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## 4 Soil microbial community assembly precedes vegetation development after 5 drastic techniques to mitigate effects of nitrogen deposition

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### 16 Abstract

18 Oligotrophic semi-natural systems are threatened by high levels of nitrogen deposition. To mitigate  
19 these effects, drastic techniques such as sod-cutting and topsoil removal are applied to reduce  
20 nitrogen loads in existing systems and expand their area on former agricultural fields. We assessed  
21 the effects of these techniques along with the influence of previous land-use, isolation and  
22 vegetation development on subsequent microbial community assembly in restored agricultural  
23 areas. Microbial community phenotypic structure was measured using PLFA-analysis, along with soil  
24 chemistry and vegetation development. Differences in soil nitrogen pools due to restoration  
25 techniques were the most differentiating factor for both microbial community assembly and  
26 vegetation development. Only after topsoil removal was resemblance of both below- and above-  
27 ground communities to well-developed heathlands increased within 10-15 years. After sod-cutting  
28 both microbial community and vegetation composition remained more similar to agricultural sites.  
29 The relative contribution of agricultural sites and heathlands in the direct vicinity had more  
30 pronounced effects on local microbial community composition than current land-use in all study sites  
31 including agricultural areas and heathlands. Vegetation development was apparently of minor  
32 importance for microbial community assembly, since characteristic belowground assembly preceded  
33 that of aboveground development in both restoration contexts.

35 **Keywords:** Heathland; Plant-soil interactions; PLFA; Restoration; Soil chemistry

### 37 1. Introduction

39 Soil community assembly is increasingly recognised as an important factor in the restoration of  
40 oligotrophic ecosystems (Harris 2009, Kardol & Wardle 2010, Van der Putten et al. 2013). The  
41 presence of specific soil community components such as mycorrhiza might be a pre-requisite for the  
42 establishment of characteristic plant species, while microbial community composition is one of the  
43 governing factors in relation to nutrient cycling and productivity (Harris 2009). However, despite an  
44 assumed strong inter-dependence between above- and below-ground community assembly, the  
45 limited number of studies available on restoration chronosequences that include both communities  
46 show variable results. Characteristically, either both communities follow the same pattern (Lozano et  
47 al. 2014) or soil community assembly lags behind vegetation development (Holtkamp et al. 2008,  
48 Jangid et al. 2011). However, to what extent vegetation and soil community assembly depend on  
49 each other is still unclear, and remains an active area of research (Harris 2009).

51 Nitrogen deposition levels in Western Europe exceed critical values for the persistence of many  
52 oligotrophic vegetation types such as heathlands and matgrass swards (Bobbink et al. 2010). With  
53 increasing nitrogen availability, eutrophic grasses outcompete oligotrophic forbs, resulting in a loss of  
54 characteristic biodiversity (Duprè et al. 2010, Maskell et al. 2010). Efforts to mitigate these effects  
55 include both habitat improvement in existing systems and expansion of their size on former  
56 agricultural areas. However, semi-natural systems and agricultural sites are situated at opposite ends  
57 of a productivity gradient. Agricultural sites contain a productive vegetation and bacteria-dominated  
58 microbial community while oligotrophic systems have low-productive vegetation and a fungal-  
59 dominated microbial community (Wardle et al. 2004, Harris 2009). Sod-cutting and topsoil removal  
60 are drastic techniques that are sometimes used to remove excess nitrogen and phosphorus from  
61 former agricultural sites, essentially transporting nutrients from the ecosystem compartment  
62 (Verhagen et al. 2001). After sod-cutting, where only the topmost layer is stripped, much organic  
63 material remains while with topsoil removal the complete organic layer is removed. After the  
64 application of such techniques a bare soil without any vegetation and a highly reduced seedbank  
65 (Klimkowska et al. 2010) remains. A key factor for the direction of vegetation development are the  
66 dispersal abilities of characteristic plant species (Van Diggelen & Marrs 2003, Cramer et al. 2008).  
67 Much less is known about the importance of dispersal in microbial community assembly (Litchman  
68 2010, Nemergut et al. 2013).

69

70 Increased nitrogen availability as a consequence of deposition not only changes abiotic conditions in  
71 favour of more competitive species, it might also weaken plant-soil interactions (Treseder 2008). In  
72 experimental studies high levels of nitrogen addition lead to a decrease in microbial biomass and  
73 respiration (Treseder 2008, Liu et al. 2014). Fungal biomass is especially reduced (Treseder 2008,  
74 Farrer et al. 2013, Wei et al. 2013), which is likely caused by both a reduced dependence of plants on  
75 mycorrhiza and a general decline in saprotrophic fungi (Treseder 2008). Bacterial biomass is generally  
76 not affected (Treseder 2008), although some studies show a decrease at high nitrogen levels (Farrer  
77 et al. 2013, Wei et al. 2013). Such negative indirect effects of nitrogen deposition are described for  
78 existing systems, but it is unknown whether constant high levels of nitrogen deposition also limit the  
79 development of characteristic fungal-dominated communities after sod-cutting or topsoil removal.

80

81 In this paper we studied whether sod-cutting and topsoil removal were effective techniques for  
82 restoring oligotrophic systems on former agricultural sites even under conditions of high nitrogen  
83 deposition. We analysed microbial community assembly in recently restored areas in relation to soil  
84 nitrogen pool, previous land-use and isolation. We assessed whether high nitrogen levels suppress  
85 fungal content and whether a characteristic vegetation development is a precondition for the  
86 assembly of an associated and concomitantly characteristic microbial community. We hypothesize 1)  
87 that soil nitrogen pool size is the dominant factor controlling microbial community assembly, and 2)  
88 that a fungal-dominated microbial community can only develop when the nitrogen pool size is  
89 reduced sufficiently.

90

## 91 **2. Materials and methods**

92

### 93 *2.1 Study sites*

94

95 We sampled 18 sites in 8 different locations in the northern part of the Netherlands between 2003  
96 and 2009 (Table 1). These sites included former agricultural areas of which 3 were restored by sod-  
97 cutting (R-SC) and 5 by topsoil removal (R-TR), 4 current agricultural meadows as starting points  
98 (Start) and 6 well-developed heathlands with a climax vegetation as target sites (Target). Of the  
99 restored areas 3 were former arable fields (F-A) and 5 were former agricultural meadows (F-M).  
100 Yearly nitrogen input of (former) agricultural sites was between 150-200 kg N ha<sup>-1</sup>, meadows were  
101 mown several times per year for silage. The degree of isolation was determined by the distance of  
102 the site to a large heathland reserve: non-isolated sites were directly adjacent to or part of a reserve,

