in these regions is more likely to occur in the form of rain rather than snow due to
53 winter warming (Wang et al., 2016). The lack of insulating snow cover could consequently
54 increase soil frost (Groffman et al., 2001a; Bokhorst et al., 2013), which could in turn have
55 complex and large impacts on soil microbiological and biochemical processes in cold forests.

56 Winter biological processes and their controls are not as well understood as growing-
57 season processes, despite the importance of winter warming and biological activity in cold
58 systems (Sanders-DeMott and Templer, 2017). Recent studies have found that soil biological
59 processes are sensitive to warming-induced changes in winter conditions, especially snow cover
60 and soil frost (Haei and Laudon, 2015; De Long et al. 2016; Li et al., 2016). Snow removal has
61 negative or neutral influences on winter soil respiration in boreal and temperate forests
62 (Groffman et al., 2006; Aanderud et al., 2013; Bokhorst et al., 2013), but changes in snow cover
63 can also alter biological processes in snow-free periods (Muhr et al., 2009; Wubs et al., 2018).
64 Snow exclusion can suppress soil respiration in the snow-free season in high-latitude
65 ecosystems (Öquist and Laudon, 2008; Zhao et al. 2017). To our knowledge, however, soil
66 biological responses to changing soil frost have rarely been investigated in both snow-covered
67 and snow-free periods in the same experiment. A better understanding of the impacts of
68 intensified soil frost on the biotic and abiotic controls over the dynamics of soil C in both snow-
69 covered and snow-free seasons is thus essential for accurately modeling and predicting potential
70 C feedbacks in a warmer world.

71 Altered soil frost may directly and indirectly affect soil C cycling, such as by affecting soil

72 temperature and moisture (Aanderud et al., 2013; Song et al., 2017), soil microbial biomass and
73 activity (Monson et al., 2006b; Sorensen et al., 2016) and substrate quality and quantity (Brooks
74 et al., 2004; Steinweg et al., 2008; Comerford et al., 2013). The direction and magnitude of
75 biological responses to increased frost may be determined by the combined effect of these
76 processes. Diverse techniques have provided insight in recent years into the impacts of winter
77 climate change on soil C cycling as the importance of winter processes has increased (Li et al.,
78 2016a). Most field-manipulation studies have focused mainly on high-latitude systems,
79 including peatlands and boreal forests (Sanders-DeMott and Templer, 2017). Soil biological
80 responses from low-latitude cold systems with unique winter conditions, such as Tibetan
81 subalpine forests, however, remain unknown.

82 The Tibetan Plateau, the Earth's 'Third Pole', has warmed substantially, especially in
83 winter (Chen et al., 2013). Winter snowfall has decreased at a rate of 0.6 mm y^{-1} in recent
84 decades (Wang et al., 2016; Xu et al., 2017). Seasonal snowpack in this region has unique
85 characteristics, such as shorter duration and shallower depth relative to high latitudes. Winter
86 soil temperature is also near the physical melting point and is sensitive to changes in snow cover
87 (Li et al., 2017). The subalpine forests of southwestern China contain a large amount of soil
88 organic C (Zhang et al., 2013), but most studies of global-change biology have only focused on
89 responses during the growing season (e.g. Xu et al., 2012; 2015; Yin et al., 2013), even though
90 warming is extremely pronounced and microbial activity is unexpectedly high during winter
91 (Wang et al., 2016; Wang et al. 2012; Tan et al., 2014). Future soil frost will also likely affect
92 the biological and environmental controls of soil C cycling in these forests, but the underlying
93 mechanisms of such processes remain unknown.

94 We conducted a snow-manipulation experiment to investigate the immediate and carry-
95 over effects of increased winter frost on soil C cycling in a spruce forest on the eastern Tibetan
96 Plateau. Specifically, we hypothesized that (1) more intense frost in the soil as a result of exclusion
97 of snow would decrease microbial activity and soil respiration in winter; (2) frost-reduced biological

98 processes would carry over into the subsequent snow-free growing season.

99 **2. Materials and methods**

100 ***2.1 Site description***

101 The field manipulation experiment was conducted in a dragon spruce (*Picea asperata*
102 Mast.) stand at the Long-term Research Station of Alpine Forest Ecosystems of Sichuan
103 Agricultural University on the eastern Tibetan Plateau of China (31°15'N, 102°53'E; 3021 m
104 a.s.l.). The mean annual precipitation and temperature are 850 mm and 3.0 °C, respectively.
105 Snow generally begins to accumulate in late November and melts in late March the following
106 year. The soil is classified as a Cambic Umbrisol (IUSS Working Group WRB, 2007). The soil
107 (0-15 cm) contains 88.5 g kg⁻¹ organic C and 5.4 g kg⁻¹ nitrogen (N) and has a pH of 6.4 (Li et
108 al., 2017).

109 ***2.2 Experimental design***

110 Winter snowfall was excluded using shelters to intensify soil frost. Shelters are considered
111 to be a useful tool for studying the responses of soil processes to winter climate change because
112 they can effectively reduce snow cover and minimize the changes in other unwanted
113 environmental conditions (Li et al., 2016a). In early November 2015, six wooden roofs were
114 set up in the spruce forest to prevent the accumulation of snow on the ground. One control plot
115 was established in the vicinity of each roof. The roofs were 2 m in height with a ground area of
116 3 × 3 m. The snow manipulation began in mid-November and ended in late March the following
117 year.

118 ***2.3 Soil sampling***

119 Soil samples were collected from the topsoil (0-15 cm) in the frost period (FP, late January),

120 early thawing period (ETP, early April) and the middle of the growing season (MGS, mid-
121 August) in the year of 2016 and 2017, respectively. Three cores (5 cm in diameter, 15 cm in
122 depth) were collected in each plot at each sampling. The three cores from each plot were
123 combined to form one composite sample. Each composite sample was passed through a 2-mm
124 sieve, and any visible living plant material was manually removed. The sieved soil was used
125 for biochemical analysis.

126 *2.4 Soil CO₂ efflux*

127 Two PVC collars (20 cm in diameter, 12 cm in height) were permanently installed in each
128 plot for measuring soil respiration. Soil CO₂ efflux was measured using a portable infrared gas
129 analyzer (Li-8100, Li-Cor Inc., Lincoln, USA) between 10:00 and 14:00 (Beijing time, China
130 Standard Time) approximately every two weeks during the experimental period. The
131 measurements were repeated twice for each collar to minimize measurement errors. Soil
132 temperature and moisture at a depth of 5 cm were also measured near each collar using
133 thermocouple and Theta probes, respectively, connected to the LI-8100. Small red flags were
134 attached to the PVC collars in the control plots to minimize disturbance during the period of
135 snow cover. During the winters of 2015/2016 (four times) and 2016/2017 (once), the surface
136 snow was removed carefully from the top of the collars when the snowpack was thicker than
137 the height of the collar. We then waited 5 min to allow the system to equilibrate before
138 measuring the CO₂ efflux. The removed snow was gently backfilled after the measurements.
139 We measured CO₂ efflux under the natural snowpack (~10 cm) in the winter of 2015/2016 in
140 the same forest stand adjacent to the snow manipulation site. CO₂ efflux was also measured
141 after removing the snow within and around the collars. CO₂ efflux did not differ significantly
142 before and after snow removal (unpublished data). Snow removal therefore likely negligibly
143 affected the quantification of immediate CO₂ efflux, at least within an interval of a few minutes.

