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1 Liming of acidified forests changes leaf litter traits but does not improve leaf litter
2 decomposability in forest streams

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11

12 **Abstract**

13 Liming, the spreading of Ca/MgCO₃ on terrestrial ecosystems, is often used as a way to
14 promote the recovery of terrestrial and aquatic ecosystems affected by anthropogenic
15 acidification. When liming is applied to a watershed, surface waters may be improved directly
16 through water chemistry changes but also indirectly through possible changes in the quality of
17 terrestrial organic matter inputs to the stream. The aim of this study was to evaluate the effect
18 of liming on leaf litter chemical and structural traits and their consequences on another trait,
19 leaf litter decomposability, giving the potential for a leaf litter to be decomposed.

20 Beech (*Fagus sylvatica*, L.) leaf litter was collected in forested sites on acid bedrock
21 (granite and sandstone), limed (9 or 21 years ago) or not (controls), as well as on two
22 calcareous reference sites. Several leaf litter traits were determined for each collection site:
23 carbon (C), nitrogen (N), phosphorous (P), calcium (Ca), magnesium (Mg), potassium (K),
24 lignin and cellulose content, and leaf mass area and toughness. To compare leaf litter
25 decomposability in aquatic ecosystem, leaf litters from all sites were placed in a reference
26 forested headwater stream over a 128-day period, in fine and coarse mesh litter bags, and leaf
27 mass loss dynamic as well as fungal biomass were evaluated.

28 Liming significantly increased leaf litter Ca and Mg contents and decreased K content
29 while other measured traits remained unchanged. Leaf litters collected in limed sites exhibited
30 significantly lower decomposition rates in coarse mesh bags but similar decomposition rates
31 in fine mesh bags. Liming had no significant effect on fungal biomass. In contrast, leaf litter
32 collected on the calcareous, reference sites, showed significantly different trait profiles (e.g.
33 higher Ca, Mg, K and P contents), leading to up to twice higher decomposition rates and
34 fungal development.

35 Our study shows that under our experimental conditions, increases in Ca and Mg leaf
36 litter contents arising from acidic forests liming did not improve leaf litter microbial

37 decomposition and even tended to reduce macroinvertebrate mediated decomposability.
38 Concomitant changes in other leaf litter traits might be necessary to stimulate leaf litter
39 decomposition. Liming with Ca/MgCO_3 might thus not improve functional recovery of stream
40 ecosystems in forests affected by acidification through an increase of litter quality.

41

42

43 **1. Introduction**

44 During the last decades of the 20th century, atmospheric acid deposition has strongly
45 affected terrestrial and aquatic ecosystems over large areas in the northern hemisphere and
46 consequently imperilled ecosystem functioning and related ecological services (Driscoll et al.,
47 2001; Lawrence et al., 2016). The unanimous acknowledgment of these deleterious impacts
48 has led to the implementation of international and national agreements aiming at decreasing
49 the emissions of SO₂ and NO_x. As a result, signs of spontaneous chemical recovery from
50 acidification have been reported in many areas throughout Europe and North America (Burns
51 et al., 2006; Driscoll et al., 2016). However, natural recovery of soils and surface waters is a
52 slow process (Lawrence et al., 2016). Liming which represents an alternative to mitigate
53 acidification and to restore acidified ecosystems has been extensively used in terrestrial and
54 aquatic ecosystems (Clair and Hindar, 2005; Lawrence et al., 2016). Lime (*e.g.* calcite:
55 CaCO₃ or dolomite: CaMg(CO₃)₂) can be spread on a whole watershed to improve the
56 chemical quality of forest soil (Forey et al., 2015) and then water chemistry in freshwater
57 ecosystems (Clair and Hindar, 2005). Such operations are expected to improve soil properties
58 (increase of base cation contents, reduction of soil acidity) and therefore mineral nutrition and
59 growth of trees (Balland-Bolou-Bi and Poszwa, 2012; Cesarz et al., 2016; de Wit et al., 2010).
60 As a result, biological nutrient cycling may be enhanced by liming and many studies reported
61 changes in measured traits on green and senescent leaves and on leaf litter (Court et al., 2018;
62 Forey et al., 2015).

63 In forest headwater streams, leaf litter input from terrestrial ecosystem is considered as
64 the major source of organic carbon (Fisher and Likens, 1973; Vannote et al., 1980; Wallace et
65 al., 1997). It has been already demonstrated that leaf litter traits impact litter processing by
66 detritivores and microbial decomposers (Cornwell et al., 2008; García-Palacios et al., 2016,
67 Zhang et al. 2019). In particular, calcium (Ca) content was noted to be a major factor

68 enhancing decomposition and impacting decomposer community composition in terrestrial
69 ecosystems (Cesarz et al., 2016; Ohta et al., 2014; Yue et al., 2016). Similarly, García-
70 Palacios et al. (2016) suggested that Ca and magnesium (Mg) contents are important drivers
71 of decomposition in streams. Other traits, principally lignin and phosphorous (P) content have
72 been pointed out as factors controlling inter- and intraspecific variation in leaf litter
73 decomposition rate in terrestrial (Aerts, 1997; Enriquez et al., 1993) and stream ecosystems
74 (Lecerf and Chauvet, 2008), by reducing (lignin) or increasing (P) the decomposition process.

75 While several studies have reported the indirect positive effect of liming on leaf litter
76 breakdown through changes in water quality (Mckie et al., 2006; Merrix et al., 2006),
77 potential indirect effects related to changes in leaf litter chemistry remain underinvestigated in
78 aquatic ecosystems. In the present study, we aimed to determine to what extent acidic forests
79 liming, performed 9 and 21 years ago, has induced changes in leaf litter quality and
80 decomposability.

81 We hypothesized that i) liming leads to changes in key leaf litter traits, ii) liming
82 enhances leaf litter decomposability and iii) higher leaf litter decomposability is related to
83 changes in leaf litter traits (especially higher Ca and Mg contents). To test these hypotheses,
84 we collected leaf litter of beech (*Fagus sylvatica*, L.) growing in three strongly acidified
85 catchments and in three contiguous limed catchments. Beech leaf litter was also collected on
86 two calcareous sites, used as positive controls in the study. Then, we measured several leaf
87 litter traits selected as potentially important for determining leaf litter decomposition. Leaf
88 litter decomposability (i.e. an integrative trait giving information on the potential for a leaf
89 litter to be decomposed) was then evaluated in an oligotrophic circumneutral stream in order
90 to only evaluate the effect of leaf litter traits on decomposition and fungal development,
91 excluding potential confounding factors related to acidification effects on invertebrate

92 organisms, fungal development, and decomposition rate (Dangles & Guerold, 2000; Baudoin
93 et al., 2008).

94

95 **2. Material and methods**

96 *2.1 Leaf litter*

97 *2.1.1. Litter selection*

98 The study was conducted in the Vosges Mountains (North-eastern France) where acid
99 deposition has strongly impacted forest ecosystems during the last century (Landmann and
100 Bonneau, 1995). Leaf litter of beech (*Fagus sylvatica*) was chosen as it is the dominant broad-
101 leaved species in the studied area. Senescent leaves were collected during fall every two
102 weeks on a net placed in the understorey, 1-m above the soil surface, preventing any contact
103 with the soil.

104 To test the effect of liming on leaf litter traits and decomposition by having a potential
105 large litter trait gradient, leaf litters were collected on six sites grouped in three areas. One
106 area is located on granite bedrock (named G) and two others on sandstone bedrock (named S1
107 and S2). In each area, one site was located in a stand that was limed with dolomite lime 9
108 years (for G and S1) or 21 years (S2) before the experiment. For each limed site (L: GL, S1L,
109 S2L; table 1), a reference litter collection site, used as control, was selected in the vicinity on
110 the same bedrock but without liming treatments (NL: GNL, S1NL, S2NL; table 1). In
111 addition, leaf litters were also collected in a fourth area located on calcareous bedrock in
112 Moselle (North East France) at two sites (named Calcareous1 and Calcareous2; table 1). This
113 aimed to provide reference data for leaf litter traits found in non-acidified sites (positive
114 control), when Ca and other mineral nutrients availability in soil is high.

115 After collection, litter was air dried and stored at room temperature during approximately
116 two months in the dark in the laboratory before being used for analyses and the decomposition
117 experiment.