103 low-isolation sites were separated from the reserve by agricultural land but were within 250 m, while  
104 there was no reserve in the direct vicinity of the highly-isolated sites. Some of the studied heathlands  
105 were highly-isolated, since they were remnants of former larger heathlands that were converted into  
106 agriculture. Critical loads of nitrogen deposition for heathlands range from 10-20 kg N ha<sup>-1</sup> year<sup>-1</sup>  
107 (Bobbink et al. 2010). In 2004 nitrogen deposition levels in the studied sites were between 23.1 and  
108 35 kg N ha<sup>-1</sup> year<sup>-1</sup> (Netherlands Environmental Assessment Agency, www.mnp.nl). In the early 1990s  
109 however, when the restoration techniques were applied, nitrogen deposition levels were 30% higher.  
110

## 111 *2.2 Soil chemistry*

112

113 Soil chemistry was measured within 2 years after application of the restoration techniques in 1994-  
114 1995 and again in 2001. Since there were only marginal differences between both sampling rounds,  
115 the 2001 data were used for analysis. The Dwingeloo sites were sampled simultaneously with the  
116 microbial community in 2009. For each sites 10 samples of 0-20 cm depth were mixed. pH(KCl) was  
117 measured in 15 g fresh soil after addition of 22.5 ml 0.11 mol/l KCl. Total nitrogen (N<sub>tot</sub>) was  
118 measured on a C/N-analyzer. Total phosphorus (P<sub>tot</sub>) was measured with a colorimetric method  
119 according to Murphey & Riley (1962). The measured parameters are compared to values for  
120 reference heathlands from De Graaf et al. (2009) and Liczner et al. (2011).  
121

## 122 *2.3 Microbial community*

123

124 Within each site, a mixed sample of 3 x 100 cm<sup>3</sup> soil cores was obtained with Kopecky rings. Aliquots  
125 of the soil were refrigerated for the analysis of microbial biomass or freeze-dried for PLFA-analysis.  
126 Except for the Dwingeloo sites, which were sampled in 2009, all sites were sampled in 2003.  
127 Microbial biomass-C was determined using the fumigation-extraction procedure (Jenkinson and  
128 Powlson 1976) using K<sub>EC</sub> of 0.45 (Vance & Jenkinson 1987; Joergensen 1996). Microbial phenotypic  
129 profiles were determined by phospholipid fatty acid (PLFA) analysis using a method modified from  
130 Frostegård et al. (1993) which is further described by Courtney et al. (2014). The mol% of indicator  
131 fatty acids was used as an indicator of the presence of groups of organisms. We determined bacterial  
132 content from the sum of PLFA's i15:0, ai15:0, 15:0, 16:1, ai16:0, 16:1ω7t, cyc17:0, i17:0, ai17:0, 17:0  
133 and cyc19:0. PLFA 18:2ω6,9 was used for fungal content.  
134

## 135 *2.4 Vegetation*

136

137 Vegetation relevés (2m x 2m) were made in 2005. The cover of each species was estimated according  
138 to the Londo scale (Londo 1976). The presence of characteristic species was calculated with a  
139 Saturation Index (SI) according to Klimkowska et al. (2007). Faithfulness values obtained from  
140 SynBioSys (Hennekens et al. 2010) were used to determine if species were characteristic, only  
141 species with a faithfulness higher than 20 to the dry heath (Calluno-Ulicetea), wet heath (Erica  
142 tetralices) or Nardetea plant communities were included. A list of these species is included in  
143 Appendix A.  
144

## 145 *2.5 Statistical analysis*

146

147 We tested the effects of restoration technique, previous land-use and isolation with a linear mixed-  
148 effect model (LME) using restricted maximum likelihood (REML) estimation. Restoration technique,  
149 previous land-use and isolation were treated as fixed factors, study area as a random effect. We  
150 considered our study areas as a collection of random samples from a theoretically large pool to  
151 which we would like to extrapolate (Bennington and Thayne 1994). This model allows us to test the  
152 main effects of restoration measure, previous land-use and isolation while correcting for variation  
153 between sites, in which we were not interested. Normal distribution and equality of variances were  
154 tested with a Shapiro-Wilkinson respectively Breuch Pagan test; if needed data were ln(x+1)

155 transformed. The overall effects on microbial community composition were tested with a  
156 multivariate LME on the first two factors of a principal component analysis (PCA) of all PLFA's,  
157 including study area as random effect. Subsequently the effects of restoration technique, land-use  
158 and isolation on microbial community composition were tested with a Linear Discriminant Analysis  
159 (LDA) including all PLFA's. Structure matrix correlations were used for interpretation. To detect  
160 differences in overall vegetation composition a Detrended Component Analysis (DCA) was used,  
161 significant differences between categories on the first two axis were tested with a LME including  
162 study area as random effect. Parallel above- and below-ground assembly was assessed by combining  
163 the first LDA-axis of both communities. Significant differences between categories on the first two  
164 axis of a LDA were tested with an Analysis of Variance (ANOVA) and a post-hoc Tukey test. R 3.2.2 (R  
165 Core Team 2016), the nlme-package for LME (Pinheiro et al. 2015) and SPSS 23 (IBM Corp) were used  
166 for statistics, Canoco 4.5 for Windows (Ter Braak and Šmilauer 2002) for DCA.

167

### 168 **3 Results**

169

#### 170 *3.1 Soil chemistry*

171

172 Nitrogen pool sizes were reduced significantly after both restoration techniques (Table 2), with even  
173 lower pool sizes after topsoil removal (LME,  $F_{3,4}$ : 40.80,  $p$ : 0.0019, Tukey test,  $p < 0.05$ ). Phosphorus  
174 pools also seemed lower after topsoil removal, but these differences were not significant (LME,  $F_{3,4}$ :  
175 4.99,  $p$ : 0.3154). pH did not differ significantly between both restoration techniques, but was lower in  
176 heathlands compared to agricultural sites (LME,  $F_{3,5}$ : 7.38,  $p$ : 0.0277, Tukey test,  $p < 0.05$ ).

177

#### 178 *3.2 Microbial biomass*

179

180 Microbial biomass was significantly reduced by both techniques compared to agricultural sites and  
181 heathlands (LME,  $F_{3,67}$ : 41.81,  $p < 0.0001$ , Figure 1), with significantly lower biomass after topsoil  
182 removal than after sod-cutting (Tukey test,  $p < 0.05$ ). Previous land-use did not affect microbial  
183 biomass reduction by both restoration techniques: microbial biomass was equally reduced in former  
184 meadows and former arable fields compared to agricultural sites and heathlands (LME,  $F_{3,67}$ : 19.20,  
185  $p < 0.0001$ , Tukey test,  $p < 0.05$ ). Low-isolated sites contained significantly lower microbial biomass  
186 compared to highly-isolated sites, non-isolated sites did not differ significantly from both other  
187 categories (LME,  $F_{2,68}$ : 8.32,  $p$ : 0.0006, Tukey test,  $p < 0.05$ ).