144 **2.5 Microclimate, extractable N and microbial respiration**

145 Air temperature 2 m above the ground in the forest stand was measured using Thermochron
146 iButton DS1923–F5 Recorders (Maxim Dallas Semiconductor Corp., USA) every 2 h during
147 the experimental period. Meanwhile, soil temperatures 5 cm below the surface were recorded
148 in the snow-exclusion and control plots, respectively. Snow depth in the control plots was
149 measured by a metal ruler approximately every two weeks during winter.

150 Soil extractable N (nitrate, NO_3^- -N, and ammonium, NH_4^+ -N) was extracted with 2 M KCl
151 (1:5 soil:solution). The extracts were shaken for 1 h and filtered with a filter paper. The
152 concentrations of NO_3^- -N and NH_4^+ -N in the extracts were determined by colorimetry (Li et al.,
153 2017).

154 The rate of soil microbial respiration was estimated using alkali absorption (Anderson et
155 al., 1982). Soil samples (50 g) were incubated in 1-L jars at 20 °C for 2 weeks. Empty jars
156 without soil were used as controls. The CO_2 produced was captured with 0.5 M NaOH in a
157 beaker suspended inside each jar. The NaOH solution was removed and titrated with 0.25 M
158 HCL solution to determine the amount of CO_2 produced. Microbial respiration was reported as
159 $\text{mg CO}_2 \text{ kg}^{-1} \text{ soil d}^{-1}$.

160 **2.6 Aggregate fraction and fine-root biomass**

161 Aggregates were isolated as described by Kristiansen et al. (2006). Two soil cores from
162 each plot were collected from the 0-15 cm layer using an auger 10 cm in diameter in the early
163 thawing periods of 2015/2016 and 2016/2017 winters. Soil samples were air-dried to optimal
164 moisture (~10-15%) that would allow limited mechanical stress to maximize brittle failure
165 along natural planes of weakness, and the samples were then gently manually crumbled to <8
166 mm. The recovered samples were transferred to a nest of sieves (2 and 0.25 mm) and shaken at
167 100 min^{-1} for 2 min. All visible roots and stones were removed, and aggregates >2 mm (large
168 macroaggregates) were collected. The same procedure was used for the material retained on the

169 0.25 mm sieve, isolating an aggregate size class 0.25-2 mm (small macroaggregates). The
170 remaining material passing through the 0.25 mm sieve was identified as aggregate class <0.25
171 mm (microaggregates).

172 Two soil cores were collected from each plot using an auger (15 cm long and 10 cm in
173 diameter) in the ETPs of 2015/2016 and 2016/2017 **winters**. Root samples were washed in the
174 laboratory on sieves (mesh size 0.1 mm) and dried to constant weight at 65 °C. Fine roots (<2
175 mm in diameter) were separated into live and dead components based on their color and
176 mechanical consistency.

177 *2.7 Assays of soil phospholipid fatty acids and enzymes*

178 Microbial biomass was estimated as the total extractable phospholipid fatty acids (PLFAs)
179 with a modified method described by White et al., (1996). Lipids from 2 g of fresh soil were
180 extracted in a chloroform-methanol-phosphate buffer mixture (1:2:0.8). The phospholipids in
181 the extracts were transformed by alkaline methanolysis into fatty acid methyl esters (FAMES),
182 which were identified by gas chromatography/mass spectrometry (GC/MS-QP2010 Series,
183 Shimadzu, Japan). Fatty acids were quantified by comparisons of the peak areas from the
184 sample with the peak areas of internal standards at 19:0 (nonadecanoic methyl ester) of the
185 known concentration. The areas were used to estimate the abundance of PLFA markers, which
186 were expressed as nmole g⁻¹ dry soil.

187 We assessed the activities of four enzymes involved in soil C cycling: two hydrolytic
188 enzymes, β -glucosidase (BG) that catalyzes one of the later steps of cellulose degradation and
189 β -N-acetyl-glucosaminidase (NAG) involved in the breakdown of chitin and fungal cell walls,
190 and two oxidases, polyphenol oxidase (PPO) that breaks down recalcitrant polymers such as
191 lignin and humic compounds and peroxidase (POD), a nonspecific enzyme that oxidizes and
192 depolymerizes lignin. The activities were measured using assay techniques described by Allison
193 and Jastrow (2006). Substrate solutions were 5 mM pNP- β -glucopyranoside for BG, 50 mM

194 pyrogallol and 50 mM EDTA for PPO, 2 mM pNP- β -N-acetylglucosaminide for NAG and 5
195 mM L-DOPA and 10 μ L of 0.3% H₂O₂ for POD. Activities were determined using a microplate
196 spectrophotometer and expressed as μ mol of substrate produced or consumed h⁻¹ g⁻¹ dry soil.

197 *Data analysis*

198 A repeated-measures ANOVA was used to test the effects of treatment, sampling date and
199 their interactions on all response variables. A Bonferroni post hoc test was used to examine the
200 treatment effect on the variables on a given sampling date when the interaction of treatment and
201 sampling date was significant ($P < 0.05$), and a paired t -test was used when the interaction was
202 not significant. All data were tested for the assumptions of an ANOVA before analysis.
203 Heterogeneous data were ln-transformed before analysis. An exponential regression model was
204 used to describe the relationship between CO₂ efflux and soil temperature during specific
205 periods (winter, growing season and entire year). All data from two winters or growing seasons
206 were used for the analyses due to the limited number of measurements. Winter was defined as
207 the period between the first day in autumn and the last day in spring when soil temperature was
208 continually below 5 °C for 5 d in the control plots. The temperature sensitivity (Q_{10}) of soil
209 respiration was estimated using van't Hoff equation (Van's Hoff, 1898). $R = \alpha \times e^{\beta \times T}$, Where R
210 is the soil respiration rate (μ mol m⁻² s⁻¹), T is the soil temperature at 5 cm (°C), α and β are
211 parameters. The Q_{10} values are calculated as: $Q_{10} = e^{10 \times \beta}$. All statistical tests were performed
212 using the Software Statistical Package for the Social Sciences (SPSS) version 17.0 (IBM SPSS
213 Statistics Inc., Chicago, IL, USA).