118 *2.1.2 Leaf litter traits*

119 Nine leaf litter traits were selected to characterize the leaf contents in some major mineral
120 elements (carbon, nitrogen, phosphorus, calcium, magnesium and potassium content), in some
121 major organic macromolecules (lignin and cellulose), as well as structural parameters (leaf
122 mass area and leaf toughness). All these traits were measured before decomposition of leaf
123 litter.

124 Leaf carbon (C) and nitrogen (N) contents were measured with a CHN elemental analyzer
125 (Carlo-Erba NA 2100). Total phosphorous (P) was measured after alkaline persulphate
126 digestion and spectrophotometric measurements using an automated analyzer (Konelab 420-
127 XTi, Thermofischer). Ca and Mg concentrations were measured at the SARM mineral analyse
128 platform (Nancy, France) by atomic absorption spectroscopy after lithium metaborate alkaline
129 fusion. Potassium (K) was measured by furnace atomic absorption spectroscopy after
130 complete mineralisation at 525°C and resuspension of ashes with 50 µL of 67% HNO₃ in 50
131 mL deionised water. Spinach (NIST 1570a, Sigma-Aldrich, France) was used as a standard
132 for mineralization efficiency evaluation.

133 Lignin and cellulose contents in leaf litter were determined with the fibre forage method
134 (Van Soest et al., 1991) as described in Gessner (2005). This method is based on the
135 determination of residual weight after the removal of the different litter components. Firstly,
136 soluble compounds were removed with boiling acid-detergent solution (cetyltrimethyl
137 ammonium bromide 20 g.L⁻¹ in a 0.5M sulphuric acid solution). In the second step of the
138 analysis, cellulose was removed with 72% (w/w) sulphuric acid. Lignin was then removed

139 with a muffle furnace at 500°C during 6 h. After each step the remaining dry mass was
140 determined.

141 For each leaf origin, four replicates of ten leaves were randomly chosen. Leaves were
142 oven dried at 65°C during two days and then weighted. Leaves were rewetted, flattened and
143 photographed for the determination of leaf area using the image analysis software ImageJ
144 (Schneider et al., 2012). The leaf mass area was calculated as the ratio between leaf mass (g)
145 and leaf area (m²).

146 Leaf toughness was measured as the strength needed to penetrate the leaf with a punch.
147 The measure was done with a custom-made penetrometer as described by Graça & Zimmer
148 (2005). The strength applied was progressively increased by adding water on a bottle fixed on
149 the punch. The weight of the filled bottle and the punch was then recorded for each point and
150 data were converted to pressure units with the equation toughness = $m \times g_0$ (with m the mass
151 of the filled bottle and the punch and g_0 the standard gravity).

152

153 2.2. Leaf-litter decomposability in stream

154 2.2.1. Study site

155 The study was conducted in La Maix stream (latitude 48°29'2.1"N, longitude
156 7°04'8.5"E), a second order stream draining a catchment lying on sandstone and located in
157 the Vosges Mountain. This study site was selected due its proximity to sandstone leaf litter
158 sampling sites and the fact that it is well known, frequently used as experimental site in many
159 studies and has a diversified community of macroinvertebrate detritivores and microbial
160 decomposers because it was not strongly impacted by acidification. The stream was
161 surrounded by a mixed forest mainly composed of beech (*Fagus sylvatica*), silver fir (*Abies*
162 *alba*) and norway spruce (*Picea abies*). Water samples were collected and analysed at the
163 beginning of the experiment and then at each sampling date. During the 128 days-experiment

164 (6 sampling dates: day 0, 21, 44, 70, 99, 128), La Maix water chemical characteristics were as
165 follows: [Mean (\pm Sd);]: pH= 7.55 (\pm 0.12), conductivity = 79.2 (\pm 6.2) μ S cm^{-1} , acid
166 neutralizing capacity (ANC) = 497.8 (\pm 60.1) μ eq L^{-1} , $[\text{Cl}^-]$ = 1.52 (\pm 0.11) mg L^{-1} , $[\text{NO}_3^-]$ =
167 2.44 (\pm 0.16) mg L^{-1} , $[\text{SO}_4^{2-}]$ = 5.52 (\pm 0.43) mg L^{-1} , $[\text{PO}_4^{3-}]$ < 0.02 mg L^{-1} , $[\text{Na}^+]$ = 0.84 mg
168 L^{-1} , $[\text{Ca}^{2+}]$ = 6.34 (\pm 0.80) mg L^{-1} , $[\text{K}^+]$ = 1.38 (\pm 0.08) mg L^{-1} and $[\text{Mg}^{2+}]$ = 3.37 (\pm 0.29) mg
169 L^{-1} . Mean temperature was 7.5°C and ranged from 4.0 °C to 12.8 °C during the course of the
170 experiment.

171

172 2.2.2. *Experimental design of the litter decomposability measurement*

173 Three hundred and twenty litter bags [8 beech litter provenance \times 5 incubation dates \times 4
174 replicates \times 2 mesh size] were filled with 3.5 (\pm 0.005) g of air dried leaf litter. Two types of
175 bags were used: coarse-mesh bags (5mm mesh size) allowing detritivore macroinvertebrates
176 to colonize leaf litter and fine-mesh bags (0.5mm mesh size) preventing macroinvertebrate to
177 colonise the litter but allowing microbial decomposers. Litter bags were immersed in La Maix
178 stream on February in 20 blocks to integrate intra-site variation. In each block, 16 litter bags
179 were immersed (8 beech litter provenances \times 2 litterbag mesh sizes): litter bags of each block
180 were thus considered as all condition replicate. Litter bags of four different blocks were
181 randomly collected after 21, 44, 70, 99 and 128 days of incubation in the stream. Each bag
182 was then placed individually in a bag filled with stream water, placed in a cooler and rapidly
183 transported to the laboratory for further processing.

184 Litter bags were opened at the laboratory within 24 hours. Residual leaf litter was gently
185 washed with stream water to eliminate particulate organic matter and sand. For each fine
186 mesh bag, five leaves were randomly sampled and one 14 mm diameter disk was cut in each
187 leaf and stored at -80°C before fungal biomass quantifications.

188

189 2.2.3. *Measured endpoints*

190 Remaining litter was dried at 65°C to constant mass (48 h), and then weighted (± 0.1 mg).
191 Ash free dry mass (AFDM) was determined after complete combustion of a sub-sample in a
192 muffle furnace (at 500°C during 6 h).

193 For each beech litter provenance and each mesh size, the decomposition rate (k) in fine
194 mesh (k_{FM}) and in coarse mesh bags (k_{CM}) was calculated by fitting an exponential model M_t
195 $= M_0 e^{-kt}$ on the remaining AFDM data of the four replicates of each date, according to
196 Bärlocher (2005).

197 Fungal biomass was evaluated by the quantification of the fungal sterol, ergosterol. We
198 used a conversion factor ergosterol-to-biomass of 5.5 mg g⁻¹ (Gessner and Chauvet, 1993).
199 The five leaf discs cut for each fine mesh bag (see above), were freeze dried before to be
200 precisely weighted (0.1 mg). Ergosterol was extracted in 5 ml methanol-KOH solution (8 g L⁻¹)
201 overnight at 4°C and then at 80°C for 30 min. The ergosterol concentration of the leaf litter
202 was then assessed using solid-phase extraction (Oasis HLB, 60mg; 3mL; Waters corporation,
203 Milford, Massachusetts, USA) and reversed phase high performance liquid chromatography
204 (Gessner and Schmitt, 1996).

205

206 2.3. *Statistics*

207 We performed ANOVAs followed by Tukey HSD post-hoc tests to compare leaf-litter
208 traits between the different provenance, and ANCOVA followed by Tukey post-hoc tests for
209 the comparison of decomposition rates. Data were log transformed when necessary to meet
210 the assumption (normality and homoscedasticity) for ANOVA. These assumptions were met
211 for all analyses. A principal component analysis (PCA) was used to investigate relationships
212 among leaf litter traits and among the different batch of litter from different sites and

213 PERMANOVA was used to test for differences in leaf quality between limed and control. For
214 the PERMANOVA, we used the *betadisper* function to check the equality of variance.

