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Alessia Guerrieri, Aurélie Bonin, Tamara Münkemüller, Ludovic Gielly,  
Wilfried Thuiller, Gentile Francesco Ficetola

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1 **Effects of soil preservation for biodiversity monitoring using environmental DNA**

2 Running title: Preserving soil for eDNA analyses

3

4 Alessia Guerrieri<sup>1, \*</sup>, Aurélie Bonin<sup>1</sup>, Tamara Münkemüller<sup>2</sup>, Ludovic Gielly<sup>2</sup>, Wilfried Thuiller<sup>2</sup>, Gentile

5 Francesco Ficetola<sup>1, 2</sup>

6

7 Affiliations:

8 <sup>1</sup>Department of Environmental Science and Policy, Università degli Studi di Milano, Via Celoria 26, 20133

9 Milano, Italy

10 <sup>2</sup>Univ. Grenoble Alpes, CNRS, Univ. Savoie Mont Blanc, LECA, Laboratoire d'Ecologie Alpine, F-38000,

11 Grenoble, France

12 \*Correspondence author. Email: [alessia.guerrieri@unimi.it](mailto:alessia.guerrieri@unimi.it)

13 **ABSTRACT**

14 Environmental DNA metabarcoding is becoming a key tool for biodiversity monitoring over large  
15 geographical or taxonomic scales and for elusive taxa like soil organisms. Increasing sample sizes and  
16 interest in remote or extreme areas often require the preservation of soil samples and thus deviations from  
17 optimal standardized protocols. However, we still ignore the impact of different methods of soil sample  
18 preservation on the results of metabarcoding studies and there is no guideline for best practices so far.  
19 Here, we assessed the impact of four methods of soil sample preservation that can be conveniently used  
20 also in metabarcoding studies targeting remote or difficult to access areas. Tested methods include:  
21 preservation at room temperature for 6h, preservation at 4°C for three days, desiccation immediately after  
22 sampling and preservation for 21 days, and desiccation after 6h at room temperature and preservation for  
23 21 days. For each preservation method, we benchmarked resulting estimates of taxon diversity and  
24 community composition of three different taxonomic groups (bacteria, fungi and eukaryotes) in three  
25 different habitats (forest, river bank and grassland) against results obtained under ideal conditions (i.e.  
26 extraction of eDNA right after sampling). Overall, the different preservation methods only marginally  
27 impaired results and only under certain conditions. When rare taxa were considered, we detected small but  
28 significant changes in MOTU richness of bacteria, fungi and eukaryotes across treatments, but MOTUs  
29 richness was similar across preservation methods if rare taxa were not considered. All the approaches were  
30 able to identify differences in community structure among habitats, and the communities retrieved using  
31 the different preservation conditions were extremely similar. We propose guidelines on the selection of the  
32 optimal soil sample preservation conditions for metabarcoding studies, depending on the practical  
33 constraints, costs and ultimate research goals.

34

35 **KEYWORDS:** eDNA metabarcoding, sample storage, MOTU richness,  $\alpha$  and  $\beta$  diversity, microbial  
36 communities, eukaryotes

37

## 38 INTRODUCTION

39 Environmental DNA (hereafter referred to as "eDNA") can be defined as the mixture of complex, sometimes  
40 degraded, DNA that microorganisms (e.g. bacteria and fungi) or macroorganisms (e.g. animals, plants) leave  
41 behind in their environment (i.e. soil, water, sediments, etc.). By studying short, taxonomically-informative  
42 DNA fragments obtained from eDNA samples, it is possible to identify the associated taxa and therefore to  
43 survey biodiversity. Coined as "eDNA metabarcoding", this approach has revolutionized several branches of  
44 ecology and environmental sciences during the last decade, by providing relatively quick and non-invasive  
45 assessments of present or past biodiversity of animals, plants and microorganisms (Taberlet, Bonin, Zinger,  
46 & Coissac, 2018). Metabarcoding is particularly valuable for monitoring biodiversity over large geographical  
47 or taxonomic scales (De Vargas et al., 2015; Delgado-Baquerizo et al., 2018; Zinger et al., 2019b).  
48 Furthermore, it gives access to biodiversity components that are elusive to conventional survey methods.  
49 For instance, it allows the rapid assessment of microbial soil biodiversity, which is extremely complex, time-  
50 consuming and imperfect when using direct observations, culturing techniques or microscopy (Giovannoni,  
51 Britschgi, Moyer, & Field, 1990; Ward, Weller, & Bateson, 1990).

