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1 **Glacier forelands reveal fundamental plant and microbial controls on ecosystem**
2 **N retention**

3

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26 **Author contributions:** FTdV, RDB, CT, and JIP conceived the study. FTdV, RDB, WW, BF, MB
27 planned and performed field work. FTdV, CT, and HG performed laboratory analyses. FTdV, CT,
28 BBP, SC, GWN, analysed data, and FTdV, CT, JIP, and RdB wrote the manuscript with significant
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34

35 **Abstract**

36 The retention of nitrogen (N) is a crucial ecosystem function of both managed and natural
37 ecosystems, and there is a long history of experimental, observational, and conceptual studies
38 identifying its major controls. Yet, the plant and soil microbial controls on the retention of
39 added N remain elusive. Here, we used three ecosystem chronosequences in front of retreating
40 Alpine glaciers to test our hypothesis that the retention of reactive N increases with succession,
41 and to identify the plant and microbial controls of ecosystem N retention. We found that the
42 uptake and retention of N was not affected by succession, despite consistent changes in plant,
43 soil, and microbial properties with increasing time since deglaciation. Instead, we found that
44 plant and microbial properties that did not change with succession controlled ¹⁵N uptake and
45 retention: low root and microbial C/N ratios, as well as high root biomass, increased plant and
46 microbial uptake of added N. In addition, high soil concentrations of nitrate and ammonium
47 reduced the uptake of N in microbes and roots, respectively. These results indicate that plant
48 and microbial N demand, as well as N limitation, are driving ecosystem N retention in glacier
49 forelands. Our findings advance our understanding of the fundamental controls on ecosystem N
50 retention and the role of plant-microbial interactions in this process. This understanding is
51 crucial for predicting and mitigating the response of terrestrial ecosystems to the ever-
52 increasing amounts of reactive N in the biosphere.

53

54

55

56 **Significance statement**

57 Human activities have massively increased the amount of reactive nitrogen in the biosphere,
58 which is leading to increased N inputs in terrestrial ecosystems. This excess N can affect plant
59 and soil communities and reduce their diversity, affect the functioning of ecosystems, and
60 exacerbate climate change. The retention of N is an important ecosystem function, yet we have
61 little understanding of the role of plants and soil microbes in this process. We show that plants
62 and soil microbes together take up a significant part of added N, and that this uptake is
63 determined by their N demand and by the availability of soil N. These findings might help
64 predict and mitigate the effects of N enrichment of the terrestrial biosphere.

65

66

67 **Introduction**

68 Nitrogen (N) retention is a fundamental and important ecosystem function, particularly in the
69 face of global nitrogen enrichment. The ability of terrestrial ecosystems to retain reactive N is
70 an indicator of the extent to which they are N-limited, as well as being a key property of natural
71 and managed ecosystems, where it regulates N losses that cause eutrophication of aquatic
72 ecosystems and exacerbate climate change (1). Ecosystem N retention and its counterpart N
73 loss are regulated by both abiotic factors, such as parent material and climate, and biotic factors,
74 such as plant and soil microbial properties (2). While many empirical and theoretical studies
75 have examined the role of plant and soil properties, and their nutrient stoichiometry, in
76 regulating ecosystem N retention, our understanding of the underpinning mechanisms remains
77 elusive. In particular, despite widespread acknowledgement of the importance of soil microbes
78 in immobilising reactive N and reducing ecosystem N leaching losses, it is still unresolved which
79 soil microbial community attributes are responsible for regulating ecosystem N retention, and
80 how these are linked to plant communities and soil physicochemical properties (3).

81

82 Early studies on terrestrial ecosystem N retention demonstrated that old-growth forests have
83 much larger N-leaching losses than early-successional forests (4). This work led to the classic
84 conceptual model of Vitousek and Reiners, who proposed that actively growing plants in early
85 to mid-successional ecosystems have high rates of N uptake, and, as such, these systems have
86 high N retention (4). In contrast, late-successional systems, with slower plant growth and
87 biomass accumulation dominated by mature trees or plant communities consisting of slow-
88 growing species, are more 'leaky' of N. However, many observational studies do not support this
89 classic model (3, 5). Recent work for instance has shown that ecosystems can differ strongly in
90 their retention of reactive N and that soil, litter, and microbial biomass pools retain more N than
91 aboveground plant biomass, and are thus more important controls on ecosystem N retention
92 than plant N uptake (2). In particular, the accumulation of soil organic matter and higher C/N
93 ratios of soil and litter have been linked to greater ecosystem N retention through increased
94 microbial N immobilisation (3, 5, 6), although the microbial community attributes associated
95 with these changes are poorly understood.

96

97 Recent studies have demonstrated the importance of interactions between plants and soil
98 microbial communities in determining ecosystem N retention. For example, field studies in
99 temperate grasslands show that N retention is greatest, and N leaching losses lowest, in
100 grasslands characterised by plant communities dominated by slow-growing, nutrient-
101 conservative species, and soil microbial communities dominated by fungi (7-10). The general
102 mechanism underpinning this observation is that soils with low N availability select for N-

103 conservative plant species and fungal-dominated microbial communities (11), which compete
104 for and immobilise significant amounts of N, thereby enhancing N retention (7). Moreover, N-
105 conservative plant species and fungi have slower growth and lower turnover rates than fast-
106 growing plants and bacteria, and thus the N taken up in their tissues is retained for longer, again
107 reducing the availability and mineralisation of N in the soil (7). In contrast, controlled common
108 garden experiments have shown that fast-growing plant species and bacteria-dominated
109 microbial communities have the highest N uptake and retention (12-14), potentially indicating
110 that different mechanisms might underlie ecosystem N retention under controlled conditions
111 compared to field conditions. In particular, under field conditions, other factors than plant and
112 microbial N uptake rates might explain ecosystem N retention, such as high soil C stocks and
113 soil C/N ratios (15), with reduced availability of N for plant and microbial uptake. Under such
114 conditions, plants might employ other strategies to acquire their nitrogen, for example reducing
115 the availability of N by releasing polyphenols, and by taking up organic N forms directly or
116 through associations with mycorrhizal fungi (16, 17). Thus, the factors affecting ecosystem N
117 retention are complex and highly interlinked, which complicates identifying the plant and
118 microbial properties that underlie increased ecosystem N retention in the real world.

119

120 Glacier forelands are excellent model systems for studying how fundamental ecological
121 processes change during ecosystem succession, and studies on these systems have led to major
122 advances in our understanding of the role of plant-soil interactions in ecosystem development
123 (18-20). As glaciers retreat, they expose barren parent material, which is then colonised by
124 microbes and plants and undergoes primary succession, thus creating a successional gradient
125 with increasing distance from the glacier front. These space-for-time substitutions show
126 predictable changes with increasing distance from the glacier forefront: plant and soil microbial
127 biomass increase, soil organic C and N accumulate, and microbial communities become more
128 dominated by fungi (21-23). In line with the increase in soil C and N with age since deglaciation,
129 the abundance of N-cycling genes related to the decomposition of organic material (24) and
130 ammonia oxidation (25) increases, while that of genes involved in denitrification decreases (25,
131 26). Moreover, while vegetation in the early stages of glacier foreland sites is limited by N and in
132 later stages by P (18), findings on nutrient limitation of soil microbes are inconsistent, and seem
133 to depend on variations in soil properties within and between glacier forelands (27-29). Thus,
134 these systems provide a unique opportunity to study how ecosystem N retention changes
135 during primary succession, while at the same time displaying enough variation within
136 successional stages to identify the major plant and microbial controls of ecosystem N uptake
137 and retention (20, 30).