215 Effect sizes were used to determine specifically the potential effect of liming on leaf litter
216 traits, litter decomposition and fungal biomass. Only the three couples of limed-control site
217 were used (GL-GNL, S1L-S1NL and S2L-S2NL, table 2). Effect sizes were determined as
218 described by Hedges et al. (1999).

219 Partial least square regressions (PLS) have been used to determine the importance of each
220 leaf litter trait on tested endpoints (k_{CM} , k_{FM} and fungal biomass at each time point). For each
221 PLS model, traits with variable importance in the projection (VIP) <0.7 were excluded and
222 VIP for other traits were recalculated. Model quality estimate by Q^2 and R^2 parameters based
223 on leave-one-out cross-validation (Lê Cao et al., 2008). Data analyses were implemented
224 within the R statistical environment (R Core Team, 2013), using the {mixOmics} and {vegan}
225 packages for multivariate analysis. The significance threshold was set at $P < 0.05$.

226

227 3. Results

228 3.1. Leaf litter traits

229 Ca and Mg contents of leaf litter were significantly increased by liming (fig. 1) from 4.08
230 (± 1.57) mg g^{-1} in not limed sites to 8.49 (± 2.49) mg g^{-1} in limed sites (G: + 143 %; S1: +
231 19%; S2: +248%) and 0.47 (± 0.06) mg g^{-1} in control sites to 0.93 (± 0.37) mg g^{-1} in limed sites
232 (G: + 198 %; S1: + 50%; S2: + 54%), respectively. Compared to their not limed counterpart,
233 we also observed a significantly lower K content (G: -25 %; S1: -41 %; S2: -36 %) and a
234 slightly higher P concentration in litter from limed site, but this difference was not significant.
235 None of the other measured leaf litter traits were affected by liming (fig. 1).

236 Leaf litter traits expressed a strong variability within litter from acid bedrock sites
237 (granite and sandstone, limed and not limed), particularly for Ca, Mg, K and P content for

238 which we observed 5-, 3-, 3- and 2-fold variation respectively (table 2). This variability in leaf
239 litter traits was higher within limed sites than within control, non-limed acid-bedrock sites
240 (fig. 2). C and N contents expressed only very low differences between sites as well as the
241 C/N ratio.

242 Cellulose content was not significantly affected by litter sampling site. Lignin content
243 was impacted by litter sampling site (table 2) but not by liming (fig. 1). No significant
244 differences in leaf toughness were observed among the litter from different sites (table 2).
245 LMA values ranged from 36.1 g m⁻² for GL litter to 63.3 g m⁻² for GNL with significant
246 differences among litter from different sites but these differences were not induced by liming
247 (fig. 1). Litter from calcareous sites (Calcareous1 and Calcareous2) were characterised by
248 high content of Ca (18.4 and 17.5 mg g⁻¹), and Mg (1.31 and 1.49 mg g⁻¹) but also
249 significantly higher P content (1.20 and 2.47 mg g⁻¹), a high K content (10.3 and 9.0 mg g⁻¹)
250 and lower fibre (lignin and cellulose) content (fig. 2, table 2). While liming led to higher Ca
251 and Mg contents, these contents remained below those observed in the litter from the
252 calcareous sites, except for Mg content in GL litter.

253 In the PCA, litter from limed and control sites cannot be distinguished by the leaf litter
254 traits measured (fig. 2). This was confirmed using a PERMANOVA, indicating no significant
255 differences between control and limed sites (p=0.3). The response to liming seemed different
256 at each liming site. Litter from granite and sandstone sites were clearly separated on the first
257 axis from litter from Calcareous1 and Calcareous2 sites (fig. 2). Only litter from the granite
258 limed site had intermediate traits on axis 1 between litter from control sites and calcareous
259 sites (fig. 2). However, contents of Mg and total C in leaf litter sampled in the two calcareous
260 sites were relatively similar to those observed from the granite bedrock limed site (GL) (table
261 2, fig. 2). Leaves sampled in the GL site showed significantly higher Ca content and lower
262 C:P ratio compared to those from the other sites on acid bedrock. As expected, litter Ca and

263 Mg were strongly positively correlated. P and K contents were also positively correlated with
264 Ca and Mg contents and negatively with lignin content. Toughness was strongly related to
265 lignin content (fig. 2).

266

267 3.2. Leaf litter decomposability

268 Liming had no significant effect on the rate of microbial decomposition (fine mesh litter
269 bags; k_{FM}) but in CM litter bags, a negative effect of liming was observed (fig. 3)..
270 Significantly different k_{FM} between leaf origins were observed, but not linked to liming (fig.
271 3, 4a). Decomposition rates of leaf litter from limed and control sites expressed only a low
272 variability. k_{FM} ranged from 0.0025 day^{-1} to 0.0032 day^{-1} and k_{CM} ranged from 0.0041 day^{-1} to
273 0.0064 day^{-1} .

274 In fine mesh (FM) litter bags litter from both calcareous sites had significantly higher k_{FM}
275 than any acid site (fig. 4a). In CM, the results were more contrasted. While Calcareous2 leaf
276 litter has a significantly higher k_{CM} (0.0112 day^{-1}) than any other site, Calcareous1 leaf litter
277 has not a significantly higher k_{CM} than most of the leaf litter from acid bedrock sites (fig. 4b).
278 Notably, Calcareous2 expressed a significantly higher k_{CM} than all the other sites, including
279 Calcareous1 (fig. 4b). However, litter from the other calcareous site (Calcareous1) in CM
280 litter bags did not decompose significantly faster than litter from acid sites (except GL and
281 S1L, fig. 4b).

282 PLS regression was used to determine the importance of each trait for decomposition.
283 When the PLS regression was done only on acid sites data, decomposition rate was best
284 explained by positive effect of K content, C content (and the C/N) and LMA but also by
285 negative effect of Ca, Mg, N content and leaf toughness in CM bags. In FM bags, P content,
286 C/P, and P/lignin ratio were all important variables, with a negative effect of P content and
287 P/lignin ratio on decomposition (table 3). However, the models for both k_{CM} and k_{FM}

288 explained only 13 and 14 % of variance, respectively. When including the calcareous sites,
289 k_{CM} was principally driven by high P content, low cellulose content and high P/lignin ratio
290 (table 3), while k_{FM} appears as positively related to Ca, K and P contents, P/lignin and
291 N/lignin ratio and negatively to cellulose content (table 3). Note that more leaf traits
292 significantly influenced k_{CM} and k_{FM} when considering calcareous sites.

293

294 3.3. Fungal biomass

295 Liming had no significant effect on fungal biomass at any sampling time (fig. 3). For
296 most of the litters tested, the maximum fungal biomass was reached after 77 days while for
297 litter from two sites (GL and S1L) the maximal biomass was reached after 44 days (fig. 5).
298 Both leaf litters from calcareous sites reached higher values than the others (fig. 3, 5) but with
299 different dynamics since leaf litter from Calcareous2 site had strongly higher fungal biomass
300 than any other litter after 21 days while leaf litter from Calcareous1 site showed not more
301 fungal biomass than the other sites at this date. In contrast, leaf litter from Calcareous1 site
302 had more fungal biomass than Calcareous2 site after 99 days (fig. 5).

303 At each sampling date, PLS models have been used based on the traits measured on leaf
304 litters (table 4). Considering only leaf litters from acid sites at 21 days, initial C/N, cellulose,
305 P and Mg contents were positively associated with higher fungal biomass while N/lignin,
306 toughness and C/P were negatively associated with it. After 44 days, fungal biomass was
307 positively related to initial Ca and Mg content and C/N ratio while C and N contents, LMA,
308 toughness and lignin content negatively affected it (table 4). At 77 and 99 days, fungal
309 biomass was mainly negatively related to N content.

310 When adding data from litter from the calcareous sites, initial Ca, Mg, P, K contents and
311 P/lignin ratio were associated with higher fungal biomass at all sampling dates. Toughness,
312 cellulose and C/P ratio were negatively related to fungal biomass at all dates and lignin

313 between 44 and 99 days. After 77 days, N content was also negatively associated with fungal
314 biomass (table 4).

315

316 **4. Discussion**

317 *4.1. Liming has a limited effect on leaf litter traits.*

318 In our experiment, we observed that liming induced a strong increase in Ca and Mg
319 contents in the collected leaf litter, supporting our first hypothesis. This is consistent with
320 literature data since previous studies have shown that liming increases Ca and Mg contents (or
321 Ca only in case of liming with calcite) of green leaves and leaf litters (Court et al., 2018;
322 Forey et al., 2015; Long et al., 2011; Moore et al., 2012). For example, Forey et al. (2015)
323 reported an increased Ca concentration in beech litter even 25 years after the liming.

324 Intraspecific variability in leaf litter traits can be due to environmental and genetic factors
325 (LeRoy et al., 2007; Ordoñez et al., 2009; Silfver et al., 2018). Our sampling sites are
326 geographically very close and we may hypothesize that traits differences between leaf litter
327 from the different sites mainly reflect differences in sites characteristics even if some
328 differences due to genetic variation cannot be excluded. Indeed, availability of soil mineral
329 nutrients is known to influence leaf litter nutrient content through a reduced foliar
330 remobilisation when nutrients are highly available in soil (Achat et al., 2018; Vergutz et al.,
331 2012).

332 Another important trait, lignin content, may be influenced by nutrient availability since
333 nutrient limitation promote the production of carbon based secondary metabolites such as

334 lignin (Koricheva et al., 1998). Changes in lignin content might affect structural traits such as
335 toughness and several studies have reported relationships between LMA and soil nutrients
336 (Hidaka and Kitayama, 2011; Poorter et al., 2009).

337 The effect of liming on soil is more complex than just an increase of available Ca and Mg
338 (Auclerc et al., 2012; Balland-Bolou-Bi and Poszwa, 2012; Court et al., 2018; Forey et al.,
339 2015) and we hypothesized that the improvement of tree mineral nutrition after liming might
340 have affected other leaf litter traits. In the present study, we observed a significant decrease of
341 leaf litter K content. Among the ten traits measured, Ca, Mg and K contents were the only
342 three leaf litter traits that were significantly impacted by liming. Neither C, N, P contents nor
343 nutrient ratios and structural traits were significantly affected by liming. A previous study has
344 reported a decrease in litter N and K contents after liming possibly due to a dilution of these
345 nutrients by the high amount of Ca (Forey et al., 2015). We just observed such an effect of
346 liming for K, but not for N content. However, N and P generally show strong resorption by
347 plants, so that little changes in nutrient availability would not strongly affect nutrient content
348 in litter (Vergutz et al., 2012). In contrast, Ca is nearly not resorpted and Mg resorption is low
349 (30%, decreasing with increasing soil Mg; Vergutz et al., 2012) so these traits in leaf litter
350 could be more affected by changes in soil nutrient availability.

351 Some studies have found changes in leaves lignin or cellulose contents due to liming
352 (Forey et al., 2015; Melvin et al., 2013) but very few studies have considered morphological
353 parameters of green or senescent leaves (Forey et al., 2015). Yet, such traits can strongly

354 affect leaf litter decomposability (Pérez-Harguindeguy et al., 2000; Quinn et al., 2000) and to
355 our knowledge, none have reported changes after liming.

356 Leaf litter traits were also measured on litters from two calcareous (Calcareous1 and
357 Calcareous2) sites used as high Ca-Mg references. These reference sites reveal a large
358 phenotypic plasticity of most of the measured traits (except cellulose and toughness, table 2).
359 Since liming aims to improve soil fertility, we expected that liming would induce changes in
360 leaf litter traits toward trait values close to those of calcareous sites. While the Mg content of
361 litter collected in the GL site approached the Mg content of litters collected on calcareous
362 sites, the Ca content in litters collected on limed sites remained 2 to 3 times lower than the Ca
363 content of litters collected on calcareous sites. The change was even in the opposite direction
364 for K content. For the other traits, leaf litter from limed sites did not exhibit intermediate
365 values between control and calcareous sites, except at the GL sites for some traits (e.g.
366 significantly higher C/P ratio in the limed plot compared to the control plot). This suggests
367 that the response to liming can be different depending on initial site characteristics, which has
368 been clearly illustrated by Rizvi et al. (2012) that reported opposite changes in humus
369 thickness at granite vs. sandstone sites after liming. The nature of soil thus largely affects the
370 importance of liming effects on leaf litter traits.

371

372 *4.2. Liming had no positive effect on leaf decomposability in the studied stream*

373 Liming aims, among others, to improve ecological functions in streams flowing in acidified
374 forests (Lawrence et al., 2016). Liming is generally considered to affect aquatic ecosystem
375 through changes in water chemistry and in biological community composition that will, in
376 turn, modify ecosystem processes such as leaf litter decomposition (Merrix et al., 2006). Our
377 hypothesis was that it could also affect ecosystem functions through enhanced litter
378 decomposability. In our experiment, microbial decomposition (in FM litter bags) as well as
379 fungal biomass remained unaffected by liming, while we observed a slight negative effect of
380 liming on total decomposition rate (in CM litter bags). When considering all sites, including
381 calcareous ones, we observed large differences in leaf litter decomposability, confirming the
382 sensitivity of the method used in this study to discriminate leaf litter qualities. However,
383 higher leaf litter decomposability was not related to liming. Our second hypothesis is
384 therefore rejected. To date, liming experiments carried out in terrestrial environments have led
385 to contrasting results. For example, in a study conducted by McCay et al. (2013), liming
386 increased leaf litter decomposability while in two other studies (Lovett et al., 2016;
387 Perkiömäki et al., 2004), liming did not significantly impact leaf litter decomposability. In
388 aquatic ecosystems, while some studies have investigated the effect of liming on
389 decomposition in limed vs. not limed streams, and shown a positive effect on microbial
390 decomposition (Mckie et al., 2006; Merrix et al., 2006), none have investigated if liming
391 induced changes in leaf litter decomposability. We therefore investigated the links between
392 traits and decomposability to explain why we did not observe the expected changes.

393

394 *4.3. Links between leaf litter traits and litter decomposability*

395 We hypothesised that liming can indirectly improve leaf litter decomposability through
396 changes in key leaf litter traits (hypothesis iii). Since liming did not increase leaf litter
397 decomposition in our study but significantly modified some leaf litter traits (increase in Ca,
398 Mg content; decrease in K content), hypothesis iii was rejected. However, in the literature,
399 some leaf litter traits are known to affect its decomposability, and intraspecific variations in
400 leaf litter traits have been shown to affect decomposition process (*e.g.* Jackrel and Wootton,
401 2014; Lecerf and Chauvet, 2008).

402 If some leaf litter traits are often pointed out as major drivers of leaf litter decomposition
403 in aquatic ecosystem (*e.g.*, P, lignin: Lecerf and Chauvet, 2008; Migliorini et al., 2018), none
404 of these studies have investigated the impact of leaf Ca and Mg contents. However, Ca is an
405 element of primary importance for aquatic invertebrates such as crustaceans (*e.g.* *Gammarus*
406 *fossarum*, which is the main detritivore macroinvertebrate in the headwater streams of the
407 studied area; Dangles and Guerold, 2000; Rollin et al., 2018) and for microbial decomposers
408 (Rosset and Bärlocher, 1985). A positive effect of higher Ca and Mg could also have been
409 mitigated by the observed reduction of litter K content with liming, a covariation that had
410 already been observed by Forey et al. (2015).

411 The fact that other traits, such as lignin and P content, known for their effects on litter
412 decomposition in coarse mesh and fine mesh litter bags in aquatic ecosystem (Lecerf and

413 Chauvet, 2008), remained unaffected by liming. This observation may also help explaining
414 our results. Here we used partial least square regression to identify leaf litter traits that drive
415 microbial decomposition (*i.e.* k_{FM}), total decomposition (*i.e.* k_{CM}) and fungal biomass at each
416 time based on litter traits. This approach showed that Ca and Mg content appeared as
417 important drivers of decomposition and fungal growth. However, this might be due only to
418 the litters from the calcareous sites where decomposition rate and fungal biomass were very
419 high, with litter traits characterised by high Ca and Mg but also high P and low lignin. GL
420 litter that has the highest Ca and Mg contents for sites overlaid by acid bedrock (table 2), has
421 relatively low k_{FM} and k_{CM} . When we focussed on acid sites, considering only litter from acid
422 bedrock sites (granite and sandstone, limed or not), Ca or Mg were associated with higher
423 fungal biomass until 44 days but did not appear to favour microbial decomposition. Moreover,
424 we observed a negative correlation between Ca content and k_{CM} .