52 Metabarcoding relies on a succession of several steps: 1) sampling; 2) preservation of the collected  
53 material until lab processing; 3) DNA extraction; 4) PCR amplification of a selected genomic region; 5) high-  
54 throughput sequencing of amplicons; and 6) analysis of sequences using bioinformatics and statistical tools  
55 (Zinger, Bonin, et al., 2019). Each step is critical to obtain robust taxonomic inventories and diversity  
56 estimates, and an increasing number of studies has assessed how methodological choices across the  
57 different steps could influence the conclusions of a study (Calderón-Sanou, Münkemüller, Boyer, Zinger, &  
58 Thuiller, 2020; Cantera et al., 2019; Chen & Ficetola, 2020; Nichols et al., 2018; Taberlet et al., 2018).

59 Despite this growing body of literature, so far little attention has been devoted to the effect of different  
60 preservation conditions of the collected environmental material before lab processing (i.e. step 2). We thus  
61 know little about the optimal storage conditions of the collected material, and how long samples can be  
62 stored to limit biases in taxonomic inventories.

63 Some recent studies have analyzed the preservation of sampling material obtained from water (see  
64 e.g. Kumar, Eble, & Gaither, 2020; Majaneva et al., 2018). Conversely, methodological analyses on the  
65 effects of sample preservation are largely scarce for soil, perhaps because the majority of metabarcoding  
66 studies have so far been performed in temperate areas where access to lab facilities is often easy  
67 (Hoffmann, Schubert, & Calvignac-Spencer, 2016; Huerlimann et al., 2020). In such cases, sample  
68 preservation is sometimes not necessary at all, or at least not over long periods of time. However, one  
69 great promise of metabarcoding is its potential for providing biodiversity data for remote areas, where  
70 biodiversity monitoring is essential but difficult. When sampling in remote or inaccessible areas (e.g.  
71 tropical and arctic areas; mountain chains), samples are rarely collected nearby lab facilities and an  
72 immediate *in situ* DNA extraction is generally not possible due to logistic constraints (but see Zinger,  
73 Taberlet, et al., 2019b for a notable exception). More generally, with the ever-increasing number of  
74 samples analyzed during a typical metabarcoding study, sample preservation is more and more  
75 indispensable, and the time lag between sample collection and subsequent molecular processing makes it  
76 particularly relevant to understand the impact of sample preservation, and to identify preservation  
77 strategies that do not bias the conclusions of studies.

78 In an ideal metabarcoding study, communities recovered from preserved samples should match  
79 those retrieved if samples had been processed immediately after sampling. However, inappropriate  
80 preservation conditions can cause both DNA degradation and the proliferation of certain taxonomic groups  
81 with respect to others, before DNA extraction (Cardona et al., 2012; Orchard, Standish, Nicol, Dickie, &  
82 Ryan, 2017). This can in turn affect taxa detection and also the relative contributions of different taxonomic  
83 groups to the estimated biodiversity. A recent review suggested that the majority of eDNA metabarcoding  
84 studies does not provide accurate information about sample treatment before processing (Dickie et al.,  
85 2018). Almost half of the studies do not report how samples were stored and conserved, and 30% of them  
86 store samples at 0-4°C, and thus at a temperature where many bacteria and fungi continue to be active and  
87 potentially affecting the whole sample. About 15% of the studies stored samples in a range of 5-35°C, which

88 can be considered a poor practice when no preservatives are added (Dickie et al., 2018), and only 10%  
89 stored them below 0°C (Dickie et al., 2018).

90 So far, the consequences of preservation practices and the resulting deviations from immediate  
91 processing and analyses have rarely been studied quantitatively. Some studies suggested that liquid-based  
92 preservatives have limited usefulness for soil eDNA (Tatangelo et al., 2014). Yet, Lauber, Zhou, Gordon,  
93 Knight, & Fierer (2010) tested the effect of storing samples from soil, human gut and skin at different  
94 temperatures and did not detect any significant effect on bacterial communities, while Orchard et al.  
95 (2017) found that storage time and temperature can affect colonization by arbuscular mycorrhizal fungi,  
96 with subsequent impacts on the reconstruction of communities. Differences between these studies may be  
97 due to their different protocols. However, they also focused on different taxonomic groups, which may  
98 react differently to storage period and temperature. Consequently, these studies are difficult to compare,  
99 highlighting the importance of formal assessments of preservation methods. Desiccation is a further  
100 approach that can efficiently conserve high-quality DNA for genomic studies (e.g. Alsos et al., 2020; Chase &  
101 Hills, 1991). Although not widely used for metabarcoding samples, desiccation through silica gel has good  
102 potential for soil, as it allows removing >25% of its weight in water in a few hours (P. Taberlet, pers.  
103 communication), is cost-effective, easy to transport, and is not an issue for aircraft transportation (no  
104 flammable or dangerous preservatives). A clear understanding of the effect of different preservation  
105 methods, especially across various groups of taxa, is thus pivotal for a robust application of eDNA  
106 metabarcoding to biodiversity monitoring in general, and that of remote areas in particular.

107 Here, using eDNA metabarcoding of different taxonomic groups in soil systems, we tested: (i) how  
108 preservation methods influence overall richness estimates and what the role of rarely observed taxa is; (ii)  
109 how preservation methods influence identified community structure and its turnover between different  
110 habitats; and (iii) what the best practices are under limited laboratory access. More specifically, we first  
111 selected three soil preservation methods (room temperature, 4°C, desiccation in silica gel) because they are  
112 commonly used in the literature (room temperature and 4°C) or because they are easy to implement in the  
113 field (desiccation and room temperature). Then, we assessed the impact of these preservation methods

114 applied to different durations in order to mimic logistic constraints (see Supplementary Material, Appendix  
115 A for details on experimental design), and compared the communities obtained with those observed in  
116 ideal conditions, i.e. when eDNA is extracted immediately after sampling (within less than one hour). We  
117 examined bacterial, fungal and eukaryotic communities to cover a broad taxonomic range, since different  
118 taxa can be differentially affected by sample preservation conditions (Cardona et al., 2012; Orchard et al.,  
119 2017).

## 120 MATERIALS AND METHODS

### 121 Soil preservation and experimental treatments

122 In April 2019, we collected soil samples from three habitats: a grassland (N 45.194° E 5.776°), a broadleaved  
123 forest (N 45.196° E 5.774°), and a vegetated river bank (N 45.195° E 5.780°). The study design was  
124 optimized to allow DNA extraction immediately after sampling, which hampered using distant study sites.  
125 All sites were within 400 m from the Laboratoire d'Écologie Alpine (LECA) in Grenoble, France. We choose  
126 habitats with some differentiation to allow different communities but not too extreme and relatively close  
127 together so that we expect some overlap between communities. This mimics what is commonly done in the  
128 field when gradients are sampled. We established five plots within each habitat; the minimum distance  
129 between nearby plots was about 20 m. Within each plot, we collected four soil samples (with a minimum  
130 distance of one meter) at a depth of 0–20 cm and then pooled them together, for a total of five pooled  
131 samples per habitat (approx. 200 g each pooled sample). Soil litter was not included in the samples. Pooled  
132 samples (15 in total) were homogenized; subsequently, from each of them we took five subsamples of 15 g  
133 of soil (total: 75 subsamples; Fig. 1).

134 The five soil subsamples of each pooled sample were subjected to five different treatments: 1) eDNA was  
135 extracted immediately after sampling (within 1 h; treatment hereafter referred to as “control”); 2) samples  
136 were preserved at room temperature (21–23°C) and eDNA was extracted 6 h after sampling; 3) samples  
137 were inserted in sterile 50-mL falcon tubes and preserved at 4°C. eDNA was extracted three days after  
138 sampling; 4) samples were inserted in hermetic, sterile boxes with 20 g of silica gel immediately after  
139 sampling, then stored at room temperature, and eDNA was extracted 21 days after sampling; 5) samples  
140 were inserted in hermetic, sterile boxes with 20 g of silica gel 6h after sampling, then stored at room  
141 temperature, and eDNA was extracted 21 days after sampling.

142 We did not test full-factorial combinations of different preservation periods and conditions, which  
143 was not feasible in terms of time and costs. We did not consider freezing or storage in liquid nitrogen,  
144 which is unrealistic when dealing with large numbers and / or volumes of samples, as is the case for large-  
145 scale metabarcoding studies. Furthermore, freezing is generally impossible in remote areas (Dickie et al.,



146 2018), where maintaining a cold chain cannot be ensured given the logistical challenges and this is often  
147 replaced by preservation in a cool box (4°C). Previous studies showed that preservative solutions have  
148 limited usefulness for soil or sediment samples ((Rissanen et al., 2010; Tatangelo et al., 2014).

149

## 150 **Molecular analyses**

151 For all sample treatments, eDNA extraction was performed in a dedicated room using the NucleoSpin® Soil  
152 Mini Kit (Macherey-Nagel, