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1 Letter

## 2 Soil inoculation steers restoration of terrestrial ecosystems

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4

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10

11 **Many natural ecosystems have been degraded due to human activities<sup>1,2</sup> and need to be restored**  
12 **in order to protect biodiversity. However, restoration can take decades and restoration activities**  
13 **are often unsuccessful<sup>3</sup> because of abiotic constraints (e.g. eutrophication, acidification) and**  
14 **unfavourable biotic conditions (e.g. competition or adverse soil community composition). A key**  
15 **question is what manageable factors prevent transition from degraded to restored ecosystems and**  
16 **what interventions are required for successful restoration<sup>2,4</sup>. Experiments have shown that the soil**  
17 **community is an important driver of plant community development<sup>5-8</sup>, suggesting that**  
18 **manipulation of the soil community is key to successful restoration of terrestrial ecosystems<sup>3,9</sup>.**  
19 **Here we examine a large-scale, six-year old field experiment on ex-arable land and show that**  
20 **application of soil inocula not only promotes ecosystem restoration, but that different origins of**  
21 **soil inocula can steer the plant community development towards different target communities,**  
22 **varying from grassland to heathland vegetation. The impact of soil inoculation on plant and soil**  
23 **community composition was most pronounced when the topsoil layer was removed, whereas**  
24 **effects were less strong, but still significant, when the soil inocula were introduced into intact**  
25 **topsoil. Therefore, soil inoculation is a powerful tool to both restore disturbed terrestrial**  
26 **ecosystems and steer plant community development.**

27

28 Plants and their associated soil communities are tightly inter-linked and influence each other during  
29 successional changes in developing ecosystems<sup>10,11</sup>. Several studies with grassland plant communities  
30 have shown that inoculation with late-successional soil communities can increase the performance of  
31 late-successional target plant species, at the expense of early-successional ruderals<sup>6-8,11</sup>. This  
32 suggests that through inoculation with late-successional soils the typically positive feedback<sup>6,11</sup>  
33 between late-successional plants and their soil organisms may be restored<sup>9</sup>. However, two important  
34 aspects of plant-soil community interactions have remained unexplored: first it has not yet been  
35 studied to what extent soil communities may also steer the direction of plant community  
36 development towards different target states, and second, how soil legacies affect soil inoculation  
37 success in the field is unknown. We define steering of community development as the ability to  
38 direct the species composition of communities towards different target states when starting from  
39 the same environmental conditions.

40

41 Intensive arable farming reduces diversity and simplifies food webs of soil communities<sup>4,12</sup>, and  
42 creates legacy effects in soil that can benefit weedy plant species for a long time<sup>4,13</sup>. Soil legacies are  
43 due to changes in abiotic and biotic soil conditions, for example because of increasing amounts of  
44 nutrients and soil-borne enemies of crop plants<sup>4</sup>. As part of restoration projects in Europe the soil  
45 legacy effects of arable land management are often diminished through removal of the organic  
46 topsoil down to the mineral layer underneath, which e.g. reduces soil fertility<sup>14,15</sup>. However, in many  
47 cases the ecosystems remain dominated by ruderal plant species even after the soil legacies have  
48 been removed<sup>7,16</sup>. This indicates that other constraints, such as seed availability and soil community  
49 composition, may not have been alleviated<sup>2,7,9</sup>. Restoration sites where soil legacies have been  
50 altered by topsoil removal may be particularly well-suited for testing the impact of soil inoculation on  
51 restoration with and without legacy effects of former agricultural management.

52

53 We tested the possibility of community steering through application of soil inocula in the field by  
54 analysing a large-scale, well-replicated, soil inoculation experiment on soils that had been used for  
55 arable farming for several decades. Topsoil was removed from large (2-5 ha) spatially-separated  
56 locations. Within each of these replicate locations we inoculated two different soil inocula and  
57 established controls where no further management was implemented. We used soil inocula from  
58 two donor sites: (i) a dry heathland and (ii) a grassland that had been restored 24 years ago. After six  
59 years, we analysed plant and soil community composition, as well as abiotic soil conditions, in order  
60 to test the hypothesis that application of the different inocula would not only promote community  
61 development, but that it would also steer the development of the plant and soil community  
62 composition into the direction of the respective donor sites. We performed an additional mesocosm  
63 experiment in order to validate that soil inoculation effects would not be due to adding plant seeds  
64 only.

65

66 Soil inoculation indeed altered both plant- and soil community composition profoundly (Fig. 1, S1, S2,  
67 Table S1, S2). The composition of the plant communities in plots inoculated with heathland and  
68 grassland soils differed markedly from each other (Fig. 1). The cover of both grassland and heathland  
69 target species were promoted by both inocula, although the heathland species responded most  
70 strongly to the heathland inoculum (Fig. 2a-d). Both early- and mid-successional species remained  
71 unaffected by soil inoculation. Moreover, inoculation led to plant communities that diverged from  
72 the controls in the direction of their respective donor community (Fig. 1b, S3; Table S3). This shows  
73 that, depending on the origin of the soil inoculum, the plant community development can be steered  
74 towards either a grassland or a heathland.

75

76 Soil inoculation also drove soil community composition towards that of the donor sites (Fig 1d, f, S3;  
77 Table S3). Inoculation with heathland soil significantly increased the biomass of both bacteria and

78 fungi (Table S4A). Both grassland and heathland soil inocula increased the abundance of arbuscular  
79 mycorrhizal fungi and the total number of nematodes (Table S4A). Heathland inoculum enhanced the  
80 diversity of springtails (Collembola) and mites (Acari), but not their total numbers (Table S4A). Most  
81 soil abiotic conditions were not affected by soil inoculation, except that in the inoculated soils  
82 percentage organic matter increased from 1.2 to 1.9-2.5 % (Table S5A).

83

84 In the mesocosm experiment, we inoculated a common field soil (mineral subsoil from the field site)  
85 with soil inocula from the two donor sites and established controls where no inoculum was added.  
86 We placed a 2-cm layer of sterilized sand on top of the mesocosm soil in order to reduce germination  
87 from the seed bank as much as possible. We subsequently sowed a standardized mixture of 30 plant  
88 species (Table S6) in all treatments and recorded the percent cover of all plant species after 30 weeks  
89 of growth. The species mixture consists of 10 representatives each for the early-, mid- and late-  
90 successional stages on sandy-soils in the region. Soil inoculation with heathland and grassland soils  
91 shifted the composition of the sown plant communities in different directions (Fig. 3, S2b). Plant  
92 species representative of the target communities benefitted from soil inoculation, while early- and  
93 mid-succession species remained unaffected (Fig. 2e-h). Furthermore, the two soil inocula led to  
94 plant communities that each shifted in the direction of their respective donor community (Fig.  
95 3). Therefore soil inoculation still steered plant community development into the direction of the  
96 vegetation composition of the donor sites, even when differences in plant propagules were excluded.

97

98 Finally, we compared the effectiveness of soil inoculation between plots with and without intact  
99 topsoil. For every treatment plot inoculated with grassland soil, we had an adjacent replicate plot  
100 where the same soil inoculum was introduced in intact topsoil with a full arable legacy. There was no  
101 such treatment for heathland soil. The plots with intact topsoil had higher soil organic matter content  
102 and nutrient availability, as well as a higher abundance of bacteria, fungi, nematodes and micro-

103 arthropods (Table S4, S5). Nevertheless, soil inoculation still had a significant effect on both plant and  
104 soil community composition of the topsoil (Fig. 4, Table S8a,b). In the inoculated plots, plant species  
105 composition was closer to the donor vegetation than the respective controls, irrespective of topsoil  
106 removal (Fig. S4). However, we found that soil inoculation influenced plant and soil community  
107 composition less strongly in plots with than without intact topsoil, particularly for the soil community  
108 (Fig. 4, Table S8c).

109

110 Our results show that soil inoculation can promote ecological restoration in the field. This has been  
111 suggested before<sup>9</sup>, however, most field tests have been done with soil transplants<sup>14,17–21</sup> and not with  
112 soil inocula<sup>16,22</sup>, which introduce considerably lower amounts of propagules. Moreover, we  
113 demonstrate that depending on the origin of the soil inoculum the restoration site could be steered  
114 to become either grassland or heathland. Glasshouse studies have indicated that the soil community  
115 is an important driver of plant community composition, and that late-successional plant species  
116 experience positive soil feedbacks, while ruderal species tend to have negative feedbacks<sup>6,8,11</sup>.  
117 However, for successful restoration plant propagules from the target community also need to be  
118 present, as many target species are dispersal limited<sup>23,24</sup>. Our mesocosm experiment shows that  
119 when differences in seed availability are alleviated, soil inoculation can still steer vegetation  
120 composition. Therefore, differences in plant community composition in the field were not solely the  
121 result of co-introducing plant propagules with the inoculum. We suggest that the co-introduction of  
122 both plant propagules and their associated soil biota restored the typically positive feedbacks among  
123 late-successional plants and their associated soil biota<sup>6,8,25</sup>. These feedbacks are thought to be major  
124 drivers of succession, suggesting that the positive effects of a single soil inoculation on the plant  
125 community may persist for prolonged periods of time.

126

127 Our study shows that soil inoculation in the field may both promote nature restoration and steer the  
128 community development depending on the origin of the soil inoculum. In our experiment the inocula  
129 application rates were quite different among grassland ( $2.5 \text{ L m}^{-2}$ ) and heathland inocula ( $1.0 \text{ L m}^{-2}$ ).  
130 However, we found the strongest effects in the treatment with the lowest amount of soil inoculum  
131 (heathland-inoculum). Therefore, we do not think that this difference was a major factor driving the  
132 results. Other studies have shown that inoculation of soils with arbuscular mycorrhizal fungi (AMF)  
133 can also promote the performance of late-successional plants over ruderal species and that local  
134 AMF strains outperform commercial strains<sup>25-27</sup>. However, several studies have argued that the  
135 transfer of whole soil communities is more effective than the addition of individual species or  
136 strains<sup>25-28</sup>. In addition, among the soil transplantation experiments in the field<sup>14,16-22</sup>, large-sized  
137 treatments tend to be more successful at restoring native plant communities than soil transfers  
138 applied at a small spatial scale. Thinly spread soil inocula, as used in our study, can restore much  
139 larger areas than when entire soil layers are translocated<sup>17-20</sup>, which may enhance the feasibility of  
140 this procedure as soil collection will disrupt current nature areas.

141

142 The success of inoculation in the field also depended on the presence of a legacy of arable  
143 cultivation: the impact of inoculation with grassland soil was greater when the topsoil was removed  
144 than when added to intact topsoil. The treatment plots with and without arable soil legacy differed  
145 substantially in their abiotic conditions, as well as soil community composition. It is, therefore, not  
146 possible to disentangle the exact causes in this experiment. However, it has been established that  
147 both low soil nutrient conditions<sup>7,29</sup> and reduced competition from resident soil communities<sup>30</sup> can  
148 enhance the impact of soil inoculation. Importantly, however, the effect of soil inoculation on  
149 community composition was still clearly present even in the intact topsoil. Further research should,  
150 therefore, test whether carefully chosen soil inocula may reduce the need for expensive topsoil  
151 removal, which has considerable environmental costs<sup>14</sup>.

152

153 We conclude that under field conditions soil inoculation can steer the course of community  
154 development on ex-arable land, irrespective of topsoil removal, but that effects are greatest when  
155 removing the topsoil prior to soil inoculation. Depending on the origin of the soil inoculum, the  
156 composition of the plant community in the recipient site was directed towards a heathland or a  
157 grassland vegetation. Based on our results we suggest that manipulation of soil communities through  
158 soil inoculation is a powerful tool for the restoration of degraded terrestrial ecosystems.

159



160 **Methods**

161 The experiment was carried out on a 160 ha former arable field (Reijerscamp, the Netherlands, GPS:  
162 52.015°N, 5.777°E), which had been used to cultivate crops for almost six decades. Reijerscamp is  
163 situated on coarse Pleistocene sand (Gleyic Placic Podzol, derived from a sandur: a glacial outwash  
164 plain) with gravel and an organic top layer of 30-35 cm, sometimes up to 50 cm thick. The site was  
165 grazed by cattle (25-30 cows throughout the year) upon completion of restoration measures and was  
166 further managed by periodic removal of tree seedlings (particularly *Betula* spp. and *Prunus serotina*).

167

168 Within the site, four separate experimental locations were selected. In each location the arable soil  
169 legacy was removed from 2-5 ha, by excavating the organic topsoil down to the mineral soil below  
170 (50-70 cm depth). In the excavation areas we established three treatments. We inoculated soil from  
171 two different nearby sources, a heathland and a grassland (H and G, respectively), in large treatment  
172 plots (on average 0.5 ha) in each of the four locations and control plots (C) were established where  
173 no further treatments were executed. In addition, in order to test the impact of the organic top-layer  
174 on inoculation success, we also inoculated plots of similar size with grassland soil outside the  
175 excavations and created controls there (i.e. without topsoil removal and soil inoculation). Hence, in  
176 total there were five experimental treatments carried out in each of the four locations (i.e.  $N = 5 \times 4 =$   
177 20 plots). The soil inocula were obtained from two nearby (<5 km distance) sites: one a grassland  
178 that had been under restoration for 24 years (Dennekamp, GPS: 52.029°N; 5.801°E) and an old dry  
179 heathland (Doorwerthse Heide, GPS: 51.995°N 5.778°E). The grassland soil was inoculated at a rate  
180 of  $\pm 2.5 \text{ L m}^{-2}$  and heathland at a rate of  $\pm 1.0 \text{ L m}^{-2}$ , with a commercial manure spreader, which  
181 resulted in a thin layer < 1 cm.

182

183 Six years after the treatments had been implemented we conducted a field sampling campaign (July  
184 2012, micro-arthropods: Sept 2012). To account for small-scale heterogeneity, we placed a randomly  
185 oriented transect across the centre of each of the 20 plots. Each transect consisted of five square

186 subplots (1 m<sup>2</sup>) each separated by five meters from the next subplot (total transect length: 25 m). In  
187 every subplot we analysed the composition of the plant and soil community and a number of abiotic  
188 soil parameters (see Supplementary Methods online). The same sampling was conducted in the two  
189 donor sites (July 2013) except that no data on microarthropods were collected. The two donor sites  
190 were each *a priori* divided into four areas of equal size using geographical stratification, and one  
191 transect was placed randomly within each area. In the heathland the selected positions of transects  
192 were slightly adjusted during sampling in the field to ensure that areas where sods had recently been  
193 cut as part of the normal management were avoided.

194

195 We analysed the data using linear mixed models for univariate response data including random  
196 effects to account for the hierarchical sampling design. We explicitly modelled heteroscedasticity in  
197 the residuals using Generalized Least Squares and post-hoc comparisons were corrected for multiple  
198 testing using the false-discovery rate. We used non-metric dimensional scaling (NMDS) to visualize  
199 differences in community composition and tested for significant differences among treatments using  
200 multiple-response permutation procedures (MRPP).

201

202 Additionally, we conducted a mesocosm experiment with mineral subsoil collected from the  
203 Reijerscamp at >70 cm depth inoculated with soil from the two donor sites (9:1 w:w). Separate soil  
204 inocula were collected from each transect in the donor fields and used as replicates. We placed a  
205 layer of sterile sand on top of the soils in order to prevent germination of seeds still present in the  
206 inoculum and sowed a standardized seed mixture of 30 species (Table S6) to ensure equal seed  
207 availability. After 30 weeks of growth we recorded the cover of all species in the treatments. As the  
208 mesocosm experiment setup was qualitatively the same as the design of the field experiment we  
209 analysed the data in the same way.

210

211 A detailed description of sample processing methods and data analysis can be found in the  
212 Supplementary Methods online. The primary data are available in Figshare (doi:  
213 10.6084/m9.figshare.3435404).

214

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283

284 **Additional information**

285 Supplementary information is available online. Reprints and permissions information is available  
286 online at [www.nature.com/reprints](http://www.nature.com/reprints). Correspondence and requests for materials should be addressed  
287 to E.R.J.W.

288

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297

### 298 **Author contributions**

299 M.B., T.M.B. and W.v.d.P. initiated and designed the field experiment, M.B. implemented  
300 the field experiment, E.R.J.W. and T.M.B. designed the mesocosm experiment. E.R.J.W.  
301 collected and analysed the data and wrote the first draft. All authors contributed to the final  
302 manuscript.

303

### 304 **Competing financial interests**

305 The authors declare to have no competing financial interests.

306 **Figures captions**

307 **Fig. 1. The effect of soil inoculation with two different soil inocula on plant and soil community**  
308 **composition in the field after topsoil removal.** The left column (panel a, c, e, g) shows the difference  
309 in community composition (Bray-Curtis distance; range 0-1, mean $\pm$ SE.) of the plant and soil  
310 community relative to control (light bars) and the difference between plots treated with the two  
311 different inocula (heathland and grassland; dark bars). Stars indicate significant differences from zero  
312 (i.e. among the two communities compared per bar), while different letters above the bars indicate  
313 significant differences among means (see Table S1). The right column shows non-metric dimensional  
314 scaling (NMDS) plots (panels b, d, f, h) of community composition in the three experimental  
315 treatments and the two donor sites (dots are means, ellipses SEs). Differences among treatments  
316 were significant in all cases (Table S2). The solid arrows indicate the direction of the effect of soil  
317 inoculation on community composition. The dotted arrows indicate the distance in community  
318 composition between the inoculated plots and their respective donor community. Stress values for  
319 each NMDS analysis are given. Stress is a lack of fit statistic (zero means perfect fit) indicating to what  
320 extent the two-dimensional plot represents the multidimensional differences in community  
321 composition (Bray-Curtis distances) in the raw data.

322

323 **Fig. 2. Changes in abundance of plant species groups in response to soil inoculation.** Shown are  
324 responses of target species for grassland (a, e) and heathland (b, f) respectively, as well as early- (c, g)  
325 and mid-succession (d, h) species. The top panels represent data from the field experiment (a-d),  
326 while the bottom panels are from the mesocosm experiment (e-h). Different letters indicate  
327 significant differences, for the overall analysis see Table S9. See Fig S2 for species membership to the  
328 different successional groups.



329 **Fig. 3. Differences in plant community composition from the control due to inoculation (10% w:w)**  
330 **with grassland and heathland soil inocula respectively in a controlled mesocosm experiment.** To  
331 alleviate differences in seed availability, inoculated and non-inoculated containers were sown with a  
332 standardized seed mixture of 30 plant species (Table S6) and a layer of sterilized sand was placed  
333 over the mesocosm soil to prevent germination from the seed bank. **a**, The difference in community  
334 composition (Bray-Curtis distance; range 0-1, mean $\pm$ SE) of the vegetation relative to control (light  
335 bars) and the difference between mesocosms treated with the two different inocula (heathland and  
336 grassland; dark bar) are shown. Stars indicate significant difference in community composition (i.e.  
337 different from zero; Table S7). **b**, Visualization of the differences in plant community composition  
338 (dots are means, ellipses SEs) using non-metric multidimensional scaling (NMDS; MRPP Pseudo-F =  
339 4.1,  $p = 0.001$ ). For each of the mesocosms their similarity to the two donor sites was calculated, and  
340 the direction of increasing similarity is plotted using dashed arrows. The solid arrows indicate the  
341 direction of the effect of soil inoculation on community composition.

342 **Fig. 4. Comparison of the effect of soil inoculation on plant (a) and soil community (b-d)**  
343 **composition in plots with (light bars) and without (dark bars) topsoil removal.** The extent to which  
344 the communities were different from the control (mean±SE, Bray-Curtis dissimilarity; range 0-1) after  
345 inoculation with soil from a heathland and a grassland is shown. Data in the light bars show the effect  
346 of inoculation with heathland and grassland soil after removal of the topsoil, data in the dark bars is  
347 the effect of inoculation with grassland soil in plots where the topsoil had not been removed. Stars  
348 indicate significant differences from zero (which equals no effect), different letters indicate  
349 significant differences among means within panels (see Table S8 for analysis).