425 These results are contrasting with some results observed in terrestrial ecosystems where
426 Ca and, in a lesser extent, Mg contents in the litter have been often pointed out as important
427 factors driving decomposition (Hobbie et al., 2006; Makkonen et al., 2012; McCay et al.,
428 2013; Yue et al., 2016). However, this positive effect of litter Ca content has been suggested
429 to be mainly linked to the high Ca demand of a major taxonomic group of leaf litter
430 consumers, earthworms (Hobbie et al., 2006), and therefore this could be specific to the
431 terrestrial environment.

432 As illustrated by the contrasting results of similar experiments carried out in terrestrial
433 environments, result of in stream decomposition can be highly dependent on environmental
434 factors. In our study, we used a circumneutral stream (pH = 7.55) with relatively high
435 concentration of Ca (6.34 mg L⁻¹) and Mg (3.37 mg L⁻¹) to determine the decomposability of
436 the leaf litters, without limitation due to the exclusion of acid sensitive microbial decomposers
437 and detritivores (Baudoin et al., 2008; Clivot et al., 2014). We cannot exclude that, in an
438 acidified stream with very low Ca, the increased content of Ca and Mg measured in the litter
439 from limed sites could have played a role in leaf litter decomposition dynamics. However this
440 remains to be investigated in further research. Furthermore, in acidified stream, numerous
441 acid sensitive detritivores such as *G. fossarum* that are generally favoured by high
442 concentrations of Ca in water (Rollin et al., 2018), are generally missing, potentially
443 precluding any stimulatory effect of higher Ca contents in leaf litter on overall litter
444 decomposition (Clivot et al., 2014).

445 Finally, K content appeared as associated with higher decomposition rate. Such
446 association of higher K content with faster decomposition have already been suggested (Yue
447 et al., 2016). This indicates that there is no positive effect of Ca or Mg when there is a
448 reduction of K, low P and a high amount of poorly labile forms of carbon such as lignin (that
449 are mostly related to nutrient poor sites; Hidaka and Kitayama, 2011; Koricheva et al., 1998).
450 Therefore, liming forest stand where leaf litter are already poor in P, K and labile carbon will
451 certainly not lead to an improved decomposability of leaf litters in stream. In some liming

452 experiments, N, P and K additions are combined with the liming (Court et al., 2015). Further
453 research is needed to determine whether adding N, P and/or K along with the calcareous
454 material can improve leaf litter decomposition rate compared to the simple liming.

455

456 **5. Conclusion**

457 In this study, we have shown that liming (done 9 and 21 years before the present study)
458 led to changes in leaf litter traits by increasing Ca and Mg content, and by a significant
459 decrease in K content. Leaf litter decomposability, however, was not improved. A slightly
460 negative effect was observed in coarse mesh litter bags, and microbially mediated leaf litter
461 decomposability was not affected. The other tested leaf litter traits remained unchanged. Our
462 experiment thus suggests that leaf litter Ca and Mg content were not the main drivers of
463 decomposition in our experimental conditions. The low P content, the reduced K content and
464 the high amount of poorly labile forms of carbon such as lignin and cellulose in the litter from
465 limed sites could have been important factors explaining their reduced decomposition. Our
466 results do not support our initial hypothesis that the liming of sites impacted by acidification
467 will systematically improve the quality of leaf litter for decomposers. Further research should
468 be conducted on factors that can potentially affect leaf litter response to liming (initial soil
469 properties especially P content, genetic factors, lime composition and applied amounts of
470 products) and its decomposability in aquatic ecosystems.

471

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476

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675

Site	Geologic substratum	Treatment with dolimie	Time since liming	Location coordinates
Granite limed (GL)	Granite	2.8 Mg ha ⁻¹	9 years	N 47°57'22.9", E 06°52'55.6'
Granite not limed (GNL)	Granite	acidified		N 47°58'22.4", E 06°53'49.5"
Sandstone site 1 limed (S1L)	Sandstone	2.5 Mg ha ⁻¹	9 years	N 48°27'58.4", E 07°05'46.9"
Sandstone site 1 not limed (S1NL)	Sandstone	acidified		N 48°27'08.2", E 07°04'49.5"
Sandstone site 2 limed (S2L)	Sandstone	2.4 Mg ha ⁻¹	21 years	N 47°59'874", E 06°29'613"
Sandstone site 2 not limed (S2NL)	Sandstone	acidified		N 47°59'853", E 06°29'53.9"
Calcareous1	Limestone (calcareous)	None		N 49°0.0'14.8", E 06°4,0'46.7°
Calcareous2	Limestone (calcareous)	None		N 49°0.0'44.5", E 06°5'30.9"

676 Table 1: Acronyms, treatment and location of the sites where leaf litters were collected.

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678 Table 2: Mean (\pm sd) leaf litter traits (Mg, Ca, P, C, N, lignin and cellulose content, toughness
679 and LMA). C/N, C/P and N/P are molar ratio. For the labels, GL and GNL represent leaf litter
680 sampled on granite watershed limed site and his no limed control, SL and SNL represent leaf
681 litter sampled on sandstone watershed limed sites (S1L, S2L) and these no limed controls
682 (S1NL, S2NL) whereas leaves coming from calcareous bedrock (without liming) were
683 annotated Calcareous1 and Calcareous2. The same letter indicates no significant differences
684 for a litter trait between beech litter provenance ($\alpha=0,05$). ^(log) indicates traits for which data
685 where log transformed before ANOVA.

Origin	GNL	GL	S1NL	S1L	S2NL	S2L	Calcareous 1	Calcareous 2
Mg (mg g ⁻¹) ^(log)	0.45 ^C (\pm 0.04)	1.34 ^a (\pm 0.10)	0.42 ^C (\pm 0.02)	0.63 ^{bc} (\pm 0.04)	0.54 ^{bc} (\pm 0.03)	0.83 ^b (\pm 0.14)	1.31 ^a (\pm 0.11)	1.49 ^a (\pm 0.16)
Ca (mg g ⁻¹) ^(log)	4.6 ^e (\pm 0.2)	11.2 ^b (\pm 0.3)	5.3 ^{de} (\pm 0.5)	6.3 ^{cd} (\pm 0.3)	2.3 ^f (\pm 0.1)	8.0 ^c (\pm 0.5)	18.4 ^a (\pm 1.2)	17.5 ^a (\pm 0.5)
K (mg g ⁻¹)	2.8 ^e (\pm 0.2)	2.1 ^f (\pm 0.3)	7.5 ^{bc} (\pm 0.2)	4.4 ^d (\pm 0.1)	6.2 ^c (\pm 0.32)	4.0 ^d (\pm 0.3)	10.3 ^a (\pm 0.8)	9.0 ^{ab} (\pm 0.8)
P (mg g ⁻¹) ^(log)	0.40 ^{cd} (\pm 0.01)	0.61 ^c (\pm 0.06)	0.33 ^d (\pm 0.01)	0.47 ^{cd} (\pm 0.04)	0.57 ^c (\pm 0.04)	0.41 ^{cd} (\pm 0.05)	1.20 ^b (\pm 0.15)	2.47 ^a (\pm 0.21)
C (mg g ⁻¹)	500 ^a (\pm 5)	471 ^{bc} (\pm 3)	492 ^a (\pm 1)	500 ^a (\pm 2)	501 ^a (\pm 2)	498 ^a (\pm 1)	477 ^b (\pm 1)	463 ^c (\pm 1)
N (mg g ⁻¹)	8.8 ^{ab} (\pm 0.2)	8.2 ^b (\pm 0.1)	8.9 ^{ab} (\pm 0.4)	9.9 ^a (\pm 0.5)	8.9 ^{ab} (\pm 0.1)	8.1 ^b (\pm 0.1)	7.8 ^b (\pm 0.3)	9.2 ^{ab} (\pm 0.3)
C/P ^(log)	3226 ^{ab} (\pm 92)	2054 ^c (\pm 240)	3865 ^a (\pm 80)	2759 ^{abc} (\pm 196)	2290 ^{bc} (\pm 157)	3187 ^{ab} (\pm 377)	1043 ^d (\pm 132)	493 ^e (\pm 46)
C/N ^(log)	66.4 ^{ab} (\pm 1.6)	66.8 ^{ab} (\pm 1.0)	65 ^{ab} (\pm 3.1)	59.2 ^b (\pm 2.7)	65.5 ^{ab} (\pm 1.0)	71.7 ^a (\pm 0.9)	71.5 ^a (\pm 2.6)	58.7 ^b (\pm 2.2)
N/P ^(log)	22.0 ^{ab} (\pm 0.3)	13.9 ^{bc} (\pm 1.4)	26.9 ^a (\pm 0.7)	21.0 ^{ab} (\pm 0.7)	12.2 ^{cd} (\pm 3.9)	20.0 ^{ab} (\pm 2.1)	5.1 ^{de} (\pm 1.5)	3.8 ^e (\pm 0.5)
Toughness (10 ³ kPa)	2.68 ^a (\pm 0.21)	1.75 ^a (\pm 0.07)	2.35 ^a (\pm 0.34)	3.16 ^a (\pm 0.54)	2.31 ^a (\pm 0.36)	2.59 ^a (\pm 0.55)	1.70 ^a (\pm 0.29)	1.82 ^a (\pm 0.41)
LMA (g.m ⁻²)	63.3 ^a (\pm 2.5)	36.1 ^b (\pm 1.3)	40.7 ^b (\pm 1.3)	53.5 ^a (\pm 2.0)	61.2 ^a (\pm 2.0)	59.3 ^a (\pm 3.8)	62.0 ^a (\pm 2.3)	58.9 ^a (\pm 3.3)
Cellulose (%)	26.70 ^{ab} (\pm 0.42)	30.25 ^a (\pm 0.01)	28.89 ^a (\pm 0.02)	29.40 ^a (\pm 0.01)	29.80 ^a (\pm 0.04)	30.60 ^a (\pm 0.01)	24.78 ^{ab} (\pm 0.01)	18.29 ^b (\pm 0.06)
Lignin (%) ^(log)	30.62 ^b (\pm 0.67)	29.69 ^b (\pm 1.45)	30.18 ^b (\pm 0.33)	35.58 ^{ab} (\pm 1.33)	37.40 ^a (\pm 1.20)	35.03 ^{ab} (\pm 1.73)	21.92 ^c (\pm 1.00)	29.27 ^{bc} (\pm 2.50)

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689 Table 3: Results from the Partial Least Square (PLS) regression of leaf litter traits on
 690 decomposition rate in coarse mesh (CM) or in fine mesh (FM) litter bags. Only traits with a
 691 variable importance in the projection (VIP) > 0.7 were kept. For each trait with VIP > 0.7,
 692 correlation coefficient (R) is shown. R² indicated the R² of the PLS model for each date. Q²
 693 indicates the R² of the model applied to the test set.
 694

	Litter from acid sites				Litter from acid and calcareous sites			
	CM		FM		CM		FM	
	VIP	R	VIP	R	VIP	R	VIP	R
	Q ² = 0.17 R ² = 0.13		Q ² = 0.26, R ² = 0.14		Q ² = 0.53, R ² = 0.34		Q ² = 0.72 R ² = 0.66	
P/lignin ratio			0.791	-0.585	1.173	0.906	1.154	0.875
Cellulose					1.201	-0.929	1.093	-0.829
P			1.078	-0.797	1.223	0.944	1.061	0.805
Ca	1.305	-0.696			0.800	0.618	1.161	0.880
C	0.772	0.380			0.841	-0.651	0.931	-0.706
C/P ratio			1.101	0.814	0.924	-0.714	0.862	-0.654
Mg	1.052	-0.561			0.713	0.551	0.873	0.662
N/lignin ratio							1.016	0.770
Lignin							0.951	-0.721
Toughness							0.706	-0.536
C/N ratio	0.714	0.381						
LMA								
N								
K	1.082	0.577			0.986	0.762	1.075	0.814

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701 Table 4: Results from the Partial Least Square (PLS) regression of leaf litter traits on fungal
 702 biomass at each sampling date. Only traits with a variable importance in the projection (VIP)
 703 > 0.7 were kept. For each trait with VIP > 0.7, correlation coefficient (R) is shown. R²
 704 indicated the R² of the PLS model for each date. Q² indicates the R² of the model applied to
 705 the test set.
 706

	Litter from acid sites							
	21 d		44 d		77 d		99 d	
	Q ² = 0.68	R ² =0.52	Q ² = 0.83	R ² =0.78	Q ² = 0.48	R ² =0.13	Q ² = 0.87	R ² =0.88
	VIP	R	VIP	R	VIP	R	VIP	R
Mg	0.715	0.447	0.908	0.658				
Ca			1.044	0.757				
P	0.763	0.477						
C			1.113	-0.807				
N			1.134	-0.823	0.974	-0.798	0.969	-0.700
K								
Toughness	0.873	-0.546	1.015	-0.736				
LMA			0.837	-0.607			0.767	0.554
Cellulose	1.041	0.650						
Lignin			1.040	-0.754				
C/N	1.182	0.739	0.863	0.626	1.025	0.839	1.236	0.892
C/P	0.709	-0.443						
P/lignin								
N/lignin	1.354	-0.846					0.973	-0.703

	Litter from acid and calcareous sites							
	21 d		44 d		77 d		99 d	
	Q ² = 0.08	R ² =0.02	Q ² = 0.91	R ² =0.91	Q ² = 0.64	R ² =0.50	Q ² = 0.46	R ² =0.20
	VIP	R	VIP	R	VIP	R	VIP	R
Mg	0.941	0.627	1.025	0.858	0.869	0.610	0.893	0.511
Ca	0.799	0.532	1.128	0.944	1.134	0.797	1.204	0.689
P	1.315	0.875	1.033	0.862	0.963	0.677	0.795	0.455
C	0.973	-0.648	1.077	-0.898	0.906	-0.637		
N					0.778	-0.547	1.164	-0.665
K	0.755	0.502	0.796	0.666	1.067	0.747	1.024	0.585
Toughness	0.738	-0.491	0.954	-0.795	1.050	-0.738	0.970	-0.554
LMA							0.971	0.555
Cellulose	1.113	-0.741	0.987	-0.823	1.010	-0.710	0.807	-0.461
Lignin			0.918	-0.765	1.122	-0.789	1.155	-0.660
C/N							0.977	0.558
C/P	1.026	-0.683	0.959	-0.800	0.900	-0.633	0.997	-0.569
P/lignin	1.181	0.786	1.106	0.922	1.110	0.780	1.023	0.585
N/lignin			0.862	0.719	0.988	0.695	0.932	0.532

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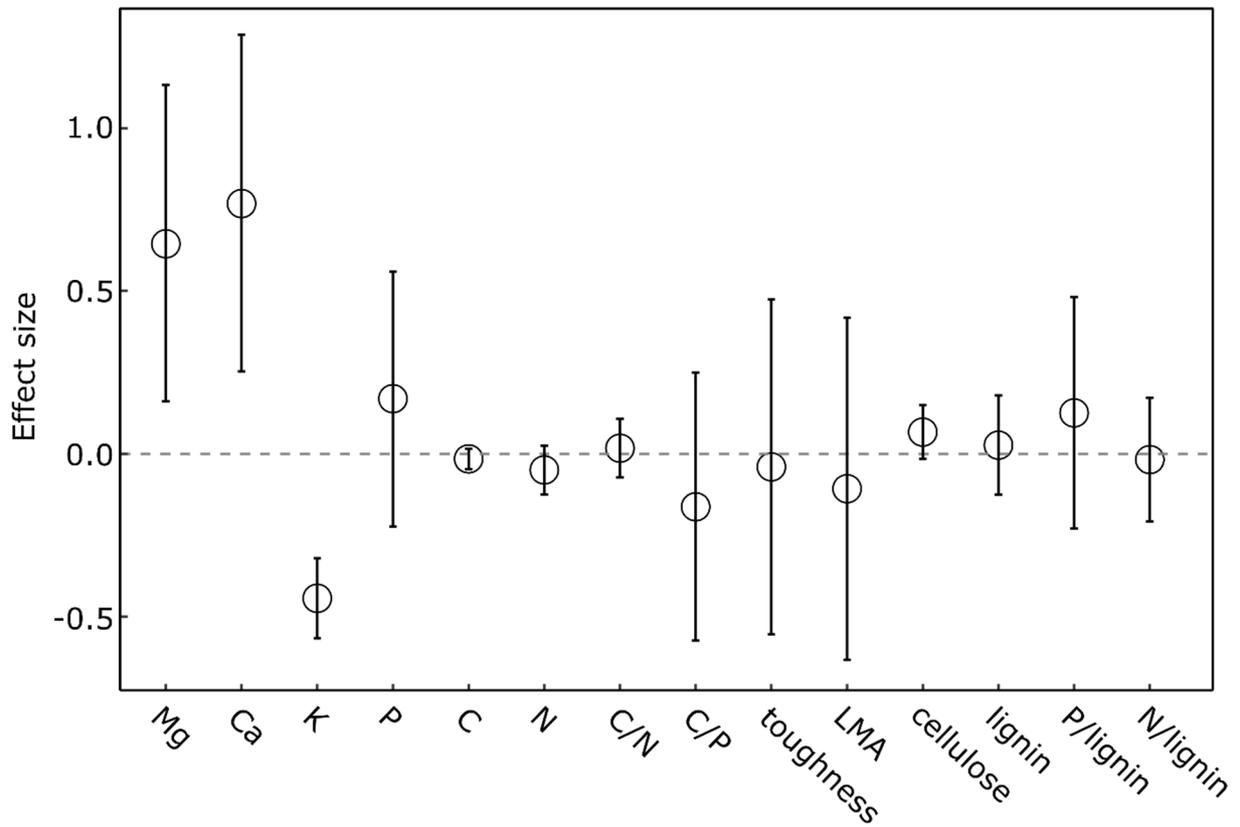
709 Figures

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711 Fig. 1. Effect of liming on the couples of sites (GNL-GL, S1NL-S1L, S2NL-S2L) on leaf

712 litter traits. Error bars represent 95% confidence intervals.

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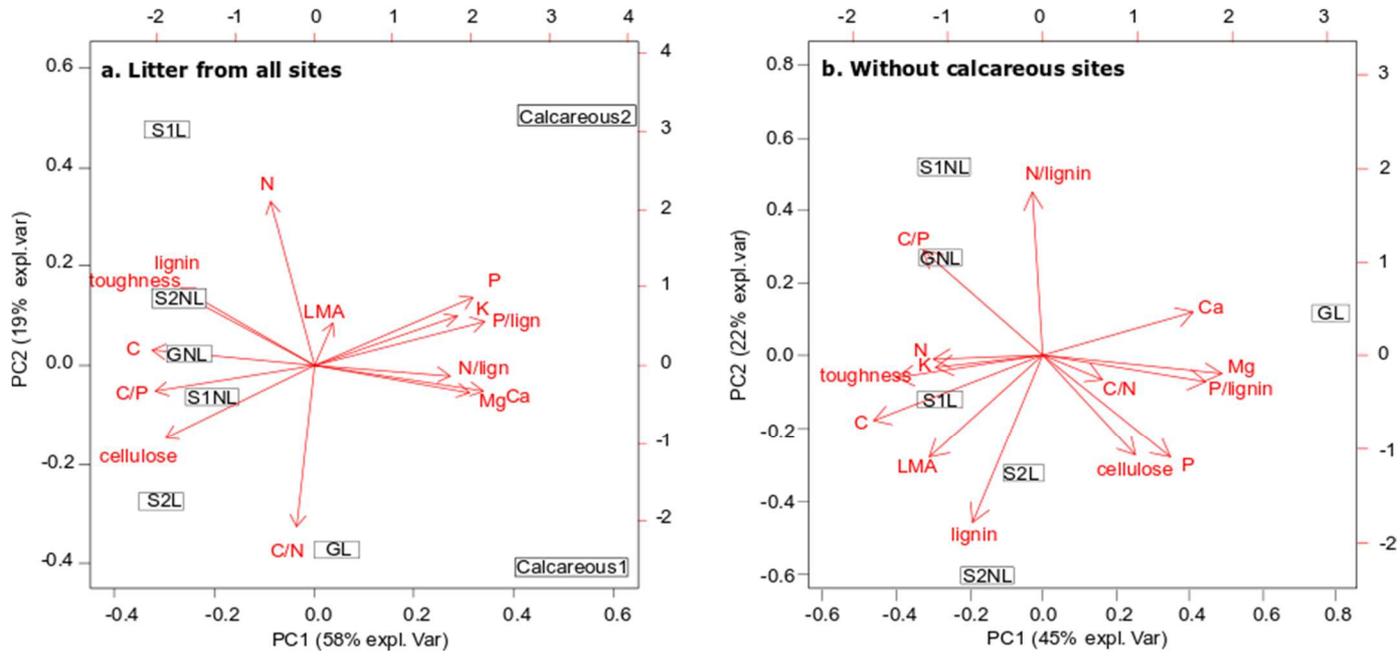
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719 Fig. 2: Variable (litter traits) and origin sites ordination from PCA. Litter origins are in black
 720 open rectangles. a: with all the sites; b: without the two calcareous sites.

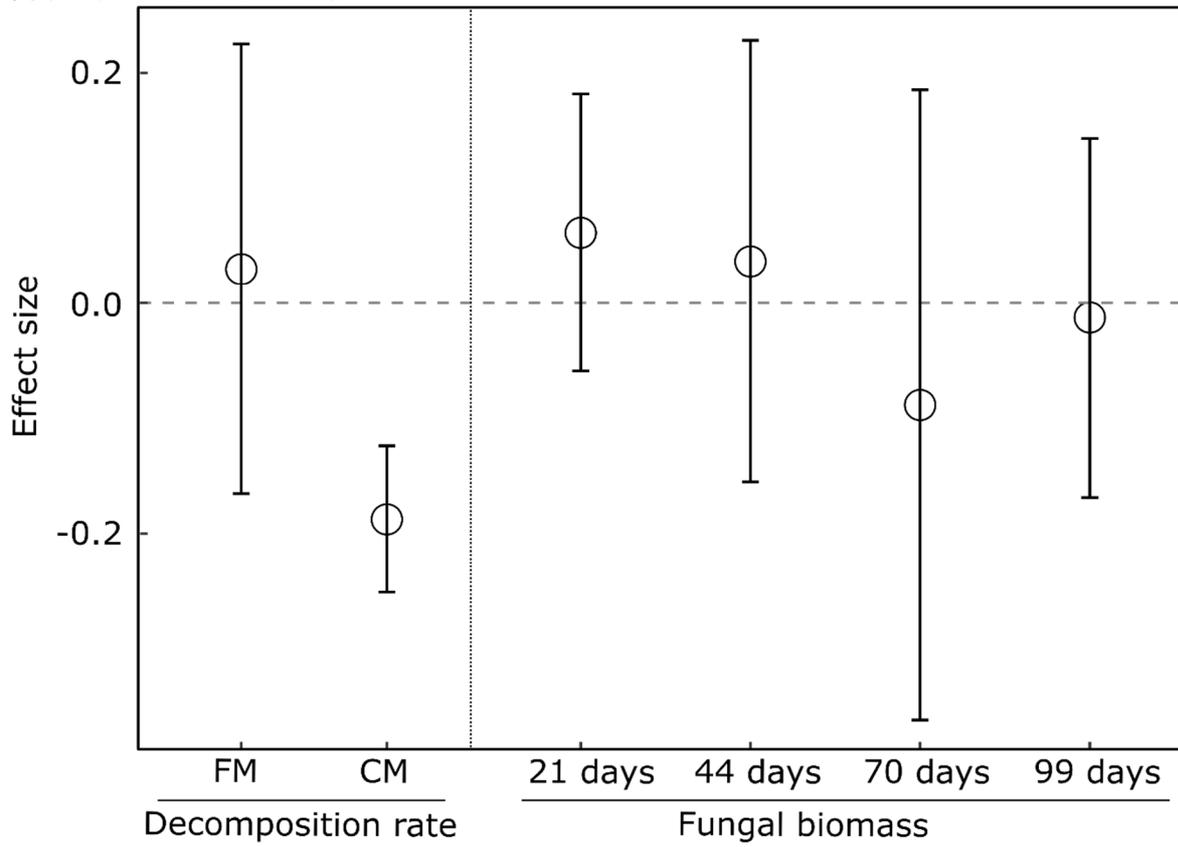


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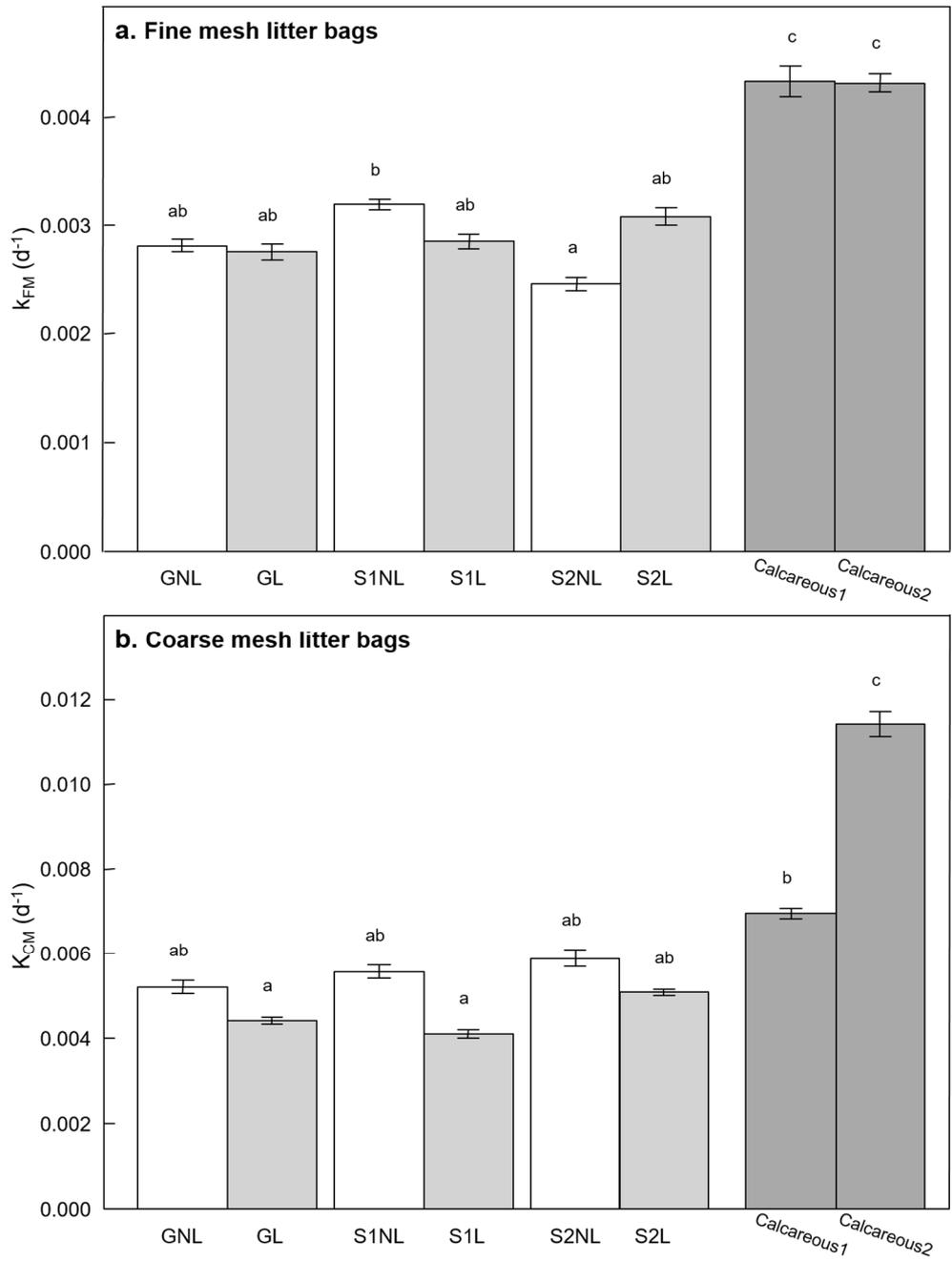
724 Fig. 3: Effect of liming on leaf litter decomposition and fungal biomass. Error bars represent
725 95% confidence intervals.



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728 Fig. 4: Decomposition rates in (a) fine mesh litter bags and (b) coarse mesh litters bags for
 729 each litter origin. White bars: control sites, light grey bars: limed sites, dark grey bars:
 730 calcareous bedrock sites. Error bars indicates standard errors. Similar letter indicates no
 731 statistical difference ($\alpha=0,05$).

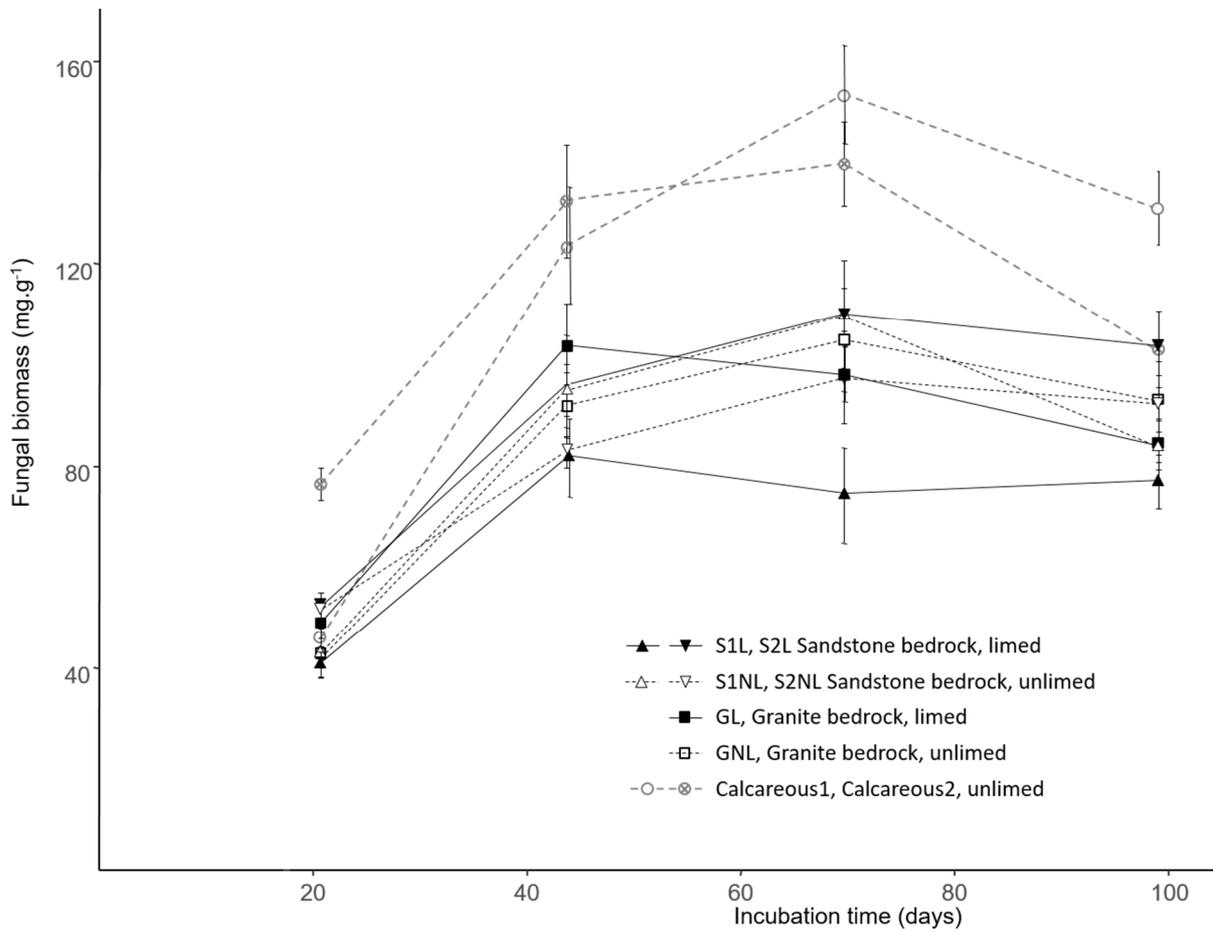


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735 Fig. 5: Fungal biomass for each litter origin between 21 days and 99 days in the stream.
 736 Dashed lines and open symbols represent sites with no liming, continuous lines and closed
 737 symbols represent sites with liming. Grey lines represent sites on calcareous bedrock, black
 738 lines sites on acid bedrock. Squares represent granite site, triangle sandstone sites, circle
 739 calcareous sites.

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