188

#### 189 *3.3 Microbial community composition*

190

191 Restoration technique (LME,  $F_{3,73}$ : 62.32,  $p < 0.0001$ ) and isolation (LME,  $F_{2,74}$ : 12.17,  $p < 0.0001$ )  
192 affected microbial community composition significantly when all measured PLFA's were combined.  
193 Although the analysis of previous land-use showed distinct differences between agricultural sites,  
194 restored areas and heathlands (LME,  $F_{3,73}$ : 49.56,  $p < 0.0001$ ), there were no significant differences  
195 between former arable fields and former meadows (Tukey test,  $p > 0.05$ ).

196

197 The sites after both restoration techniques ordinated between the agricultural sites and heathlands  
198 on the first linear discriminant (Figure 2), with significant differences between all categories  
199 (statistics in Appendix B). Microbial community composition after sod-cutting showed a greater  
200 resemblance to agricultural sites, and after topsoil removal it ordinated closer to the heathlands. The  
201 fungal PLFA (18:2 $\omega$ 6,9) was positively correlated with the first discriminant, while several bacterial  
202 PLFA's (ai15:0, 16:1 $\omega$ 7t, c17:0, i17:0) showed a negative loading. The second linear discriminant  
203 separated the restored sites from older soils. Microbial community composition in all degrees of  
204 isolation differed significantly from each other on the first discriminant (Tukey test,  $p < 0.05$ ), with a  
205 negative loading of the fungal PLFA (18:2 $\omega$ 6,9) and a positive loading of several mainly bacterial  
206 PLFA's (15:0, i16:0, 18:0 isomer and 19:2). The second linear discriminant separated microbial

207 community composition of low-isolated sites from the other two categories (Tukey test,  $p < 0.05$ ),  
208 with a positive loading of the fungal PLFA and a negative loading of several bacterial PLFA's (ai15:0,  
209 i15:0, 16:1 $\omega$ 7t, ai17:0, i17:0 and c17:0).

210  
211 Bacterial and fungal content showed the same pattern as the PLFAs loadings on the first linear  
212 discriminant for restoration techniques (Figure 3). The fungal content was significantly higher in  
213 heathlands and after topsoil removal compared to the agricultural sites and sod-cutted areas (LME,  
214  $F_{3,73}$ : 28.35,  $p < 0.0001$ , Tukey test,  $p < 0.05$ ). The lowest bacterial content was found after topsoil  
215 removal, although these sites did not differ significantly from heathlands (LME,  $F_{3,73}$ : 16.38,  $p < 0.0001$ ,  
216 Tukey test,  $p < 0.05$ ). Bacterial content after sod-cutting was similar to both agricultural sites and  
217 heathlands (Tukey test,  $p < 0.05$ ). Although the analysis of previous land-use showed significant  
218 differences between agricultural sites, restored areas and heathlands, there were no significant  
219 differences in fungal or bacterial content between former arable fields and former meadows (Tukey  
220 test,  $p < 0.05$ ). In highly-isolated areas bacterial content was lower compared to non-isolated sites,  
221 while sites with low-isolation did not differ from both other categories (LME,  $F_{2,74}$ : 5.19,  $p$ : 0.0078,  
222 Tukey test,  $p < 0.05$ ). On the contrary, fungal content was significantly higher in highly-isolated sites  
223 compared to low- and non-isolated sites (LME,  $F_{2,74}$ : 9.99,  $p$ : 0.0001, Tukey test,  $p < 0.05$ )

224

### 225 *3.4 Vegetation*

226

227 Vegetation development showed a generally similar pattern to microbial community assembly,  
228 although differences between restoration techniques were less distinct and not significant (Figure 4).  
229 The saturation index differed significantly between agricultural sites and heathlands but not between  
230 both restoration techniques (LME,  $F_{3,7}$ : 5.19,  $p$ : 0.0337, Tukey test,  $p < 0.05$ ). Characteristic heathland  
231 species were absent in agricultural sites and after sod-cutting, while their presence was highly  
232 variable after topsoil removal. Three out of five sites after topsoil removal had a similar number of  
233 characteristic species as heathlands, while in the other two sites these species were still absent. The  
234 absolute cover of characteristic heathland species showed a similar pattern (LME,  $F_{3,7}$ : 37.83,  
235  $p$ : 0.0001), with a significant higher cover of heathland species after topsoil removal compared to  
236 sod-cutting, but still significantly lower compared to heathlands (Tukey test,  $p < 0.05$ ). The absolute  
237 cover of agricultural species showed the opposite pattern, although differences between both  
238 restoration techniques were not significant (LME,  $F_{3,7}$ : 7.03,  $p$ : 0.0161, Tukey test,  $p < 0.05$ ). A DCA of  
239 vegetation composition showed a clear separation between agricultural sites and heathlands on the  
240 first axis (LME,  $F_{3,7}$ : 21.37,  $p$ : 0.0007, Tukey test,  $p < 0.05$ , Figure 5). Although highly variable,  
241 vegetation composition after topsoil removal differed significantly from both agricultural sites and  
242 heathlands (Tukey test,  $p < 0.05$ ). Vegetation composition after sod-cutting did not differ significantly  
243 from agricultural sites.

244

### 245 *3.5 Parallel above-below-ground development*

246

247 Both above- and below-ground distinct differences in community composition related to restoration  
248 technique were found on the first axis of the multivariate analysis. A combination of the first LDA-  
249 axis of vegetation and microbial community composition shows a pattern of increasing resemblance  
250 to heathlands (Figure 6). With sod-cutting the resemblance only slightly increased below-ground,  
251 while vegetation composition remained in the same domain of the ordination for the agricultural  
252 context. After topsoil removal below-ground resemblance to heathlands increased in all sites  
253 irrespective of highly variable above-ground development. After both techniques microbial  
254 community composition showed a greater resemblance to heathlands than vegetation composition,  
255 and seemed to precede vegetation development.

256

## 257 **4 Discussion**

258

#### 259 4.1 Effects of soil nutrient pools

260

261 Differences in nitrogen availability had a pronounced effect on microbial community assembly,  
262 especially with respect to fungi. Fungal content was higher at low nitrogen availability and low at  
263 higher nitrogen levels, which is similar to the pattern observed in nitrogen addition studies (Treseder  
264 2008, Wei et al. 2013, Liu et al. 2014, Freedman et al. 2015). Bacterial content, however, showed the  
265 opposite pattern, with higher content at high nitrogen availability. This resulted in a low  
266 fungal/bacterial ratio after sod-cutting and a high fungal/bacterial ratio after topsoil removal,  
267 reflecting characteristic differences between fertile and oligotrophic systems (Wardle et al. 2004,  
268 Harris 2009). The expected negative effects of high nitrogen deposition levels on fungi (Treseder  
269 2008, Farrer et al. 2013, Wei et al. 2013) did not prevent the development of a fungal-dominated  
270 community, apparently soil nitrogen pool size was still the dominant factor for microbial community  
271 assembly. Soil nitrogen pools after topsoil removal lie within the range of the target system in  
272 comparison to the meta-analysis of De Graaf et al. (2009). After sod-cutting nitrogen availability was  
273 much higher than the maximum range for heathlands. Phosphorus pools after both restoration  
274 techniques were still larger than the upper bounds for heathland habitats (De Graaf et al. 2009),  
275 leading to conditions where oligotrophic systems can only be supported after topsoil removal  
276 because of highly reduced nitrogen soil pools. N:P ratios in the vegetation after topsoil removal  
277 ranged from 3.8 to 8.5 (van Diggelen, unpublished data), which suggests that productivity is limited  
278 by nitrogen (Koerselman & Meuleman 1996). Despite high levels of nitrogen deposition, soil nitrogen  
279 pools remained still low in the first decades, maintaining suitable conditions for oligotrophic systems.  
280 However, optimal conditions for oligotrophic system development might change after a few decades.  
281 Constant high levels of nitrogen deposition may lead to increased nitrogen availability, which in  
282 combination with the large phosphorus pools increases productivity and favours a shift towards  
283 more eutrophic species (Duprè et al. 2010, Maskell et al. 2010). This shift could be enhanced by  
284 indirect effects of high nitrogen deposition levels, such as weakening the interaction between  
285 mycorrhiza and host plants (Treseder 2008). The establishment of an interaction between heather  
286 (*Calluna vulgaris*) and ericoid mycorrhiza is considered essential in heathland restoration (Read et al.  
287 2004, Diaz et al. 2008).

288

#### 289 4.2 Impact of cultural legacy and isolation

290

291 Several studies have reported differences in microbial community composition between arable fields  
292 and agricultural meadows (Francisco et al. 2016, Griffiths et al. 2016). Interestingly, we found no such  
293 differences in microbial community composition between former arable fields and former meadows:  
294 none of the most differentiating PLFAs (Francisco et al. 2016) differed significantly between both  
295 categories. Similar to reduced soil fauna densities after sod-cutting and topsoil removal (Frouz et al.  
296 2009), most of the original microbial biomass was also removed after application of these  
297 techniques. Apparently the cultivation legacy is most prominent in the upper soil layer, and is  
298 removed with the application of both restoration techniques.

299

300 Characteristic plant species often have difficulties to reach highly-isolated sites, leading to  
301 differences in vegetation composition between isolated and well-connected sites (Cramer et al. 2008,  
302 Myers & Harms 2009). Remarkably, isolated microbial communities from highly-isolated sites  
303 differed less from heathlands in fungal/bacterial ratio than those in low- or non-isolated sites. Higher  
304 initial availability of organic material might promote fungal establishment more than bacteria, as  
305 fungi are more dependent on organic material availability (Schmidt et al. 2014). The effects of  
306 isolation on microbial community composition were independent from land-use category. This  
307 suggests that across radical different systems the relative contribution of agricultural sites and  
308 heathlands in the direct vicinity had a more profound effect on local microbial community  
309 composition than actual land-use.

310

#### 311 *4.3 Dependence of microbial community assembly on vegetation development*

312

313 Studies on simultaneous development of both vegetation and soil communities after land  
314 abandonment reported either similar trajectories of faster vegetation development (Jangid et al.  
315 2011, Lozano et al. 2014), while after topsoil removal soil community assembly lags behind  
316 vegetation development (Holtkamp et al. 2008, Frouz et al. 2009). Contrary to these studies, we  
317 found more pronounced patterns in microbial community assembly after the application of both  
318 restoration techniques. Microbial community composition after topsoil removal was more similar to  
319 heathlands, while vegetation composition was still highly variable. A similar pattern was found after  
320 sod-cutting, where vegetation composition remained very similar to agricultural meadows while  
321 microbial community composition already showed more resemblance to reference heathlands. Both  
322 techniques minimize above- and below-ground competition with removal of 1) the vegetation, 2) soil  
323 seedbank (Török et al. 2008) and 3) most of the soil community. The first phases in vegetation  
324 development are determined mainly by dispersal rates of immigrating species and seed pressure  
325 from remaining species (Myers & Harms 2009). Since the seedbank that remains contain mostly  
326 ruderal and agricultural species (Klimkowska et al. 2010), seed pressure of the latter species is  
327 presumably high in all restored areas. After shallow sod-cutting these common species can gain  
328 dominance fast, while they are almost absent after topsoil removal, leaving a ‘window of  
329 opportunity’ for oligotrophic target species to establish. Disturbances such as sod-cutting or topsoil  
330 removal increase the probability of dramatic shifts in microbial community composition, assumed to  
331 be caused either by selective pressures or neutral processes (Litchman 2010, Nemergut et al. 2013).  
332 Contrary to other studies (Holtkamp et al. 2008, Frouz et al. 2009, Jangid et al. 2011, Lozano et al.  
333 2014), microbial community assembly preceded vegetation development in the present situation.  
334 The clear effects of both soil nitrogen availability and regional species pool on microbial community  
335 assembly suggest that here interactions between the abiotic environment and the local microbial  
336 community play a determining role.

337

#### 338 *4.4 Implications for mitigating effects of nitrogen deposition*

339

340 Our results show that in former agricultural sites only topsoil removal can mitigate the effects of  
341 enhanced nitrogen availability sufficiently fast. When nitrogen availability in the soil is reduced,  
342 conditions are suitable for the development of characteristic communities both above- and below-  
343 ground, even under constantly high levels of nitrogen deposition. Vegetation development can be  
344 facilitated by enhancing dispersal via hay transfer (Kiehl et al. 2010, Klimkowska et al. 2010), while  
345 soil inoculation might enhance below-ground development (Wubs et al. 2016). Unfortunately, in the  
346 mid- to long-term the combination of a large phosphorus pool and a high nitrogen deposition likely  
347 will shift both above- and below-ground communities backwards towards a degraded state (Duprè et  
348 al. 2010, Maskell et al. 2010). With topsoil removal suitable starting conditions can be created, but  
349 under conditions of high nitrogen deposition management activities such as sod-cutting remain  
350 essential to conserve these systems in the mid to long term.