214

215 **3. Results**

216 *3.1 Treatment effect on winter soil conditions*

217 The mean and minimum air temperatures were -2.1 and -14.1 °C during the winter of

218 2015/2016 and $-0.9\text{ }^{\circ}\text{C}$ and $-6.4\text{ }^{\circ}\text{C}$ during the winter of 2016/2017, respectively (Figure 1 a).
219 The maximum snow depth was 40 cm in the winter of 2015/2016 but only 23 cm in the winter
220 of 2016/2017. The mean air temperature in the winter of 2015/2016 ($-2.1\text{ }^{\circ}\text{C}$) was comparable
221 to the seven-year average of $-2.4\text{ }^{\circ}\text{C}$ for 2010-2016. The mean air temperature in the winter of
222 2016/2017 ($-0.9\text{ }^{\circ}\text{C}$), however, was the highest in the last seven winters and $1.5\text{ }^{\circ}\text{C}$ higher than
223 the mean.

224 The snow-exclusion treatment successfully created a more intense frost regime in both
225 winters (Figure 1 a). The minimum daily mean soil temperatures were $-2.2\text{ }^{\circ}\text{C}$ (2015/2016) and
226 $-2.4\text{ }^{\circ}\text{C}$ (2016/2017) in the snow-exclusion plots but were only $-0.5\text{ }^{\circ}\text{C}$ (2015/2016) and $-1.3\text{ }^{\circ}\text{C}$
227 (2016/2017) in the control plots. The numbers of days with differences in soil temperature
228 $\geq 0.5\text{ }^{\circ}\text{C}$ between the treatment and control plots were 42 and 56 for the winters of 2015/2016
229 and 2016/2017, respectively. Such differences were mainly during mid- and late winter when
230 snow cover was $>10\text{ cm}$. Soil temperature fluctuated more in the treatment than the control
231 plots. Volumetric soil moisture was similar between the treatment and control plots across the
232 two years ($F=3.364$, $P = 0.116$, Figure 1 b).

233 **3.2 Soil CO_2 efflux**

234 The snow exclusion lowered CO_2 efflux early in the winter of 2015/2016 and in mid-winter
235 of 2016/2017. The snow exclusion reduced CO_2 efflux by averages of 15% and 19% in the
236 winters of 2015/2016 and 2016/2017, respectively, and these reductions were statistically
237 significant ($F = 11.13$, $P < 0.01$ for the winter of 2015/2016; $F = 9.143$, $P < 0.05$ for the winter
238 of 2016/2017). The snow-exclusion manipulation, however, did not affect CO_2 efflux during
239 the snow-free growing seasons ($F = 1.065$, $P = 0.323$ for 2016; $F = 1.354$, $P = 0.305$ for 2017).
240 Mean CO_2 efflux differed marginally between the frost regimes in the winter of 2015/2016 ($t =$
241 2.006 , $P = 0.076$; Table 1), but differed significantly between the regimes in the winter of
242 2016/2017 ($t = 3.909$, $P < 0.01$). Mean CO_2 efflux nevertheless did not differ significantly
243 between the treatment and control plots in either growing season ($t = -1.584$, $P = 0.335$ for 2016;

244 $t = -0.285, P = 0.465$ for 2017).

245 Soil CO₂ efflux increased exponentially with soil temperature throughout the study period
246 (Figure 3a-c). Soil temperature explained 82-83% of the variation in CO₂ effluxes during the
247 growing seasons (Figure 3b) but explained only 52-53% of the variations in the winters (Figure
248 3a). Soil temperature explained 90-91% of the variance in CO₂ effluxes when the data for the
249 two years were pooled (Figure 3c). The temperature sensitivity (Q_{10}) of the CO₂ efflux was
250 23.3, 3.2 and 4.4 in the treatment plots and 22.6, 3.3 and 4.7 in the control plots for winter,
251 growing season and the entire year, respectively. Q_{10} did not differ significantly between the
252 frost regimes for each period modeled (all $P > 0.05$).

253 **3.3 Soil PLFAs and microbial respiration**

254 The intensified frost tended to decrease the soil PLFAs biomarkers. PLFA content was
255 lower in the snow-exclusion treatment in than the control plots in the FPs of 2015/2016 ($t = -$
256 $2.072, P < 0.05$) and 2016/2017 ($t = -3.686, P < 0.05$; Figure 4) but did not differ significantly
257 in the MGSs of 2016 ($t = 1.368, P = 0.245$) or 2017 ($t = 0.035, P = 0.895$).

258 Microbial activity, measured as basal respiration without roots, was estimated by
259 determining CO₂ emission. The intensified frost tended to decrease soil microbial respiration
260 in the winter. The snow-exclusion treatment negatively affected soil microbial respiration in
261 the FP of 2015/2016 ($t = -0.918, P < 0.05$; Figure 5) and in the ETP of 2016/2017 ($t = -5.821,$
262 $P < 0.01$) but had no effect in the MGSs of 2016 and 2017 (both $P > 0.05$)

263 **3.4 Soil enzymes**

264 The activities of the soil enzymes varied significantly with sampling date (all $P < 0.01,$
265 Figure 6a-d). The snow-exclusion treatment tended to inhibit activities in the winter. Activity
266 was significantly lower in the treatment than in the control plots for BG in the ETP of 2015/2016
267 ($t = -1.975, P < 0.05$; Figure 6a) and for PPO in the ETP of 2016/2017 ($t = -2.643, P < 0.05$;

268 Figure 6b). The intensified frost decreased POD activity in the FPs of 2016 and 2017 (both P
269 < 0.05 , Figure 6c) and decreased NAG activity in the ETPs of 2016 and 2017 (both $P < 0.05$,
270 Figure 6d) but did not significantly affect the activities of the enzymes in the MGSs of 2016 or
271 2017.

272 **3.5 Soil extractable N**

273 Frost treatment, sampling date and their interaction all significantly affected soil $\text{NH}_4^+\text{-N}$
274 concentration (all $P < 0.05$, Figure 7a). The snow-exclusion treatment increased $\text{NH}_4^+\text{-N}$
275 concentrations in the FP and ETP of 2015/2016 (all $P < 0.01$) but not in the winter of 2016/2017
276 (both $P > 0.05$). Likewise, the intensified frost increased $\text{NO}_3^-\text{-N}$ concentrations in both winters
277 ($F = 16.575$, $P < 0.01$; Figure 7b). $\text{NO}_3^-\text{-N}$ concentrations were significantly higher in the
278 treatment than the control plots in the ETP of 2015/2016 ($t = 2.309$, $P < 0.05$) and in the FP of
279 2016/2017 ($t = 5.017$, $P < 0.01$). Neither $\text{NH}_4^+\text{-N}$ nor $\text{NO}_3^-\text{-N}$ concentration, however, differed
280 between the frost regimes in the MGS of 2016 and 2017 (both $P > 0.05$).

281 **3.6 Aggregate fraction and fine-root biomass**

282 The relative distribution of the aggregate-size classes of the bulk soil was in the order small
283 macroaggregates (0.25-2 mm) > large macroaggregates (>2 mm) > microaggregates (<0.25 mm)
284 irrespective of frost regime ($F = 221.75$, $P < 0.001$; Table 2). The snow-exclusion treatment
285 did not affect the distribution of aggregates in the size classes ($F = 0.159$, $P = 0.897$), the live
286 fine-root biomass ($F = 0.202$, $P = 0.663$; Table 3) or the dead fine-root biomass ($F = 0.171$, P
287 $= 0.688$) in the ETP.

288

289 **4. Discussion**

290 We investigated the impact of intensified soil frost on soil C cycling in a Tibetan subalpine
291 spruce forest using a field experiment manipulating frost. Our main objective was to determine

292 whether differences in winter frost conditions induced immediate and carry-over effects on soil
293 CO₂ efflux. In line with the first hypothesis, snow exclusion resulted in more intensive soil frost,
294 which decreased soil respiration in the winter season. However, contrary to what we expected,
295 increased winter frost did not have carry-over effects on soil respiration in the subsequent
296 growing season. Several potential mechanisms have been tested to account for the underlying
297 the changes of CO₂ efflux during the snow-covered and snow-free seasons.

298 Firstly, frost manipulation could decrease winter CO₂ efflux, in part, by the direct effect of
299 temperature, because snow exclusion during the winter decreased soil temperature. A slight
300 reduction in soil temperature over the lower range is especially important to CO₂ production,
301 because the temperature sensitivity of microbial processes is extremely high at low
302 temperatures (Davidson and Janssens, 2006; Schütt et al., 2014). Soil temperature in our study
303 accounted for only 52-53% of the variance in winter CO₂ efflux. CO₂ efflux was very sensitive
304 to small changes in soil temperature throughout the winter, particularly near 0 °C. *Q₁₀ was much*
305 *higher in the winter* than in the growing season regardless of frost regime. *A several studies*
306 *have described a surprisingly higher temperature sensitivities for winter soil respiration in*
307 *temperate and boreal forests (Monson et al., 2006a; Muhr et al., 2009; Wang et al., 2010). Q₁₀,*
308 *however, was estimated over a narrow temperature span (~5-6 °C), and the temperature-CO₂*
309 *relationship was as weak as in temperate forests (Schindlbacher et al., 2007; Wang et al., 2010;*
310 *Schindlbacher et al., 2014).*

311 Other factors co-varying with soil temperature may also likely regulate winter CO₂ efflux.
312 The intensified frost may have produced a stronger ‘freezing drought’, which would likely limit
313 microbial activity and the extracellular diffusion of substrates (Rivkina et al., 2000). Soil
314 moisture did not differ significantly between the treatment and control plots during the winters,
315 suggesting that soil moisture was not likely responsible for the decreased winter CO₂ efflux.
316 The lack of significant differences in CO₂ efflux during the snow-free seasons was likewise
317 partially attributed to the lack of significant differences in both soil temperature and moisture

318 between the treatment and control plots during the growing season.

319 Secondly, root activity is extremely low during dormant seasons, and winter soil
320 respiration in cold ecosystems is primarily derived from microbial decomposition (Muhr et al.,
321 2009; Wang et al., 2010). Winter soil respiration is thus largely determined by the biomass and
322 activity of soil microbes (Lipson et al., 2002; Moorhead et al., 2014). Soil microbes are very
323 susceptible to soil frost (Monson et al., 2006b; Aanderud et al., 2013), which can kill a
324 substantial proportion of the organisms by the rupture of cell membranes by ice crystals
325 (Sulkava and Huhta, 2003; Jusselme et al., 2016; Gavazov et al., 2017). We also found that the
326 increased frost significantly reduced soil microbial PLFAs, implying a lowered potential for
327 the microbial community to metabolize soil C in the winters. A significant decline in the cross-
328 winter microbial PLFAs also implied that seasonal frost would kill the soil microbes in the
329 spruce forest irrespective of frost manipulation. Our measurements of microbial basal
330 respiration, excluding plant roots, also indicated a similar decline with winter frost, consistent
331 with *in situ* soil CO₂ efflux. Frost-induced decreases in winter soil respiration may thus largely
332 be attributed to the lower microbial biomass and activity. Soil PLFAs and basal respiration in
333 the middle of the growing season nevertheless did not differ significantly between frost regimes.
334 These observations may partially account for the neutral effect of increased frost on CO₂ efflux
335 during the snow-free growing seasons.

336 Soil enzymes play very important roles in the cycling of soil C and nutrients. Little
337 attention has been paid to enzymatic activities in studies of winter climate change, despite the
338 importance of soil enzymes in soil C cycling. A recent study found that enzymatic activities
339 were negatively correlated with the intensity of soil frost in mixed-hardwood forests (Sorensen
340 et al., 2016), and another experiment also found that snow removal decreased the activity of
341 soil invertase in an alpine spruce-fir forest (Tan et al., 2014). We assayed the activities of four
342 enzymes involved in soil C cycling to further assess the functional capacity of soil. The
343 intensified frost tended to reduce soil enzymatic activities. Soil enzymatic activities are strongly

344 temperature-dependent (Tabatabai, 1982), so a decrease in soil temperature caused by snow-
345 exclusion may, to some extent, reduce soil enzymatic activities directly. The lower activities
346 may also partly be attributed to the smaller population size of the microbes, which are an
347 important source of enzyme synthesis. Soil enzymes, as proximate agents of the decomposition
348 of soil organic C, can break down plant and microbial cell walls and catabolize macromolecules
349 into soluble substrates for microbial assimilation (Sinsabaugh et al., 2008). Frost-induced
350 decreases in enzymatic activities may thus constrain this decomposition, which could also
351 partly account for the lower winter CO₂ efflux. Conversely, intensified frost did not affect
352 activities in the snow-free growing seasons, which may account for the lack of significant
353 responses during the subsequent growing season.