138

139 Here, we aimed to identify the fundamental plant and microbial controls on ecosystem N uptake
140 and retention using three distinct glacier forelands in the European Alps as model systems. We
141 hypothesised that ecosystem N retention increases with ecosystem succession as a result of
142 increased abundance of slow-growing, N conservative plants which associate with fungal-
143 dominated soil microbial communities, and the build-up of soil organic matter. Specifically, we
144 hypothesised that plant communities dominated by N-conservative species would select for N-
145 conservative microbial communities, which, together with an increase in soil C/N ratio, would
146 increase microbial immobilisation of N and decrease N leaching losses. We also identified
147 changes in taxonomic and functional microbial community composition associated with this
148 hypothesised increase in microbial N immobilisation. In particular, we assessed changes in the
149 abundance of soil fungi, bacteria and ammonia-oxidising archaea and bacteria, which play a key
150 role in nitrification. This was achieved by collecting intact soil cores from five locations in each
151 of five successional stages (16, 38, 60, 112, and 157 years old) and one reference site
152 (deglaciated around 2,000 years ago) in three well-documented, geographically distinct glacier
153 forelands in the European Alps: the Damma Glacier in Switzerland (21), and the
154 Ödenwinkelkees and Rotmoosferner glaciers in Austria (31) (see Fig. S1 for an overview of
155 sampling scheme, sites, and site characteristics). A stable isotope approach was used to trace
156 the fate and retention of added N in these intact soil cores: we added the equivalent of 20 kg N
157 ha⁻¹ (as a 0.05 M ¹⁵NH₄¹⁵NO₃ solution) to each core and assessed the effect of soil age on the
158 uptake and retention of ¹⁵N in soil, roots, shoots, and microbial biomass and the amount leached
159 after a simulated rainfall event (30 mm) 48 h after ¹⁵N addition (7). Detailed analyses of soil,
160 vegetation, and microbial community properties of paired soil samples were used to explain
161 ecosystem N uptake and retention (i.e. the total amount of ¹⁵N retained in soil, plant, and
162 microbial pools after a rainfall event) across glaciers and soil age. This approach enabled us to
163 identify the fundamental plant and microbial drivers of ecosystem N retention, and how these
164 change with ecosystem succession in glacier forelands.

165

166 **Results**

167 Soil and plant properties

168 Measured soil properties changed predictably across the three glacier forelands. Soil pH
169 decreased with soil age, while total soil C and N stocks and dissolved organic C (DOC)
170 concentration increased (Table 1, Table S1). In contrast, soil C/N ratio and the availability of
171 inorganic (NH₄⁺ and NO₃⁻) and organic (DON) N forms did not change significantly with soil age.
172 Of the plant properties measured, only root N increased significantly with soil age. Other than
173 soil pH, which was overall lowest in the Damma and highest in the Rotmoosferner glacier

174 foreland, none of the soil and plant properties differed between the three glacier forelands
175 (Table 1, Table S1, S2).

176

177 Microbial communities

178 Microbial community properties showed strong and consistent changes across primary
179 succession gradients. Microbial biomass C and N, the total amount of soil DNA, bacterial (16S
180 rRNA), fungal (18S rRNA), and ammonia-oxidising archaea (AOA) gene abundances (assessed
181 using qPCR) all increased with soil age (Table 1, Table S3, S4). While the fungal-to-bacterial
182 gene abundance ratio did not change, the ratio of ammonia-oxidising archaea to ammonia-
183 oxidising bacteria (AOB) increased with soil age. In addition to measuring changes in gene
184 abundance, we used amplicon sequencing of the 16S rRNA gene to assess taxonomic changes in
185 bacterial community composition, and used the resulting taxonomic assignments to predict
186 abundances of bacterial traits and assess changes in bacterial functional community
187 composition, using the beta version of the R package Tax4Fun (32). In contrast to the absolute
188 abundance of AOA genes, predicted relative abundances of ammonia-oxidising and ammonia-
189 transporter genes did not increase with soil age (Fig. S2). Predicted relative abundances of
190 genes associated with free-living and symbiotic N-fixation genes and chitin degradation
191 increased with soil age (Fig. S2). Because our qPCR analysis of AOB gene abundances (see
192 Methods) resulted in many missing values, and because AOB gene abundances were positively
193 correlated with predicted ammonia-oxidising gene abundances and negatively correlated with
194 ammonia-transporter gene abundance ($P < 0.0001$ and R-squared = 0.316, and $P = 0.018$ and R-
195 squared = 0.119, respectively, Fig. S3), we used these predicted gene abundances for our follow-
196 up models (see Methods).

197

198 Both taxonomic and functional bacterial community composition changed with soil age and
199 patterns were similar across the three glaciers (Fig. 1). We compared the variance explained in
200 taxonomic and functional community composition through running a PCA on arcsine-
201 transformed relative abundances of bacterial OTUs and KEGG orthologs. The first two axes of PCA
202 for functional community composition explained more variance than those for taxonomic
203 community composition – 46.5% vs. 13.8% – and soil age, glacier, and their interaction all
204 explained more variance in functional community composition than in taxonomic community
205 composition (Fig. 1). The PCA of functional community composition clearly discriminated young
206 soils from the oldest for both Damma and Ödenwinkelkees glaciers. The functional profile also
207 changed with soil age in the Rotmoosferner glacier foreland, but here discrimination was less
208 marked. Damma and Ödenwinkelkees microbial communities followed very similar shifts in
209 taxonomic composition with soil age, converging to similar structures in the oldest soils, while

210 the Rotmoosferner community was clearly distinct from the other two glacier forelands (Fig. 1).
211 Despite these clear changes in microbial community structure, neither Shannon diversity nor the
212 Gini coefficient of taxonomic bacterial community composition changed with soil age, and OTU
213 richness increased with soil age only in the Rotmoosferner glacier foreland (Fig. S4). The relative
214 abundance of several phyla changed significantly with soil age (Fig. S5). In particular, the relative
215 abundance of *Proteobacteria* and *Actinobacteria* decreased with soil age, while that of
216 *Acidobacteria*, *Planctomycetes*, *Verrucomicrobia* and *Chloroflexi* increased.

217

218 ¹⁵N uptake, retention, and leaching

219 Roots and microbial biomass were the ecosystem N pools that took up and retained the largest
220 amount of added ¹⁵N; across all soil ages and glaciers they retained on average 0.56 g and 0.62 g
221 ¹⁵N m⁻², respectively, or 28% and 31% of the ¹⁵N added (Fig. 2). These amounts varied strongly
222 across soil age and glacier, though the apparent increase in ¹⁵N uptake by roots with soil age
223 was not significant ($P = 0.094$; Fig. 2). Similarly, microbial ¹⁵N uptake and shoot ¹⁵N uptake were
224 not affected by soil age, although the latter tended to decrease with soil age ($P = 0.079$; Fig. 2).
225 On average, 0.13 g, or 6%, of the added ¹⁵N leached from the soil with the simulated rainfall
226 event, but the amount of ¹⁵N leached did not change with ecosystem succession. As a result,
227 ecosystem ¹⁵N retention was not affected by soil age.

228

229 Despite the absence of an effect of soil age on ecosystem ¹⁵N uptake and retention, a significant
230 part of the variation in ¹⁵N uptake in plant and microbial pools was explained by ecosystem
231 properties that did change with soil age. Microbial ¹⁵N uptake increased with a larger microbial
232 biomass N pool and with decreasing microbial C/N ratio (Table 2). In addition, microbial ¹⁵N
233 uptake decreased with greater soil nitrate concentrations. The model for shoot ¹⁵N uptake
234 explained more variation than the model for microbial ¹⁵N uptake (40% vs. 28%); shoot ¹⁵N
235 uptake increased with a larger shoot N pool and decreased with soil total N. The model for root
236 ¹⁵N uptake included more variables and explained the largest amount of variation (48%) of ¹⁵N
237 uptake in biotic ecosystem pools. Root ¹⁵N uptake increased with higher root C/N ratio and root
238 biomass, but the interaction between the two meant that high root C/N ratios increased root ¹⁵N
239 uptake at low root biomass and decreased root ¹⁵N uptake at high root biomass (Fig. S6). In
240 addition, root ¹⁵N uptake decreased with increasing soil ammonium concentrations. Total
241 ecosystem ¹⁵N retention increased with increasing microbial biomass N and was also explained
242 by PC2-axis scores of functional bacterial community composition; this model explained 25% of
243 variation. Finally, 32% of the variation in ¹⁵N leaching was explained by a combination of soil
244 C/N ratio and taxonomic and functional bacterial community composition, where leaching
245 increased in soils with increasing soil C/N ratio.

246

247 The volume leached from the intact soil cores increased slightly with higher soil moisture
248 content and the amount of ^{15}N leached weakly increased with the volume leached ($P = 0.013$
249 and $R\text{-squared} = 0.068$ and $P = 0.063$ and $R\text{-squared} = 0.041$, respectively; Fig. S7). However, the
250 amount of ^{15}N leached decreased when soil moisture was higher ($P = 0.004$ and $R\text{-squared} =$
251 0.095 , Fig. S7). The total recovery of ^{15}N in plants, soil, and leachates together was variable and
252 not affected by soil age and glacier, and, in general, did not amount to the full 2 g m^{-2} of ^{15}N
253 added (Fig. S8). The average recovery of added ^{15}N was 78.4 ± 29.9 (mean \pm SD) % across all soil
254 ages and glaciers, and therefore, for ^{15}N tracer experiments, relatively complete.

255

256 ^{15}N enrichment of ecosystem N pools

257 Similar to total ^{15}N uptake in ecosystem N pools, ^{15}N enrichment (i.e. the percentage of ^{15}N
258 enrichment in each ecosystem N pool, which is independent of the size of the pool) of ecosystem
259 N pools varied little with soil age. Neither microbial, root, nor shoot ^{15}N enrichment changed
260 with successional stage (Fig. S9). In contrast, soil ^{15}N enrichment decreased with soil age across
261 the foreland of all three glaciers. Microbial ^{15}N enrichment increased with decreasing microbial
262 biomass C/N ratio and with a decreasing predicted relative abundance of ammonia oxidisers
263 (Table S5). The model for ^{15}N enrichment of shoot N only explained 8.3% of the variation and
264 showed that shoot ^{15}N enrichment increased with higher root C/N ratio. In contrast, 68% and
265 69% of variation in root and soil ^{15}N enrichment was explained by the selected models,
266 respectively. ^{15}N enrichment of the root N pool decreased with a larger root N pool size and
267 increasing root biomass. Similar to total root ^{15}N uptake, root ^{15}N enrichment also decreased
268 when soil ammonium concentrations increased. Soil ^{15}N enrichment decreased with an
269 increasing size of the soil N pool.

270

271 Direct and indirect controls on plant and microbial ^{15}N uptake and ^{15}N leaching

272 Structural equation modelling (SEM) is a robust statistical method to test how well
273 experimental data fit a hypothesised causal structure that is based on prior knowledge (33). To
274 test our hypothesis that N-conservative plants (with high tissue C/N ratio) select for N-
275 conservative microbial communities (with high microbial biomass C/N ratio), which would in
276 turn increase microbial immobilisation, we fitted our data to a previously hypothesised and
277 tested SEM (14). Importantly, in a previous common garden experiment, this SEM showed that
278 low microbial biomass C/N ratios increased microbial N immobilisation, which contrasts with
279 our current hypothesis (14). The data fitted this model well and confirmed that plant C/N ratio
280 and root biomass determine plant and microbial ^{15}N uptake and leaching (Fig. 4). Shoot C/N
281 ratio, root C/N ratio, and root biomass were all correlated. Roots with a high tissue C/N ratio

282 were linked to microbial communities with a high C/N ratio, which, in contrast to our
283 hypothesis, in turn immobilised *less* ¹⁵N. In addition, high root biomass increased plant ¹⁵N
284 uptake, which decreased ¹⁵N leaching. ¹⁵N leaching was only controlled by plant ¹⁵N uptake, not
285 by microbial ¹⁵N immobilisation.

286

287 **Discussion**

288 Using the forelands of three geographically distinct retreating glaciers in the European Alps as a
289 model system, we were able to disentangle the plant and microbial properties that regulate
290 ecosystem N uptake and retention during ecosystem succession. Many ecosystem properties
291 changed in a predictable way with soil age across the three glaciers but, in contrast to our
292 hypothesis, neither microbial or plant ¹⁵N uptake, nor ecosystem ¹⁵N retention increased with
293 soil age. However, total ecosystem ¹⁵N retention and microbial ¹⁵N immobilisation, the second
294 largest sink for added ¹⁵N, increased with a greater microbial biomass N pool, which itself
295 increased with soil age across all three glacier forelands. Plant ¹⁵N uptake, rather than microbial
296 ¹⁵N uptake, decreased the amount of ¹⁵N lost via leaching and, in contrast to our expectation, ¹⁵N
297 leaching increased with higher soil C/N ratios. Moreover, we found that microbial ¹⁵N uptake, a
298 measure of microbial immobilisation of N, was primarily determined by the C/N stoichiometry
299 and biomass of soil microbial communities, with only a minor role for changes in taxonomic or
300 functional composition of bacterial communities.

301

302 Both taxonomic and functional bacterial community composition, and many soil microbial
303 community properties known to influence soil N availability, were strongly affected by soil age.
304 The AOA:AOB gene abundance ratio increased with soil age, as previously reported in a salt
305 marsh successional gradient (34), possibly as a consequence of a preference of AOA for acidic
306 soils (35). Of the predicted relative abundances of genes involved in the N cycle, only symbiotic
307 and free-living N fixation increased with soil age, which is in line with legumes being more
308 abundant at late-successional stages (Fig. S1). These findings again indicate increased N
309 limitation with succession (36, but see 37). However, in contrast to our expectation, the ratio of
310 fungi-to-bacteria, which has been shown to increase during ecosystem succession (22), and has
311 been linked to increased microbial N immobilisation (7), did not increase. Moreover, none of
312 these microbial community properties explained microbial ¹⁵N immobilisation or the ¹⁵N
313 enrichment of microbial biomass. Only the predicted relative abundance of ammonia-oxidisers,
314 which did not change with ecosystem succession, explained microbial ¹⁵N enrichment; ¹⁵N
315 enrichment of microbial biomass N decreased with an increasing abundance of ammonia-
316 oxidisers, suggesting that their abundance is indicative for microbial communities with low N
317 affinity. While we found a strong correlation between measured AOB gene abundances (which

318 had too many missing values to use in our predictive models) and predicted ammonia-oxidiser
319 abundance, functional gene inference by tools like Tax4Fun that we used here has limited
320 reliability in environmental samples (38). Still, taken together, these findings are in line with
321 our observation that microbial ^{15}N immobilisation and ^{15}N enrichment did not increase during
322 ecosystem succession.

323

324 Rather than taxonomic and functional microbial community properties, whole-community
325 stoichiometry explained microbial uptake and enrichment of ^{15}N , irrespective of glacier foreland
326 and soil age. Microbial ^{15}N uptake increased with a greater microbial biomass N pool and with
327 lower microbial biomass C/N ratio, indicating that microbial communities with high N demand
328 immobilised most added ^{15}N . This finding is consistent with observations in controlled
329 experiments (12, 14), but contradicts field observations that fungal-dominated microbial
330 communities of high C/N ratio immobilise more added N than bacterial-dominated microbial
331 communities of low C/N ratio (7, 39). And while soil C stocks or C/N ratios did not explain
332 microbial ^{15}N immobilisation, microbial ^{15}N immobilisation decreased with increasing soil
333 nitrate concentrations, indicating that N limitation did drive microbial ^{15}N uptake (Table 2). A
334 similar pattern was found for root and shoot ^{15}N uptake. Shoot ^{15}N uptake increased with a
335 larger shoot N pool, and root ^{15}N uptake was highest with high root biomass and decreased with
336 higher soil ammonium concentrations, again indicating that ^{15}N uptake was driven both by N
337 demand and N limitation. This was confirmed by our structural equation model, which showed
338 that across these distinctly different glacier forelands, ecosystem N uptake was determined by
339 plant and microbial N demand. Thus, our results indicate that high microbial N immobilisation
340 by fungal-dominated microbial communities of high C/N ratio (7, 39) are a result of ecosystem
341 N limitation, rather than of an innate high affinity of fungi for N.

342

343 Despite the finding that both plant and microbial ^{15}N uptake were driven by their N demand, we
344 found no evidence for competition between plants and microbes for ^{15}N , as indicated by the lack
345 of a significant negative effect of microbial ^{15}N uptake on plant ^{15}N uptake (Fig. 4). Microbes
346 have been shown to be stronger competitors for N than plant roots in N-limited systems on
347 short time scales (10, 40), and it has been suggested that through selecting for specific soil
348 microbial communities, plants can alter the microbial sink strength for N (41). We did indeed
349 find that high root C/N ratios, indicative of conservative plant nutrient acquisition strategies
350 (Fig. S4), were associated with microbial communities with a high biomass C/N ratio that,
351 however, immobilised less ^{15}N . Still, high root C/N ratios were only linked to reduced root N
352 uptake when root biomass was high, which suggests that when both root and microbial C/N
353 ratios were low, plants might have increased their N uptake through investing more in root

354 biomass. In alpine, N-limited systems like the glacier forelands studied here, the preferential
355 uptake of different forms of N can alleviate N competition between plants and microbes. This
356 chemical niche partitioning has primarily been found between inorganic and organic N forms,
357 but also between various forms of organic N (amino acids) (10, 40, 42). Our results point to a
358 partitioning of the uptake of inorganic N forms: high soil concentrations of nitrate were
359 associated with lower microbial ¹⁵N uptake, while high soil ammonium concentrations were
360 associated with lower root ¹⁵N uptake, respectively. This finding suggests that microbes were
361 limited by nitrate and plants by ammonium, thus when more of these forms were present,
362 microbial and root uptake of added ¹⁵N was reduced. This is consistent with the knowledge that
363 subalpine grassland plants, especially more conservative species, have higher ammonium than
364 nitrate uptake rates, (43), and suggests coordination between plant and microbial N uptake
365 strategies beyond broad-scale tissue N requirements, as has been found before (44, 45).

366

367 As expected, roots and microbes together accounted for the largest proportion of ¹⁵N retention.
368 After rapid initial uptake by microbes, N is gradually released through microbial turnover and
369 taken up by roots, while root N is translocated to aboveground biomass (7, 40), and eventually
370 transferred to soil organic matter pools via shoot, root, and microbial turnover (46). Despite
371 these shifts in ecosystem N allocation, the total amount of N retained in plants and microbes has
372 been shown to remain constant for two months after ¹⁵N addition, indicating that initial
373 ecosystem N retention is indicative of longer-term N retention (6). The average total amount of
374 ¹⁵N retained in our systems was relatively high (73%) compared to N retention in temperate
375 grasslands (2, 7, 47), and comparable to shrubland N retention (2). However, it is important to
376 note that we only collected intact soil cores that included plants, whereas plant cover was
377 sparse during early successional stages. Thus, our method will have resulted in an
378 overestimation of ecosystem N retention per square meter in the field, in particular for the most
379 recently deglaciated soils. Because of the large contribution of plant and microbial ¹⁵N uptake to
380 ecosystem N retention, it is therefore plausible that whole-ecosystem N retention increases with
381 succession.

382

383 Our study identifies fundamental plant and microbial controls on ecosystem N retention, and
384 establishes that plant and microbial N demand, as well as N limitation, are driving ecosystem N
385 retention in glacier forelands. Importantly, these findings are in line with experimental studies
386 in grasslands and forests, as well as with recent adaptations to Vitousek and Reiners conceptual
387 model of nutrient retention (3-5). While increasing (predicted) abundances of free-living and
388 symbiotic N fixation genes indicated increasing N limitation with soil age, we did not find an
389 increase in N retention with ecosystem succession, likely because the main determinants of

390 plant and microbial ^{15}N uptake were not affected by soil age. Our findings highlight the
391 importance of plant and microbial N uptake for ecosystem N retention and advance our
392 understanding of the fundamental controls on ecosystem N retention. This understanding is
393 crucial for predicting the effects of increasing atmospheric deposition of reactive N on
394 terrestrial ecosystems, as well as for increasing the retention of N in natural and managed
395 ecosystems.

396

397 **Materials and Methods**

398 Sample collection

399 Soil was collected in geographically distinct glacier forelands located in Ödenwinkelkees
400 (Austria, 47°12' E, 12°64' N, 2,060-2,161 m altitude), Rotmoosferner (Austria, 46°83' E, 11°04'
401 N, >2,296 m altitude) and Damma (Switzerland, 46°64' E, 8°46' N, 1,939-2,050 m altitude).
402 Mean annual temperatures are 0.2 ± 0.6 °C, -1.0 ± 0.5 °C and 2.2 ± 0.5 °C and mean annual
403 precipitation $2,258 \pm 665$ mm, 904 ± 150 mm and $2,400 \pm 385$ mm for the Ödenwinkelkees and
404 Rotmoosferner (both: mean of 2000-2017), and Damma, respectively. Intact soil cores (6.4 cm
405 diam., 15 cm depth, vegetation intact, bottom covered with mesh) and adjacent vegetation and
406 soil samples ('field soil' hereafter, 15 cm depth) were collected from five transects at each site,
407 at distances from the glacier forefront corresponding to an average time since deglaciation of
408 16, 38, 60, 112 and 157 years, as well as from plots located in each glacial valley but outside the
409 foreland *sensu stricto*, and deglaciated more than 1,000 year ago (reference soils). This resulted
410 in five replicates for each of five successional sites, plus one reference site for each glacier, i.e.
411 90 sample units. Soils (not intact cores) were sieved to 4 mm, shipped and stored at 4 °C until
412 further analysis. Intact cores and collected vegetation were immediately shipped and stored
413 under the same conditions (see below) until the start of the ^{15}N addition experiment and
414 vegetation sample processing.

415

416 ^{15}N addition experiment

417 After shipping to The University of Manchester, intact cores (with vegetation intact) were
418 placed in controlled growth chambers (Percival USA, 9 °C, which is the average September
419 temperature at the sites, 8 h/16 h day/night cycle, at constant moisture content) in a
420 randomised block design and left to acclimatise for one week. Thereafter 4 x 1 ml of a 0.05 M
421 98.2% enriched $^{15}\text{NH}_4^{15}\text{NO}_3$ solution was injected in the top 5 cm of each soil core in 4 evenly
422 spaced locations (total of 6.43 mg ^{15}N per core, equivalent to 20 kg N ha⁻¹ or 2 g m⁻²). Forty-eight
423 hours after ^{15}N injection, the cores were leached with 97 ml demineralised water (equivalent to
424 a 30 mm rainfall event). Past work has shown that the amount retained in the system 48 h after
425 ^{15}N addition is a good indicator of longer-term ecosystem N retention (7). After leaching, the

426 cores were dismantled, and soil and aboveground and belowground vegetation were separated,
427 and the volume of leachates recorded.

428

429 Leachate, soil, and vegetation analyses

430 Gravimetric soil moisture contents of core and field soils were determined by drying for 24 h at
431 105 °C, and for the core soils with recorded weight and volume of stones larger than 4 mm, used
432 to determine core bulk density and fine soil (all material <4 mm) content. Leachates and field
433 soil were analysed for dissolved inorganic and organic nitrogen (DON) and dissolved organic C
434 (DOC) as described in De Vries *et al.* (7). Microbial biomass C and N in field soil and column soil
435 were determined by chloroform-fumigation extraction as described by Brookes *et al.* (48). Root
436 and shoot biomass from field samples and intact cores were separated, weighed, dried (60°C for
437 48 h), weighed again and analysed for total C and N on an Elementar Vario EL elemental
438 analyser. Dried and ground field and core soil samples were also analysed for total C and N on
439 the Elementar Vario EL elemental analyser. Leachates and microbial extracts from soil cores
440 (enriched in ¹⁵N) and field soil (natural abundance of ¹⁵N) were freeze-dried, and, with dried
441 and ground root, shoot, and soil material from both cores and field samples, analysed for
442 ¹⁵N/¹⁴N ratios at the Life Sciences Mass Spectrometry Facility, Lancaster node, CEH Lancaster.
443 Enough sample to provide as near as possible to 84 µg elemental nitrogen was weighed using a
444 high precision micro-balance, (Sartorius Ltd) and then sealed into a 6 x 4 mm tin capsule.
445 Samples were then combusted using an automated Carlo Erba NA1500 elemental analyser
446 (Carlo erba, part of Thermo Scientific, USA) coupled to a Dennis Leigh Technology Isotope Ratio
447 Mass-Spectrometer (Dennis Leigh Technologies, Sandbach, Cheshire, UK.). A working standard
448 of n-carbobenzyloxy-l-aspartic acid (CBA - Sigma-Aldrich Co. Ltd.) which is calibrated against
449 the certified reference material IAEA-N1 (Ammonium sulphate – NIST number 8547, Bureau of
450 Analysed Samples Ltd, Middlesbrough, UK) was analysed after every 12th sample resulting in an
451 analytical precision of 0.63‰ for the natural abundance samples. Results are expressed in δ
452 notation; i.e. $\delta^{15}\text{N} = [(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] \times 1000$ (‰) where R is the ratio of ¹⁵N to ¹⁴N in
453 the sample and standard, accordingly. All δ¹⁵N results are expressed relative to the international
454 standard of atmospheric air. δ¹⁵N values from ¹⁵N enriched (core soil) and natural abundance
455 (field soil) samples were then used to calculate ¹⁵N excess atom percent values in the ¹⁵N
456 enriched samples, after which total amounts of ¹⁵N in ecosystem pools (microbial biomass,
457 shoots, roots, soil, and leachates) were calculated using their total N pool sizes, which were then
458 scaled to g m⁻² using the bulk density and the surface area of the cores. ¹⁵N retention was
459 calculated as the sum of ¹⁵N in soil, microbial biomass, and aboveground and belowground
460 vegetation.

461

462 DNA extraction, qPCR, preparation of amplicons, and sequencing

463 Nucleic acids were extracted from 0.5 g field soil as previously described (49) with
464 modifications (50) and raw extracts were stored at -20 °C. The abundances of fungi, bacteria
465 and ammonia oxidising archaea and bacteria were estimated by real-time quantitative PCR
466 (qPCR). Fungal abundance was assessed using 18S ribosomal gene primers (Fung5F and
467 FF390R, Lueders et al. 2004) (51) rather than the ITS region to avoid potential bias from the
468 variable length of ITS amplicons. Bacterial abundance was assessed using 16S rRNA gene
469 primers 968F/1401R (52). Archaeal and bacterial ammonia oxidiser abundances were assessed
470 using *amoA* primers Crenamo23F/Crenamo616R (53) and amo1F/amo2R (54), respectively.
471 Illumina paired-end sequencing of the hypervariable V3-V4 region of bacterial 16S rRNA gene
472 amplicons was performed at the Centre for Genome-Enabled Biology and Medicine (Aberdeen,
473 UK). DNA quantity, measured fluorometrically using the Qubit dsDNA BR Assay Kit
474 (ThermoFisher, UK), increased with soil age and varied from 1.3 to 190 ng g⁻¹ soil (Fig. S1). We
475 removed samples with less than 5 ng DNA; for each of the remaining samples, PCR (25 cycles
476 each) was performed using 5 ng template DNA, using the forward and reverse primer sequences
477 5'-CCTACGGGNGGCWGCAG-3' (Bakt_341F) and 5'-GACTACHVGGGTATCTAATCC-3' (Bakt_805R)
478 (55) and Illumina overhang adapters 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3' and
479 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3', respectively. Replicate amplification
480 products were pooled and processed with AMPure purification system (Agencourt Bioscience
481 Corporation, US). Pure pooled amplicons were then indexed with 8 PCR cycles and fragment
482 size determined on a 2200 TapeStation (Agilent, Germany). Equimolar concentrations of
483 samples were pooled and sequenced on an Illumina MiSeq sequencer with V2 2x150 bp paired-
484 end chemistry including PhiX Control V3 (Illumina, US) and generated 7,576,266 quality-
485 checked reads.

486

487 Sequence cleaning and clustering

488 Sequence analysis was performed using a custom pipeline. Briefly, Illumina-generated forward
489 and reverse reads were first paired using the *PANDaseq* Assembler and trimmed to remove
490 primer sequences (56). Trimmed assembled sequences were dereplicated by *de novo* clustering
491 at 97% similarity (57) and remaining singletons (1 read in the total dataset) were removed.
492 This generated 14,622 OTUs, with an average of 68,000 reads per sample. Average read number
493 increased significantly with soil age (from 38,909 ± 7,129 to 76,545 ± 9,350). Read numbers in 5
494 samples of young soils were very low (<4,632) and were discarded from the analysis. A
495 reference V3-V4 database was generated by performing *in silico* PCR on the SILVA 115 database
496 with the same set of primers using the *TaxMan* webserver (58). Double-annotated sequences
497 and sequences with no species annotation were removed from the database, leading to a

498 reference sequence set containing 172,576 unique, fully annotated sequences. Taxonomic
499 assignment was achieved by clustering the 14,622 representative sequences against the
500 reference database using the *Uclust* algorithm (57) in combination with a custom Linux script
501 considering all possible hits at 97% similarity, dividing the number of reads of each OTU in a
502 sample by the number of potential hit sequences, to distribute the uncertainty of the annotation.
503 OTU number was used as measure of bacterial richness and computed by custom R scripts using
504 the *vegan* (59) and *ineq* (60) packages.

505

506 Inference of the functional profile using Tax4Fun

507 We selected 46 functional traits, grouped into 7 functional categories and 16 functions, related
508 to trophic niche, growth strategy, versatility, adaptation to abiotic stress and biotic interactions
509 (Table S6). For each trait, 1 to 50 functional indicators (i.e. enzymes catalysing that particular
510 functional trait) were selected, based on literature review and exploration of web-based KEGG
511 and MetaCyc resources (versions available online in May 2017) [55, 56]. Finally, for each
512 indicator, 1 to 56 KEGG ortholog genes, encoding that particular indicator, were listed for 1,849
513 KEGG orthologs (Table S7). The known biological functions of peptides and enzymes encoded
514 by each of these KEGG proxies was examined individually. KEGG pathways often include
515 orthologs that are not directly linked to the function to which they refer; e.g. the KEGG Quorum
516 Sensing pathway contains many genes that actually encode chemotaxis or pathogen virulence.
517 In addition, individual KEGG pathways related to organic compound metabolism, often contain
518 both synthesis and degradation enzymes, which were discriminated in the present analysis.

519

520 Functional profiles, consisting of the relative abundance of KEGG orthologs, were inferred for
521 each sample from the processed 16S rRNA amplicon sequences, using the beta version of the R
522 package Tax4Fun (<http://tax4fun.gobics.de/>), which uses available genome annotation of the
523 nearest neighbour of each OTU, based on 16S rRNA gene sequence similarity in the SILVA
524 database (32). Tax4Fun used $73.1 \pm 0.1\%$ of the nucleotide sequences within a sample for
525 prediction (i.e. $26.9 \pm 0.1\%$ of the OTUs were not used for functional inference).

526

527 Statistical analyses

528 All variables were scaled to g m^{-2} using the bulk density, stone content, and area of the intact
529 cores. All statistical analyses were done in R version 3.5.2 (61) and all data were checked for
530 normality and log-transformed where necessary. The effect of individual glacier and time since
531 deglaciation (soil age, this excluded the never-glaciated Reference sites), and their interaction,
532 on soil, vegetation, microbial properties, and ^{15}N pools was tested using linear mixed effects
533 models with an error term for block to account for the non-independence of the 5 replicate

534 samples per soil age. This was done using the function `lme` in the R package *nlme* (62). To
535 compare the drivers of taxonomic and functional community composition, principal component
536 analyses (PCA) of inferred Tax4Fun KEGG functional and taxonomic profiles were followed by
537 between class analysis (BCA), calculating the between- and within-group inertia percentages
538 and testing the significance of between- and within-group variances using a Monte-Carlo test
539 with 999 iterations using R package *ade4* (63). PC scores of taxonomic and functional bacterial
540 community composition were used in subsequent analyses to explain ¹⁵N uptake and retention
541 in ecosystem N pools.

542

543 We used a manual model selection procedure, again using the `lme` function in *nlme*, to identify
544 the main drivers of ¹⁵N uptake in, and enrichment of, individual ecosystem N pools, entering
545 terms individually first, retaining significant terms, and testing them in combination and in
546 interaction, following De Vries et al. (11). In these models, an error term was included where
547 soil age (including Reference sites) was nested in glacier. Model selection was done based on
548 AIC using maximum likelihood estimation and the final most parsimonious model was run using
549 restricted maximum likelihood estimation. Finally, structural equation modelling was done
550 using the package *piecewiseSEM* (64), and regressions included an error term of soil age
551 (including Reference sites) nested in glacier (as above). Model fit was assessed using Fisher's C
552 statistic.

553

554 **Data Availability**

555 Data and code are available from [10.6084/m9.figshare.13913024](https://doi.org/10.6084/m9.figshare.13913024). This Targeted Locus Study
556 project has been deposited at DDBJ/ENA/GenBank under the accession KCJZ00000000. The
557 version described in this paper is the first version, KCJZ01000000.

558

559

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- 734
- 735

736 **Tables and figures**

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738 **Table 1.** ANOVA table of lme models for the effect of soil age, glacier, and their interaction,
 739 on soil, plant and microbial properties. Significant effects ($P < 0.05$) are highlighted in bold,
 740 significant trends ($P > 0.1$) in italics.

	<i>Soil age</i>		Direction of change with soil age	<i>Glacier</i>		<i>Glacier x soil age</i>	
	F _{1,9}	P		F _{2,9}	P	F _{2,9}	P
Soil moisture (g g ⁻¹)	0.96	0.353		1.04	0.391	0.62	0.559
Soil pH	21.75	<0.0001	↓	65.04	<0.0001	2.69	0.121
Soil C (g m ⁻²)	16.18	0.003	↑	<i>4.12</i>	<i>0.054</i>	2.15	0.172
Soil N (g m ⁻²)	31.30	0.0003	↑	1.74	0.229	1.20	0.345
Soil C/N	0.76	0.405		1.51	0.273	0.31	0.739
NH ₄ ⁺ concentration (g m ⁻²)	0.66	0.439		3.98	0.058	1.58	0.258
NO ₃ ⁻ concentration (g m ⁻²)	1.67	0.229		1.89	0.207	1.68	0.240
DON concentration (g m ⁻²)	1.33	0.279		0.04	0.957	1.21	0.341
DOC concentration (g m ⁻²)	15.86	0.003	↑	0.41	0.675	0.12	0.884
Shoot biomass (g m ⁻²)	3.15	0.110		1.09	0.377	1.27	0.327
Root biomass (g m ⁻²)	3.51	0.093	(↑)	2.17	0.170	0.31	0.738
Shoot N (g m ⁻²)	1.39	0.267		0.45	0.652	1.49	0.276
Root N (g m ⁻²)	10.10	0.011	↑	2.12	0.176	1.90	0.205
Shoot C/N	0.75	0.409		0.22	0.808	0.74	0.505
Root C/N	1.17	0.307		2.32	0.153	0.28	0.760
Microbial biomass C (g m ⁻²)	25.39	0.0007	↑	1.70	0.236	0.05	0.946
Microbial biomass N (g m ⁻²)	10.46	0.010	↑	1.97	0.196	0.04	0.965
Microbial C/N	1.39	0.267		1.15	0.358	0.24	0.793
DNA (ng m ⁻²)	6.57	0.031	↑	0.60	0.571	0.26	0.778
16S (gene copies m ⁻²)	20.78	0.001	↑	0.09	0.910	0.13	0.880
18S (gene copies m ⁻²)	18.98	0.002	↑	0.64	0.548	0.28	0.765
18S/16S	1.39	0.269		6.02	0.022	2.30	0.156
AOA (gene copies m ⁻²)	48.21	0.0001	↑	7.94	0.010	<i>3.70</i>	<i>0.067</i>
AOB (gene copies m ⁻²)	2.58	0.147		15.02	0.002	0.90	0.446
AOA/AOB	14.41	0.009	↑	0.21	0.819	0.37	0.707

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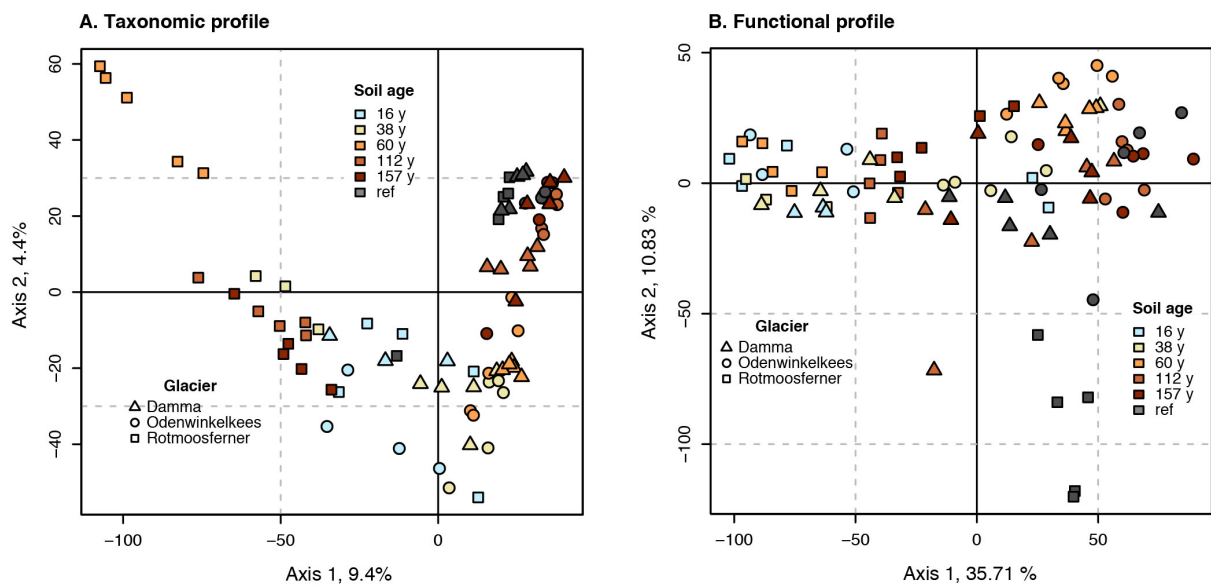
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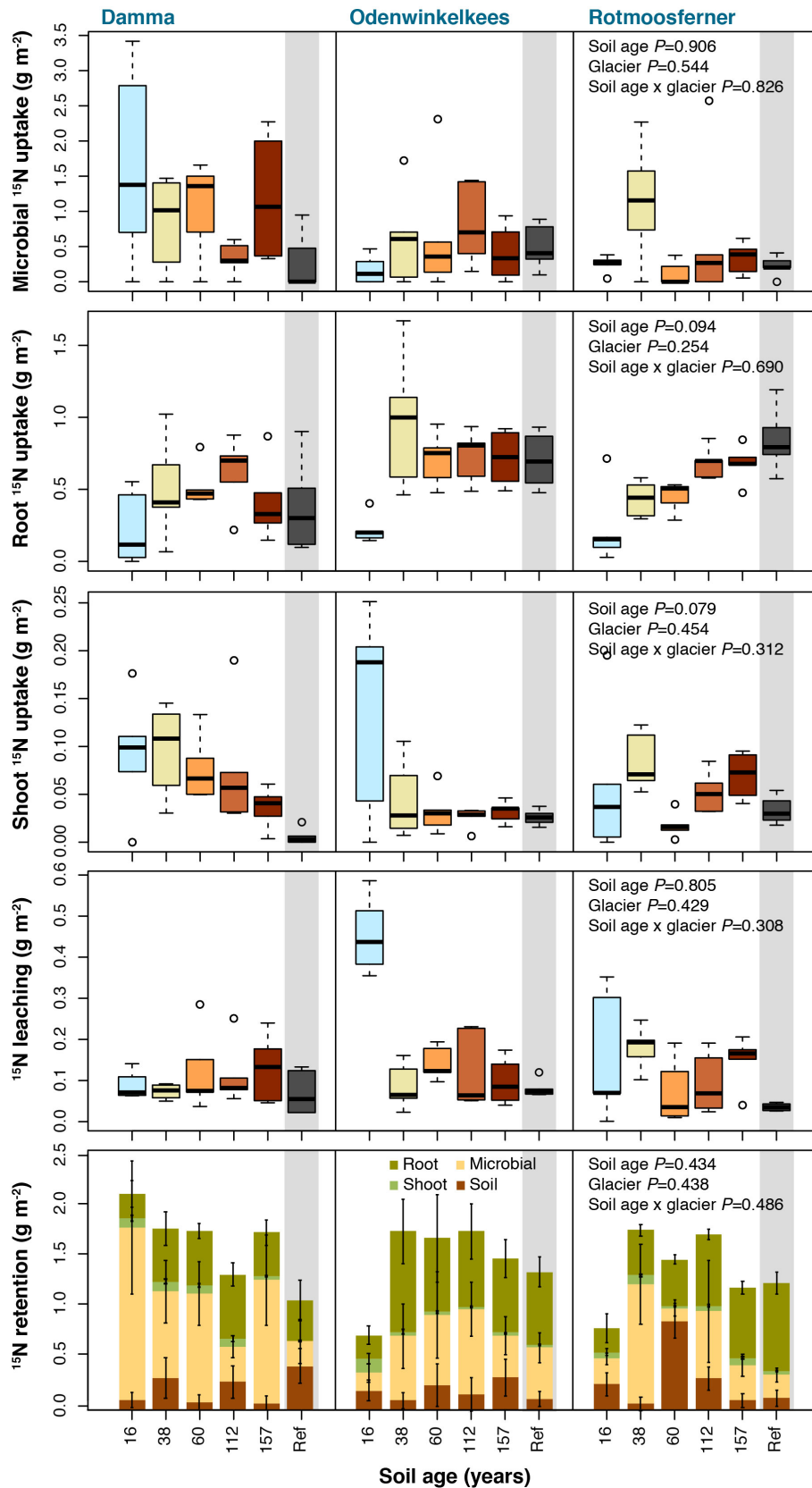


C. Variance explained by soil age and/or glacier (%)

	Taxonomy	Function
Soil age	12	23
Glacier	8	14
Soil age x glacier	40	59

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Fig. 1. PCA of taxonomic (A) and functional (B) microbial community composition as affected by soil age and glacier.



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Fig. 2. Uptake, leaching, and retention of ¹⁵N 48h after addition in different ecosystem N pools as affected by soil age and glacier.

Table 2. Selected linear mixed effects models for ¹⁵N uptake, retention, and leaching. All biomass, C, and N parameters are expressed in g m⁻².

	<i>In Microbial ¹⁵N uptake (g m⁻²)</i>		<i>In Shoot ¹⁵N uptake (g m⁻²)</i>		<i>Root ¹⁵N uptake (g m⁻²)</i>		<i>¹⁵N retention (g m⁻²)</i>		<i>In ¹⁵N leaching (g m⁻²)</i>	
	Parameter value	<i>P</i>	Parameter value	<i>P</i>	Parameter value	<i>P</i>	Parameter value	<i>P</i>	Parameter value	<i>P</i>
Intercept	+0.143	0.138	+0.129	<0.0001	-0.053	<0.0001	+0.948	<0.0001	-3.355	<0.0001
Soil C and N pools			-0.028 * ln Soil N	<0.0001					+0.316 * ln Soil C/N	0.017
Microbial C and N pools	+0.056 * Micr N -0.076 * ln Micr C/N	<0.0001 0.0033					+0.088 * Micr N	<0.0001		
Plant C and N pools			+0.028 * ln Shoot N	0.006	+0.011 * Root C/N +0.0005 * Root biom -0.000007 * Root C/N * Root biom	<0.0001 <0.0001 0.0007				
Soil dissolved C and N pools	-0.127 * ln NO ₃ ⁻	0.0031			-0.282 * ln NH ₄ ⁺	0.0077				
Microbial community properties							+0.007 * Func PC2	0.013	+0.068 * Tax PC2 +0.008 * Func PC2 -0.026 * Tax PC2 * ln Soil C/N	0.008 0.080 0.006
df	59		65		68		61		59	
AIC	50.4		-286.9		57.5		157.5		246.4	
R-squared	0.280		0.401		0.481		0.248		0.318	

Abbreviations: Micr N – microbial biomass N; Micr C/N – microbial biomass C/N ratio; Func PC2 – PCA axis 2 scores for functional microbial community composition; Tax PC2 – PCA axis 2 scores for taxonomic microbial community composition

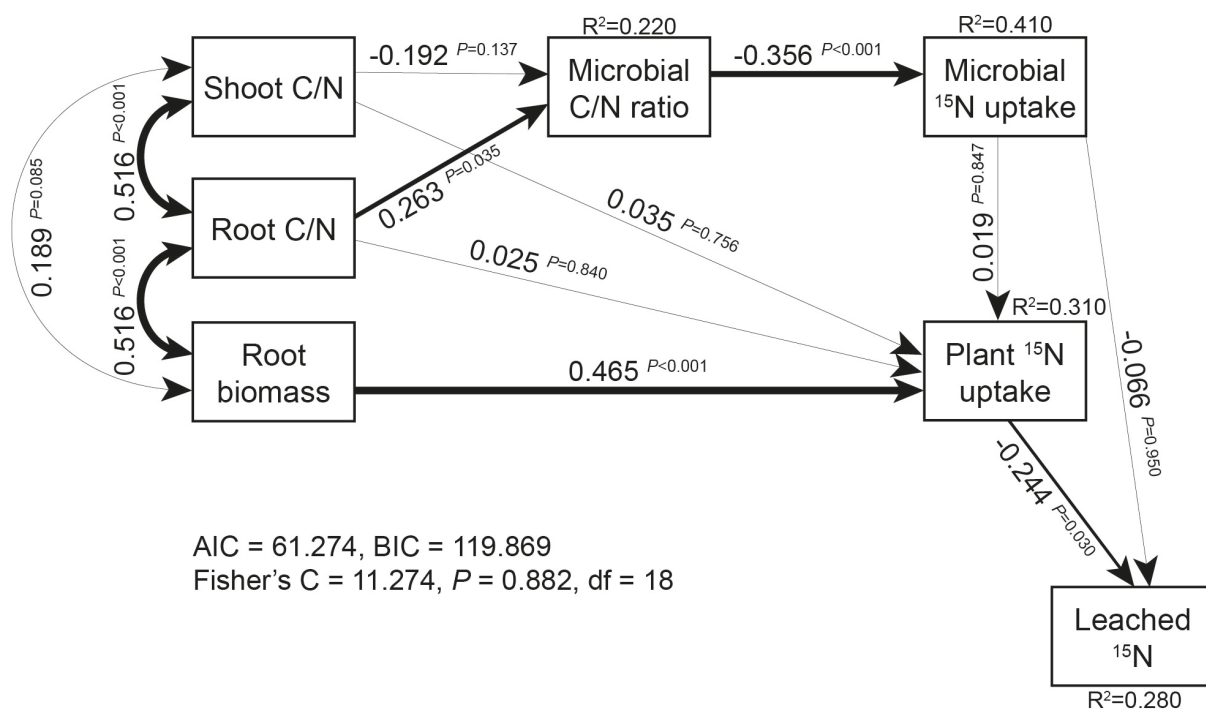


Fig. 3. Structural equation model explaining plant and microbial ^{15}N uptake and ^{15}N leaching across all glaciers and soil ages.