351

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353

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358

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524 **Tables and figures**

525

526 *Table 1. Description of the study sites with area, location, restoration technique, years since*  
 527 *restoration, previous land-use and degree of isolation.*

| Category | Restoration technique | Area        | Restoration period (years) | Previous land-use | Isolation | Latitude   | Longitude |
|----------|-----------------------|-------------|----------------------------|-------------------|-----------|------------|-----------|
| Start    | Agricultural          | Delleburen  | -                          | -                 | Not       | 52.957060° | 6.154318° |
| Start    | Agricultural          | Dwingeloo   | -                          | -                 | Not       | 52.808550° | 6.422350° |
| Start    | Agricultural          | Dwingeloo   | -                          | -                 | Not       | 52.799900° | 6.413317° |
| Start    | Agricultural          | Eexterveld  | -                          | -                 | Not       | 53.014232° | 6.708168° |
| R-SC     | Sod-cut               | Delleburen  | 10                         | Meadow (F-M)      | Not       | 52.957987° | 6.149869° |
| R-SC     | Sod-cut               | Eemboerveld | 12                         | Arable (F-A)      | Highly    | 53.017892° | 7.093543° |
| R-SC     | Sod-cut               | Eexterveld  | 9                          | Meadow (F-M)      | Low       | 53.015188° | 6.702981° |
| R-TR     | Topsoil removal       | Bakkeveen   | 13                         | Meadow (F-M)      | Low       | 53.081547° | 6.280386° |
| R-TR     | Topsoil removal       | Delleburen  | 10                         | Meadow (F-M)      | Not       | 52.958867° | 6.152861° |
| R-TR     | Topsoil removal       | Eexterveld  | 9                          | Meadow (F-M)      | Low       | 53.013391° | 6.702926° |
| R-TR     | Topsoil removal       | Ennemaborg  | 12                         | Arable (F-A)      | Highly    | 53.182255° | 7.004271° |
| R-TR     | Topsoil removal       | Tichelberg  | 23                         | Arable (F-A)      | Highly    | 53.022717° | 7.005042° |
| Target   | Heathland             | Appelbergen | -                          | -                 | Highly    | 53.137292° | 6.640562° |
| Target   | Heathland             | Delleburen  | -                          | -                 | Not       | 52.958914° | 6.145421° |
| Target   | Heathland             | Delleburen  | -                          | -                 | Not       | 52.962556° | 6.138043° |
| Target   | Heathland             | Dwingeloo   | -                          | -                 | Not       | 52.806733° | 6.405417° |
| Target   | Heathland             | Dwingeloo   | -                          | -                 | Not       | 52.789417° | 6.422683° |
| Target   | Heathland             | Eexterveld  | -                          | -                 | Highly    | 53.008915° | 6.701301° |

528

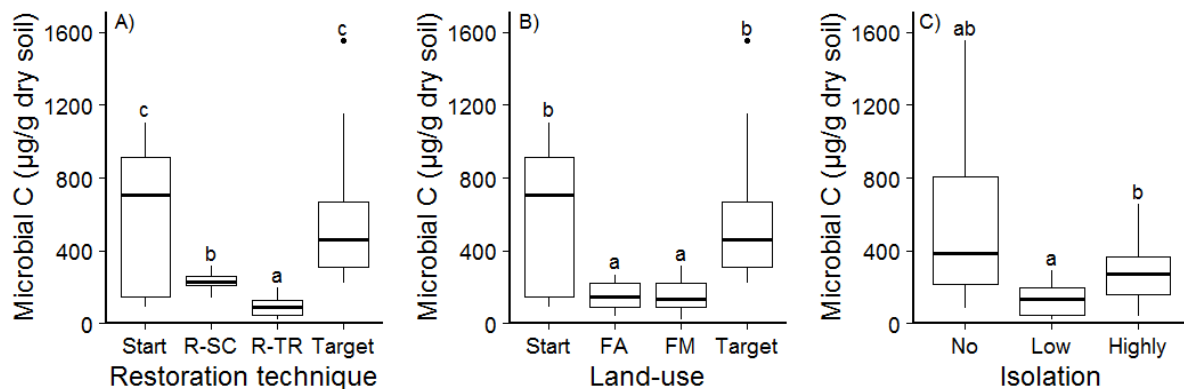
529

530 *Table 2. Soil chemistry of the study sites compared to values of meta-analyses for heathlands (De*  
 531 *Graaf et al. 2009, Liczner et al. 2011). Outcomes of a Tukey test are given between brackets, only the*  
 532 *study sites were included in the analysis.*

|   | Start (Agricultural) | R-SC (Sod-cut)  | R-TR (Topsoil removal) | Target (Heathlands) | Values meta-analysis heathlands |
|---|----------------------|-----------------|------------------------|---------------------|---------------------------------|
| <b>N<sub>total</sub> (g/100g soil)</b>  | 1.42±0.24 (a)        | 0.24±0.12 (b)   | 0.03±0.01 (c)          | 0.38±0.19 (bc)      | 0.02 (0.00-0.09)                |
| <b>P<sub>total</sub> (mg/100g soil)</b> | 22.09±3.76 (a)       | 25.68±10.32 (a) | 8.48±3.95 (a)          | 24.84±3.84 (a)      | 0.12 (0-0.90)                   |
| <b>pH (KCl)</b>                         | 4.73±0.32 (a)        | 5.20±0.75 (ab)  | 4.60±0.19 (ab)         | 3.50±0.32 (b)       | 4.3 (4.0-5.4)                   |

533

534



535

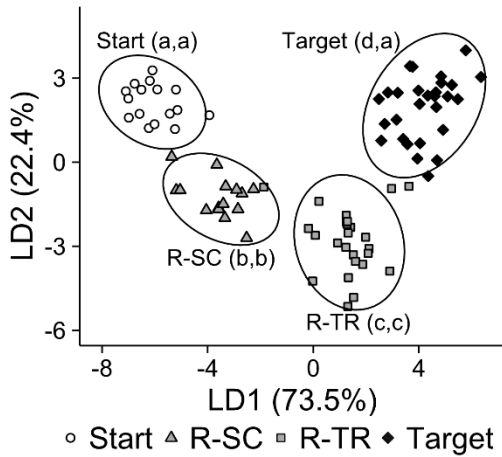
536 **Figure 1. The effects of restoration technique (A), land-use (B) and isolation (C) on microbial biomass.**  
 537 **Start: agricultural, R-SC: sod-cut, R-TR: topsoil removal, Target: heathlands, F-A: former arable and F-**  
 538 **M: former meadow. Boxplots show median, 1<sup>st</sup> and 3<sup>rd</sup> quartiles and 1.5\*IQR whiskers, the letters**  
 539 **indicate Tukey outcomes.**

540

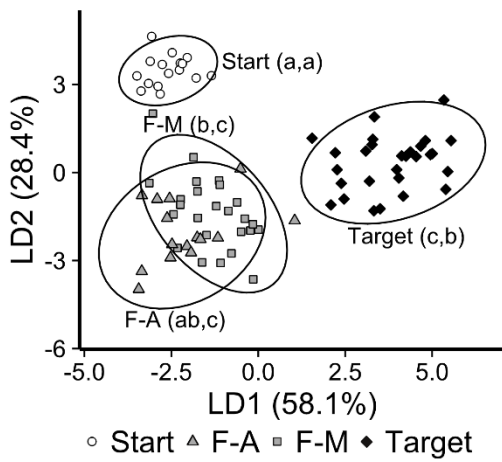
541 (note Figure 1: TIFF-format only preview, .EPS and .PDF source files included. Two-column graph)

542

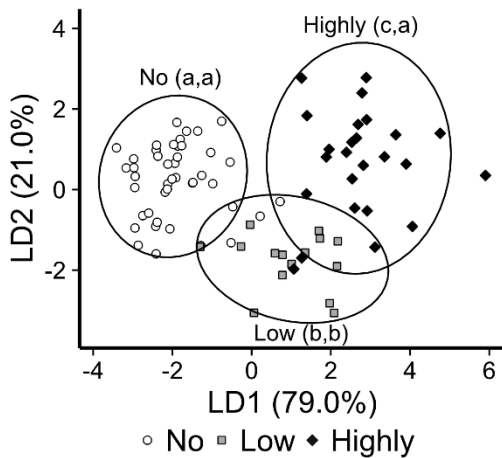
A) Restoration techniques



B) Land-use



C) Isolation



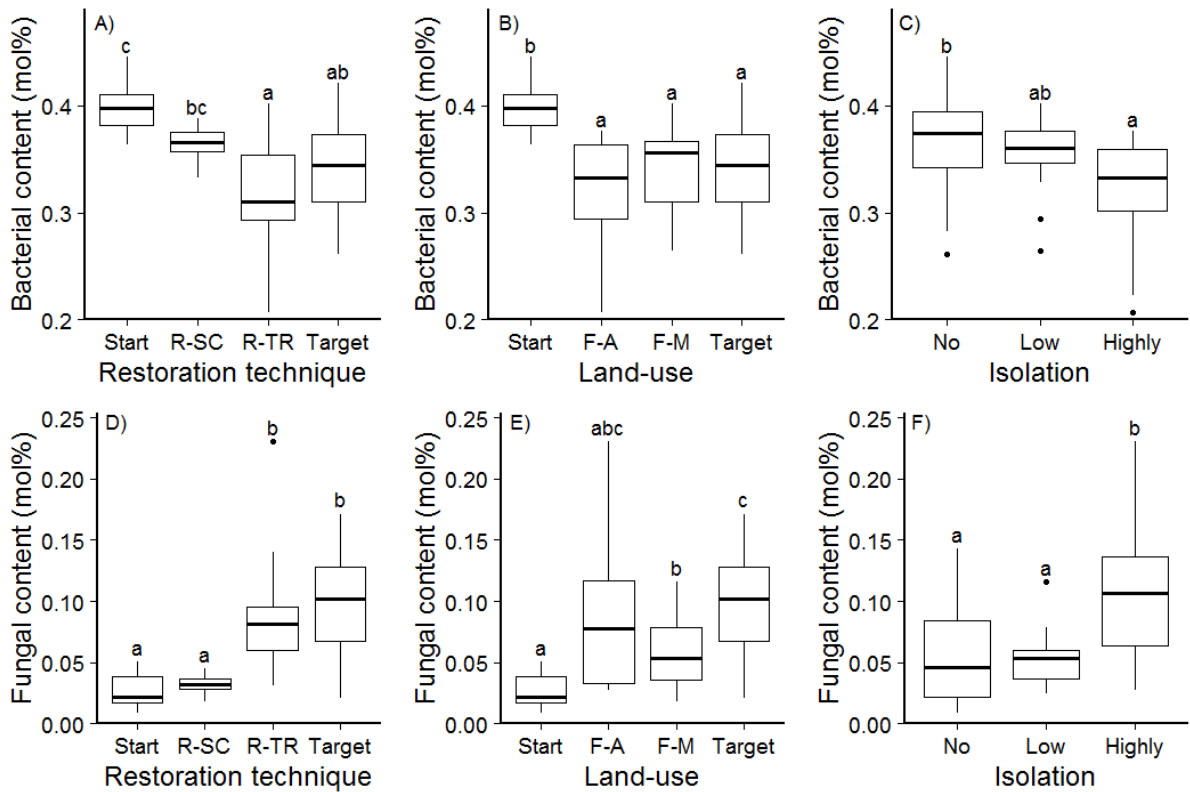
543

544 Figure 2. The first two linear discriminants of microbial community composition based on all PLFA's  
 545 for restoration technique (A), land-use (B) and isolation (C). Percentages view the amount of  
 546 variation explained by each axis. Tukey outcomes for LD1 and LD2 are given after each group  
 547 between brackets. Ellipses represent 95% confidence intervals. Start: agricultural, R-SC: sod-cut, R-  
 548 TR: topsoil removal, Target: heathlands, F-A: former arable and F-M: former meadow.

549

550 (note Figure 2: TIFF-format only preview, .EPS and .PDF source files included. One-column graph)

551



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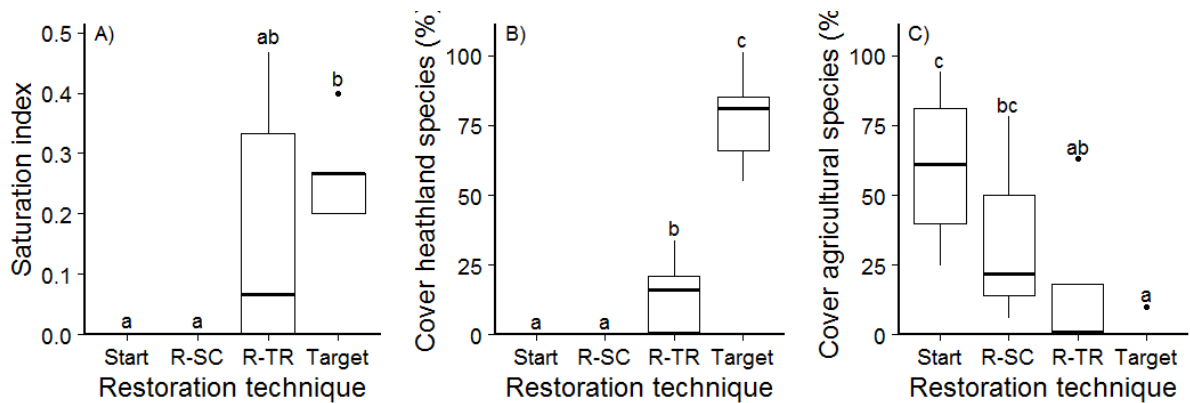
553 Figure 3. The contents of bacteria (A-C) and fungi (D-F) for restoration technique (A,D), land-use (B,E)  
 554 and isolation (C,F). Start: agricultural, R-SC: sod-cut, R-TR: topsoil removal, Target: heathlands, F-A:  
 555 former arable and F-M: former meadow. Boxplots show median, 1<sup>st</sup> and 3<sup>rd</sup> quartiles and 1.5\*IQR  
 556 whiskers, the letters indicate Tukey outcomes.

557

558 (note Figure 3: TIFF-format only preview, .EPS and .PDF source files included. Two-column graph)

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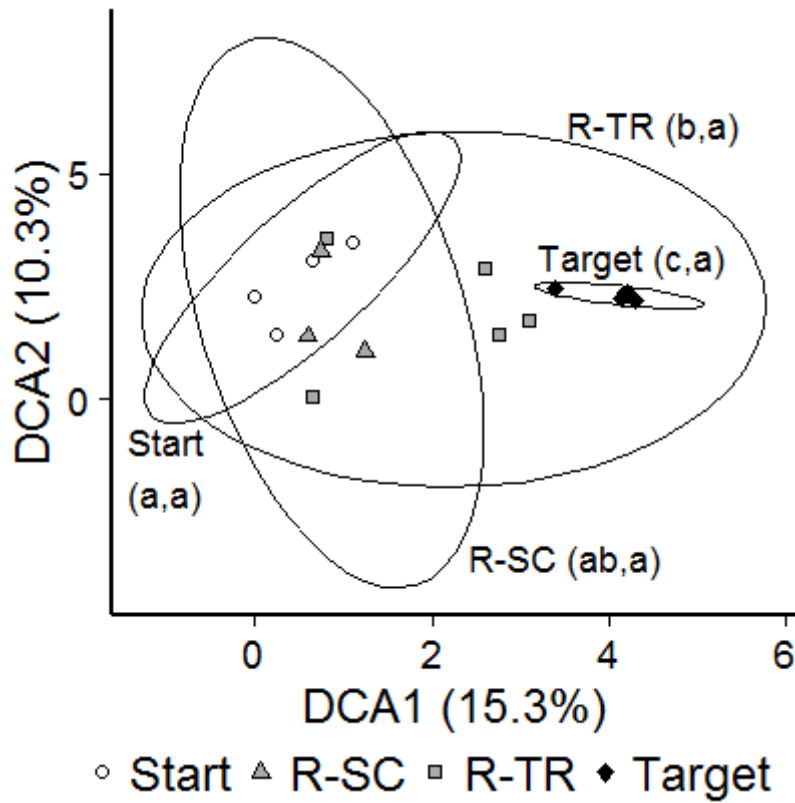
561

562 Figure 4. The effect of restoration technique on Saturation index (A), cover of characteristic  
 563 heathland species (B) and cover of agricultural species (C). Start: agricultural, R-SC: sod-cut, R-TR:  
 564 topsoil removal and Target: heathlands. Boxplots show median, 1<sup>st</sup> and 3<sup>rd</sup> quartiles and 1.5\*IQR  
 565 whiskers, the letters indicate Tukey outcomes.

566

567 (note Figure 4: TIFF-format only preview, .EPS and .PDF source files included. Two-column graph)

568



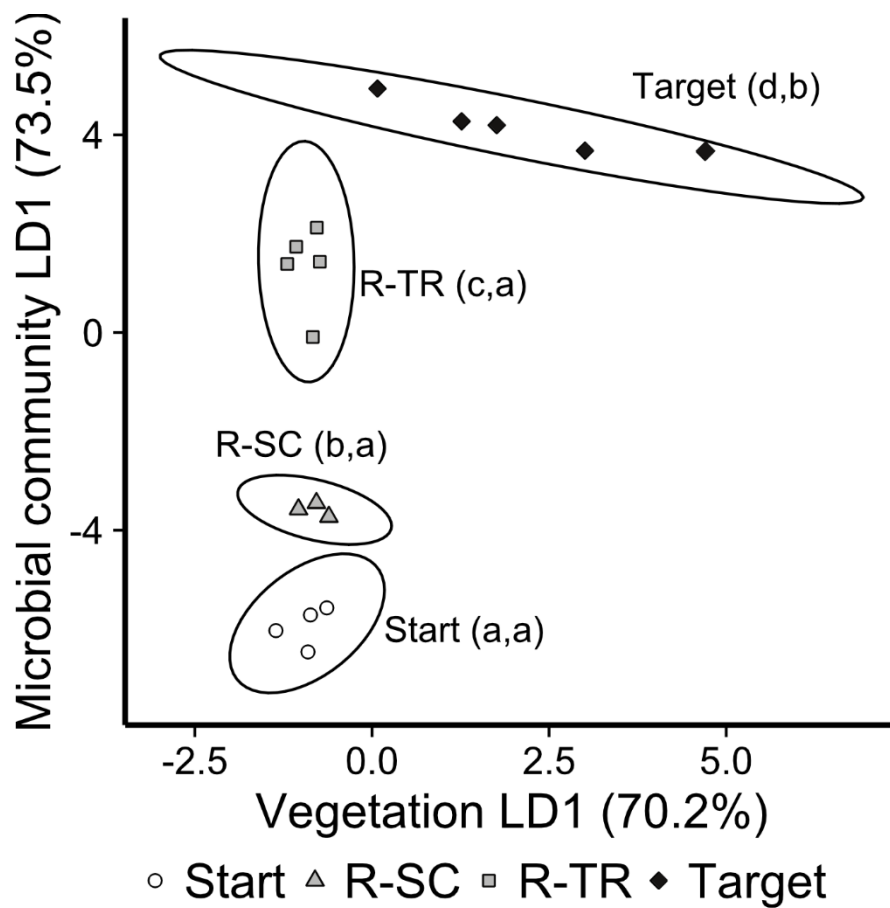
569

570 Figure 5. A Detrended Component Analysis (DCA) of the effects of restoration technique on vegetation  
 571 composition. Percentages view the amount of variation explained by each axis. Tukey outcomes for  
 572 DCA1 and DCA2 are given after each group between brackets. Ellipses represent 95% confidence  
 573 intervals. Start: agricultural, R-SC: sod-cut, R-TR: topsoil removal and Target: heathlands.

574

575 (note Figure 5: TIFF-format only preview, .EPS and .PDF source files included. One-column graph)

576



577

578 Figure 6. The first Linear Discriminant of vegetation composition versus the first Linear Discriminant  
 579 of microbial community composition for restoration techniques. Tukey outcomes for microbial  
 580 community and vegetation are given after each group between brackets. Ellipses represent 95%  
 581 confidence intervals. Start: agricultural, R-SC: sod-cut, R-TR: topsoil removal and Target: heathlands.  
 582

583 (note Figure 6: TIFF-format only preview, .EPS and .PDF source files included. One-column graph)  
 584



585 Appendix A

586

587 *Faithfulness values obtained from SynBioSys (Hennekens et al. 2010) of characteristic heathland*  
588 *species to the dry heath (Calluno-Ulicetea), wet heath (Erica tetralices) or Nardetea plant community*  
589 *observed in the vegetation relevés. Only species with a faithfulness higher than 20 were included.*

| <b>Species</b>                 | <b>Plant community</b> | <b>Faithfulness</b> |
|--------------------------------|------------------------|---------------------|
| <i>Calluna vulgaris</i>        | Calluno-Ulicetea       | 24.28               |
| <i>Carex oederi</i>            | Nardetea               | 21.97               |
| <i>Carex panicea</i>           | Nardetea               | 30.66               |
| <i>Carex pilulifera</i>        | Nardetea               | 23.31               |
| <i>Dactylorhiza maculata</i>   | Nardetea               | 42.86               |
| <i>Erica tetralix</i>          | Ericetum tetralicis    | 26.26               |
| <i>Festuca ovina</i>           | Nardetea               | 50.00               |
| <i>Galium saxatile</i>         | Nardetea               | 41.89               |
| <i>Genista anglica</i>         | Nardetea               | 36.22               |
| <i>Genista tinctoria</i>       | Calluno-Ulicetea       | 22.49               |
| <i>Juncus squarrosus</i>       | Nardetea               | 39.47               |
| <i>Luzula campestris</i>       | Nardetea               | 24.53               |
| <i>Nardus stricta</i>          | Nardetea               | 59.96               |
| <i>Potentilla erecta</i>       | Nardetea               | 33.73               |
| <i>Trichophorum cespitosum</i> | Nardetea               | 29.85               |

590

591

592 Appendix B

593

594 Statistics of all analysed parameters with application of ln(x+1) transformation, statistical test and

595 post-hoc Tukey test. Statistical tests: ANOVA: Analysis of Variance and LME: Linear Mixed-Effect

596 Model. Abbreviations factors: RT: restoration technique, PL: previous land-use and IS: isolation.

597

| Measurement                 | Parameter                     | ln(x+1)<br>trans-<br>for-<br>med | Statistical analysis |      |        | Tukey   |       |       |        |        |
|-----------------------------|-------------------------------|----------------------------------|----------------------|------|--------|---------|-------|-------|--------|--------|
|                             |                               |                                  | Test                 | df   | F      | p       | RT    | Start | R-SC   | R-TR   |
|                             |                               |                                  |                      |      |        | PL      | Start | F-A   | F-M    | Target |
|                             |                               |                                  |                      |      |        | IS      | No    | Low   | Highly |        |
| Soil chemistry<br>(only RT) | Ntotal                        | yes                              | LME                  | 3,4  | 40,80  | 0,0019  | a     | b     | c      | bc     |
|                             | Ptotal                        | yes                              | LME                  | 3,1  | 4,99   | 0,3154  | a     | a     | a      | a      |
|                             | pH (KCl)                      | no                               | LME                  | 3,5  | 7,38   | 0,0277  | a     | ab    | ab     | b      |
| Microbial<br>biomass        | Restoration technique         | yes                              | LME                  | 3,67 | 41,81  | <0,0001 | c     | b     | a      | c      |
|                             | Previous land-use             | yes                              | LME                  | 3,67 | 19,20  | <0,0001 | b     | a     | a      | b      |
|                             | Isolation                     | yes                              | LME                  | 2,68 | 8,32   | 0,0006  | ab    | a     | b      |        |
| PLFA-Total<br>bacteria      | Restoration technique         | yes                              | LME                  | 3,73 | 16,38  | <0,0001 | c     | bc    | a      | ab     |
|                             | Previous land-use             | yes                              | LME                  | 3,73 | 10,11  | <0,0001 | b     | a     | a      | a      |
|                             | Isolation                     | yes                              | LME                  | 2,74 | 5,19   | 0,0078  | b     | ab    | a      |        |
| PLFA-Fungi                  | Restoration technique         | yes                              | LME                  | 3,73 | 28,35  | <0,0001 | a     | a     | b      | b      |
|                             | Previous land-use             | yes                              | LME                  | 3,73 | 17,28  | <0,0001 | a     | abc   | b      | c      |
|                             | Isolation                     | yes                              | LME                  | 2,74 | 9,99   | 0,0001  | a     | a     | b      |        |
| PLFA-PCA                    | Restoration technique         | no                               | LME                  | 3,73 | 62,32  | <0,0001 |       |       |        |        |
|                             | Previous land-use             | no                               | LME                  | 3,73 | 49,56  | <0,0001 |       |       |        |        |
|                             | Isolation                     | no                               | LME                  | 2,74 | 12,17  | <0,0001 |       |       |        |        |
| PLFA-LDA                    | RT LD1                        | no                               | ANOVA                | 3    | 435,51 | <0,0001 | a     | b     | c      | d      |
|                             | RT LD2                        | no                               | ANOVA                | 3    | 132,68 | <0,0001 | a     | b     | c      | a      |
|                             | PL LD1                        | no                               | ANOVA                | 3    | 208,34 | <0,0001 | a     | ab    | b      | c      |
|                             | PL LD2                        | no                               | ANOVA                | 3    | 101,73 | <0,0001 | a     | c     | c      | b      |
|                             | IS LD1                        | no                               | ANOVA                | 2    | 180,84 | <0,0001 | a     | b     | c      |        |
|                             | IS LD2                        | no                               | ANOVA                | 2    | 30,67  | <0,0001 | a     | b     | a      |        |
|                             |                               |                                  |                      |      |        |         |       |       |        |        |
| Vegetation<br>(only RT)     | Saturation index              | yes                              | LME                  | 3,7  | 5,19   | 0,0337  | a     | a     | ab     | b      |
|                             | Cover heathland<br>species    | yes                              | LME                  | 3,7  | 37,83  | 0,0001  | a     | a     | b      | c      |
|                             | Cover agricultural<br>species | yes                              | LME                  | 3,7  | 7,03   | 0,0161  | c     | bc    | ab     | a      |
| Vegetation-<br>DCA RT       | RT DCA1                       | no                               | LME                  | 3,7  | 21,37  | 0,0007  | a     | ab    | b      | c      |
|                             | RT DCA2                       | no                               | LME                  | 3,7  | 0,57   | 0,6537  | a     | a     | a      | a      |
| Vegetation-<br>LDA RT       | RT LD1                        | no                               | ANOVA                | 3    | 11,06  | 0,0007  | a     | a     | a      | b      |
|                             | RT LD2                        | no                               | ANOVA                | 3    | 3,97   | 0,0327  | a     | ab    | b      | ab     |

598