354 Thirdly, soil frost may also have affected the decomposition of soil C in winter by altering
355 nutrient availability. Intensified frost can increase the mortality of roots and microbes (Henry,
356 2007; Repo et al., 2014; Blume-Werry et al., 2016), which are important substrates for soil
357 microbial metabolism during winter (Schimel et al., 2004). Dead roots and microbes are also
358 main N sources during winter in cold systems (Chapin III et al., 1988; Tierney et al., 2001). In
359 an earlier study we observed that soil at -5 °C could release considerable extractable N in the
360 soils of this spruce forest, possibly due to the effect of freezing on microbial mortality (Xu et
361 al., 2014). The snow-exclusion treatment in the present study stimulated the production of soil
362 extractable N in the two winters, likely due mainly to the increased microbial mortality. An
363 increase in N availability but a decrease in soil PLFAs throughout the winter, irrespective of
364 the frost regime, may also support this conclusion. Live and dead fine-root biomass also notably
365 did not differ significantly between frost regimes later in the winter, further suggesting that the
366 increased N availability was mainly attributed to microbial mortality rather than root injury. An
367 increase in N availability coincided with a decrease in CO₂ efflux, implying that the cycling of
368 soil C could be decoupled from N availability during winter under intensified frost.

369 In addition to microbial and root mortality, substrate availability could have been affected

370 by the physically disruptive effects of frost on soil aggregates (Chai et al., 2014). Freezing can
371 break down macroaggregates into microaggregates (Oztas and Fayetorbay, 2003).
372 Microaggregates with a larger surface area have more contact points, which can potentially
373 increase the amount of substrate decomposed by microorganisms (Grogan et al., 2004). Snow
374 removal increased the fraction of microaggregates in a northern hardwood forest, implying that
375 soil substrate could become more accessible to soil microorganisms (Steinweg et al., 2008).
376 Our observations, however, did not provide further evidence that more intense frost could
377 disrupt aggregates in the soil of this Tibetan spruce forest. The intensified frost did not affect
378 the distribution of aggregates among the size classes, suggesting that frost-associated changes
379 to aggregates may not importantly affect soil respiration in the spruce forest during the winter
380 and growing season.

381 Lastly, the flux of CO₂ derived from decaying litter accounts for a considerable part of
382 total soil respiration during winter (Uchida et al., 2005). CO₂ flux derived from aboveground
383 litter accounts for an average of 14.2% of total soil respiration in this spruce-forest stand (Xiong
384 et al., 2015). In a previous study we also found that the mass loss of spruce needles over the
385 winter constituted 18.3-28.8% of the net loss rates for the entire year (Xu et al., 2016). The lack
386 of snow cover at this experimental site decreased the temperature of the surface soil by an
387 average of 1.4 °C during the winter (Li et al., 2017), implying that litter decomposition was
388 most likely inhibited by the lower temperatures. A growing number of studies have documented
389 that thick snow covers can provide relatively stable conditions for biological activity, favoring
390 the decomposition of plant litter (Christenson et al., 2010; Bokhorst et al., 2013; Saccone et al.,
391 2013). The rates of decomposition of litter from subalpine tree species in this area similarly
392 decrease with decreasing snow depth (Ni et al., 2014; He et al., 2015). The lower rate of litter
393 decomposition due to the lack of snow cover may therefore also have contributed to the lower
394 winter soil respiration in the snow-exclusion plots, but further supporting evidence is needed.

395

396 **5. Implications**

397 The climate on the Tibetan Plateau has changed considerably in recent decades, especially
398 in winter (Wang et al., 2016). Winter snowfall has tended to decrease substantially due to strong
399 winter warming (Xu et al., 2017). The decrease or absence of insulating snow cover associated
400 with climate change may thus increase the duration and intensity of soil frost in the future in
401 this special region. The importance of soil frost, large storage of soil C and sensitivity of snow
402 cover to winter warming indicate that understanding the potential effects of projected frost
403 increases on soil C cycling in the subalpine forests of western China is essential. To our
404 knowledge, our study is the first to identify the effects of changes in soil frost on soil C cycling
405 in a Tibetan forest. Our results generally indicate that more intense soil frost decreases winter
406 soil respiration and biological activities. Winter soil CO₂ emission was lower in the snow-
407 exclusion than in the control plots during the two winters of the study. Intensified soil frost,
408 however, did not affect soil CO₂ efflux and biological activities during the subsequent growing
409 season, suggesting that a short-term change in snow cover does not produce large carry-over
410 effects in snow-free periods. If the observed effects apply to natural conditions, intensified soil
411 frost would decrease the amount of soil C released to the atmosphere from subalpine forests
412 during winter, but additional supporting evidence is needed.

413 This study was conducted during two contrasting winters (cold winter and thick snow
414 cover in 2015/2016 and mild winter and thin snow cover in 2016/2017) so offered a good
415 opportunity for determining the effect of the lack of snow cover in winters with different
416 weather on soil C cycling in the Tibetan spruce forest. The decrease in soil respiration due to
417 frost in the first winter occurred early but then disappeared, suggesting that soil biological
418 processes may begin to acclimate to the frost late in the winter. Soil respiration early in the mild
419 winter of 2016/2017 did not differ significantly between the treatment and control plots, mainly
420 due to the absence of an insulating snow cover. CO₂ effluxes, however, were lower in the
421 treatment plots after the formation of a steady snow cover (>10 cm). In addition to variable

422 snowfall, extreme winter events (e.g. warm weather and snow storms) may become more
423 frequent and likely under scenarios of future climate, indicating the complexity and uncertainty
424 of winter climate change in this specific region. The comparably strong climate change and
425 variable winter snowfall on the Tibetan Plateau bring great challenges and opportunities for
426 studying winter climate change and its impacts on the structure and function of Tibetan
427 ecosystems. Long-term monitoring is strongly needed for exploring the natural winter
428 variations and underlying mechanisms of the observed phenomena to help models for providing
429 more realistic predictions of future winter conditions.

430 The frost intensity due to the lack of snow cover was low at our experimental forest site,
431 unlike in temperate and boreal forests (e.g. Groffman et al., 2006; Muhr et al., 2009; Sorensen
432 et al., 2016), but the difference in temperature minima was nearly 2 °C, likely due to the site-
433 specific characteristics, such as winter snowfall, air temperature, properties of soil heat transfer
434 and albedo. Such soil frost, however, had large impacts on soil respiration, microbial PLAFs,
435 enzymatic activity and N availability, suggesting that Tibetan forest soils will be sensitive to
436 changing soil frost in the future. The direction and magnitude of the response of soil respiration
437 to intensified soil frost may largely depend on the interaction between less snowfall and warmer
438 temperature in winter. Winter warming may offset the negative effects induced by frost to some
439 extent. Seasonal snow cover in cold regions plays a key role in decoupling soil from cold winter
440 weather, but soil temperature is often insensitive to a small change in air temperature. Changes
441 in snow cover will thus likely have a stronger influence on soil biological processes than winter
442 warming itself (Groffman et al., 2001a), and long-term changes in soil frost may also have
443 carry-over effects on soil C dynamics during the subsequent growing season in cold systems
444 (Zhao et al., 2017). More research is warranted to integrate potential factors and separate their
445 relative importance for a better understanding and ability to predict potential C feedbacks in
446 snowy regions under a warmer future.

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448

449 **6. Conclusions**

450 This study explored the immediate and carry-over effects of intensified soil frost on soil C
451 cycling in a subalpine spruce forest on the Tibetan Plateau of China. Our results suggested that
452 the lack of snow cover increased the intensity of soil frost, which decreased soil respiration in
453 the winters but did not affect it during the subsequent growing seasons. Frost decreased
454 microbial biomass and activities in the winters but not in the snow-free growing seasons. More
455 intense soil frost did not affect the size distribution of soil aggregates or the fine-root biomass.
456 Predicted soil frost due to winter climate change may thus decrease winter soil respiration by
457 direct environmental effects (e.g. soil temperatures) and indirect biological processes (e.g.
458 microbial biomass and activities) in the subalpine forests on the Tibetan Plateau. Intensified
459 soil frost did not cause cross-seasonal effects on soil CO₂ efflux or biological activities in the
460 subsequent growing seasons. Our observations underscore the ecological importance of
461 seasonal snowpack and microbe-associated C processes in subalpine forest soils. These findings
462 improve our understanding of the response of soil C dynamics to winter climate change in this
463 region experiencing large decreases in winter snowfall.

464

465

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475

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477 **Competing interests**

478 The authors declare no competing financial interests.

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695 **Table 1** Mean soil CO₂ efflux ($\mu\text{mol m}^{-2} \text{s}^{-1}$, means \pm SEs) during the winter and growing
696 season.

Year	Period	Treatment	Control
2016	Winter	0.47 \pm 0.08	0.55 \pm 0.08
	Growing season	2.07 \pm 0.28	2.19 \pm 0.29
2017	Winter	0.43 \pm 0.06	0.53 \pm 0.07
	Growing season	2.23 \pm 0.26	2.25 \pm 0.30

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699 **Table 2.** Relative distribution of the aggregate size classes (% , means \pm SEs) in the snow-
700 exclusion treatment and control plots in the early thawing period of 2016 and 2017.

Year	Size	Treatment	Control
2016	>2 mm	34.9 ± 5.7	32.9 ± 6.9
	0.25-2 mm	59.9 ± 4.7	58.6 ± 4.2
	<0.25 mm	5.2 ± 2.0	8.5 ± 2.8
2017	>2 mm	35.3 ± 6.4	36.7 ± 4.5
	0.25-2 mm	53.9 ± 3.8	50.7 ± 2.2
	<0.25 mm	10.8 ± 3.6	12.6 ± 2.8

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702 **Table 3.** Content of live and dead fine roots (g m⁻², means ± SEs) up to a depth of 15 cm in the
703 snow-exclusion treatment and control plots in the early thawing period of 2016 and 2017.

Year	Fine root pool	Treatment	Control
2016	Live	243.7 ± 45.6	251.7 ± 35.6
	Dead	92.8 ± 14.4	79.4 ± 26.3
2017	Live	221.2 ± 38.5	215.6 ± 28.9
	Dead	94.3 ± 22.6	88.1 ± 16.4

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713 **Figure legends**

714 **Figure 1.** Air temperature, soil temperature and snow depth (a) and soil moisture (b) in the
715 snow-exclusion treatment and control plots during the experimental period.

716 **Figure 2.** Soil CO₂ efflux (means ± SEs) in the snow-exclusion treatment and control plots
717 during the experimental period. Significant differences between the control and treatment on a
718 given date are indicated by asterisks ($P < 0.05$).

719 **Figure 3.** Exponential relationships between soil CO₂ efflux and soil temperature (means ± SEs)
720 in the treatment and control plots for winter, growing season and entire year, respectively.

721 **Figure 4.** Total soil phospholipid fatty acids (means ± SEs) in the snow-exclusion treatment
722 and control plots. Significant differences between the control and treatment on a given date are
723 indicated by asterisks ($P < 0.05$). FP, frost period; ETP, early thawing period; MGS, middle of
724 the growing season.

725 **Figure 5.** Soil microbial respiration (means \pm SEs) in the snow-exclusion treatment and control
726 plots. Significant differences between the control and treatment on a given date are indicated
727 by asterisks ($P < 0.05$). FP, frost period; ETP, early thawing period; MGS, middle of the growing
728 season.

729 **Figure 6.** Activities of (a) β -glucosidase, (b) polyphenol oxidase, (c) peroxidase and (d) β -N-
730 acetyl-glucosaminidase (means \pm SEs) in the snow-exclusion treatment and control plots.
731 Significant differences between the control and treatment on a given date are indicated by
732 asterisks ($P < 0.05$). FP, frost period; ETP, early thawing period; MGS, middle of the growing
733 season.

734 **Figure 7.** Soil ammonium (a) and nitrate (b) concentrations (means \pm SEs) in the snow-
735 exclusion treatment and control plots. Significant differences between the control and treatment
736 on a given date are indicated by asterisks ($P < 0.05$). FP, frost period; ETP, early thawing period;
737 MGS, middle of the growing season.

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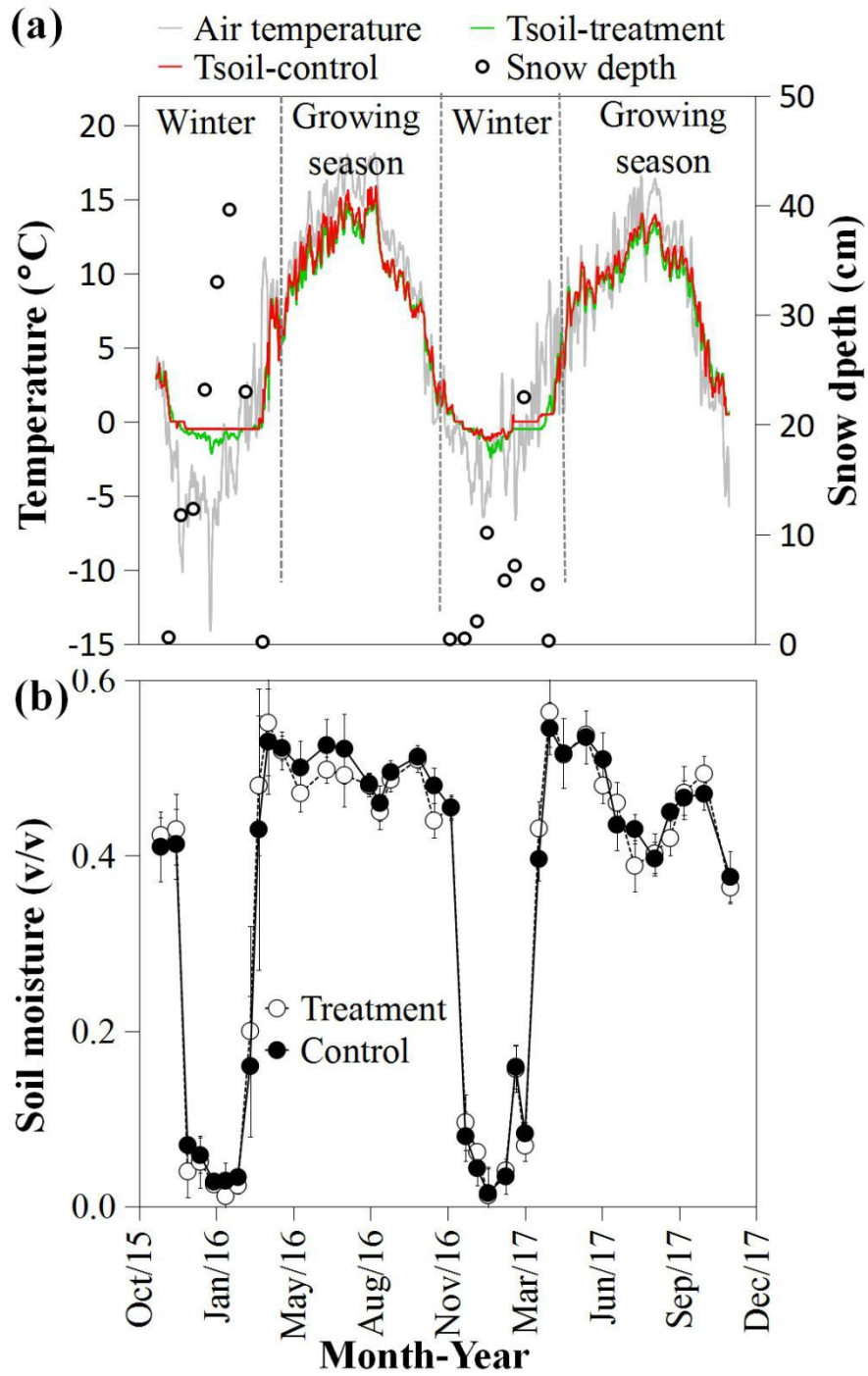
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750 **Figure 1**

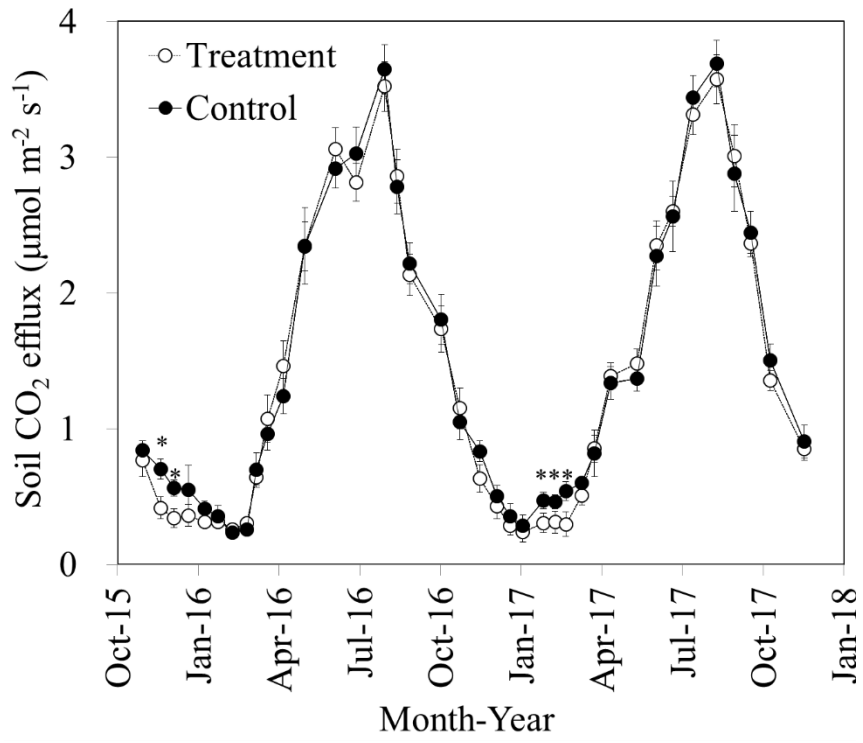


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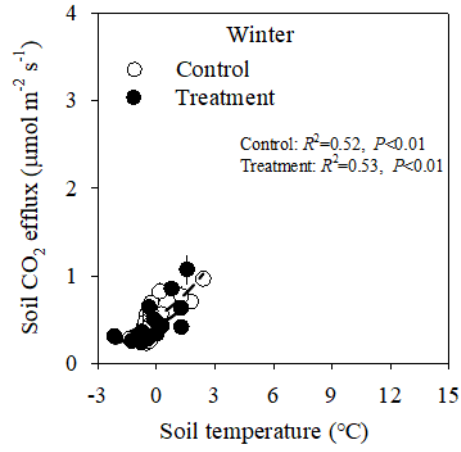
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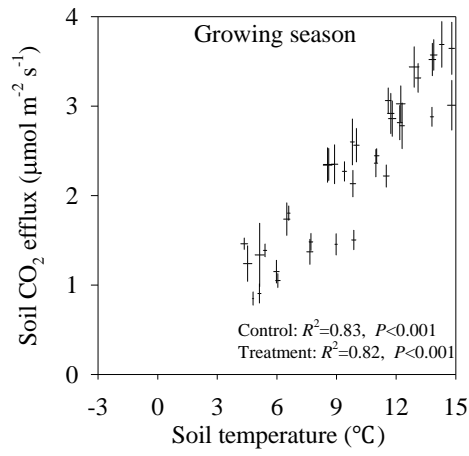
754 **Figure 2**



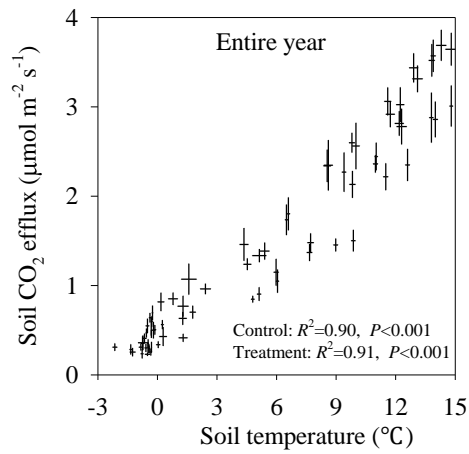
771 **Figure 3**



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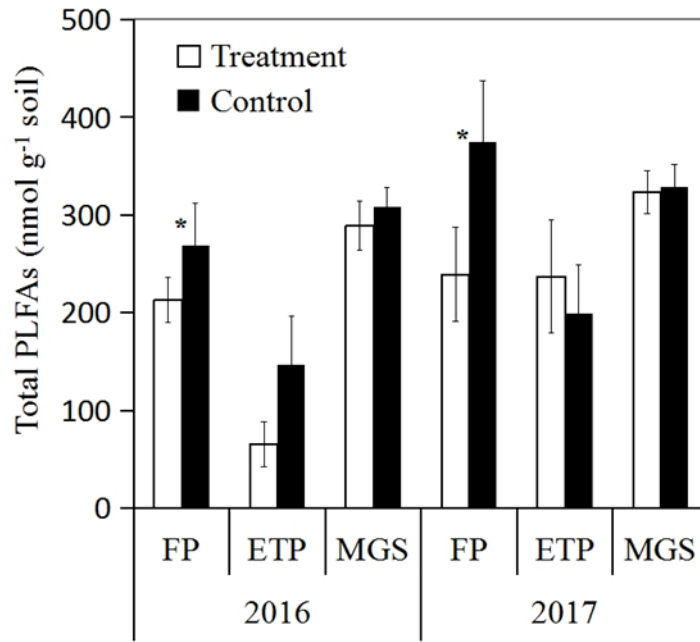
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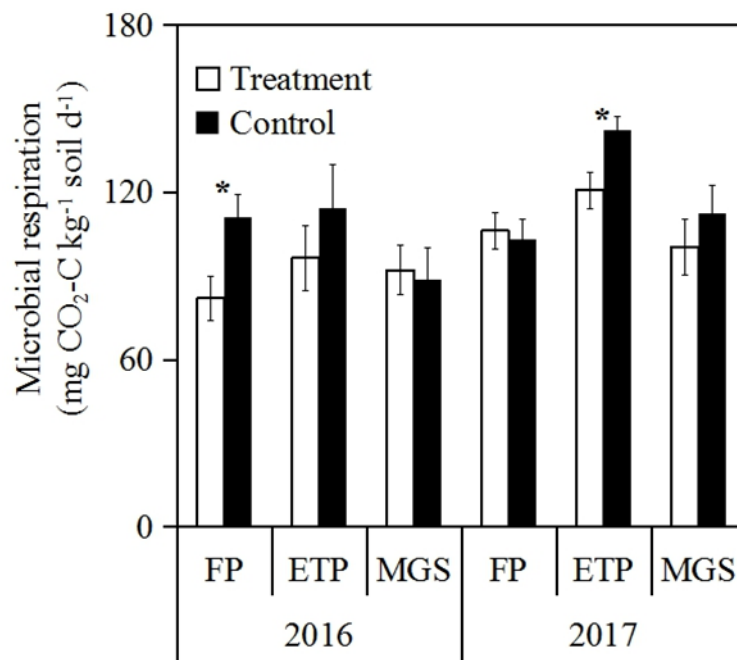
776 **Figure 4**



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779 **Figure 5**



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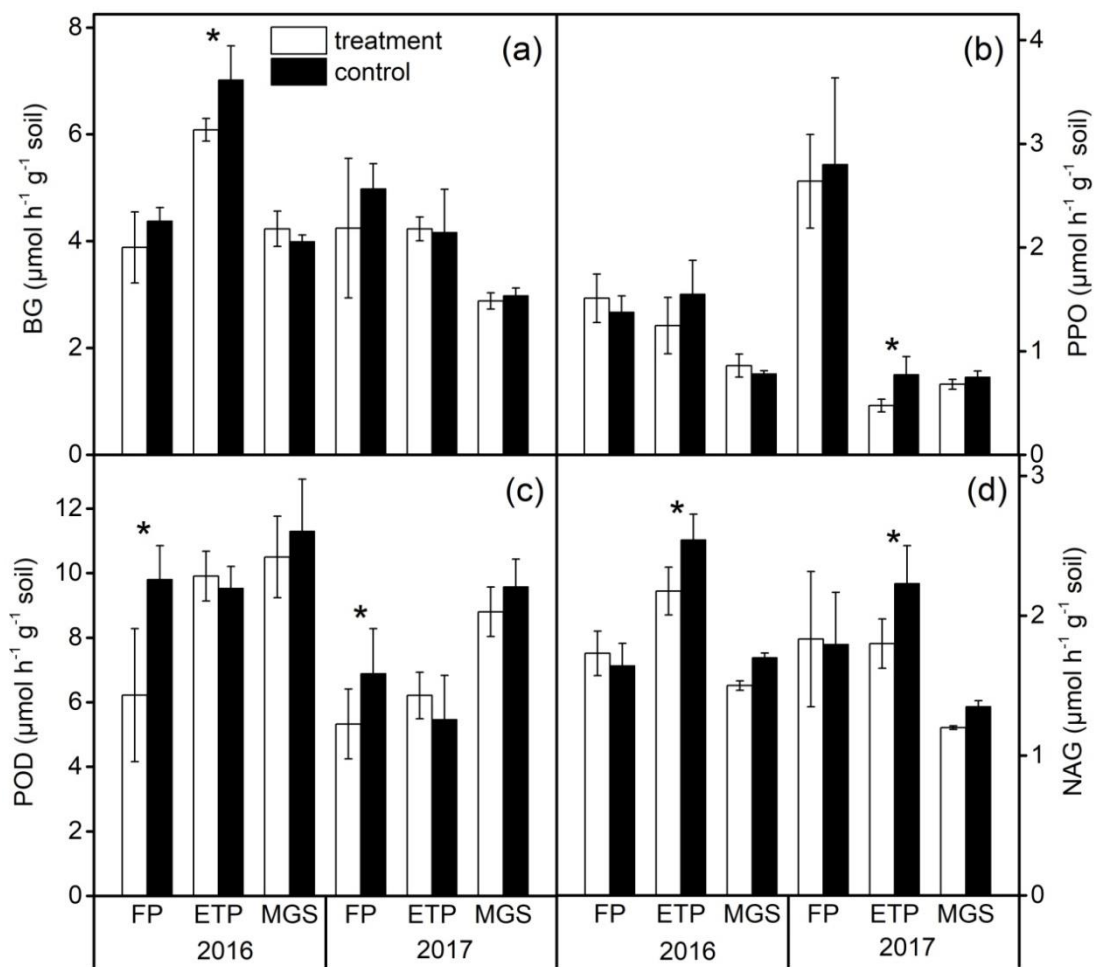
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787 **Figure 6**

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791 **Figure 7**

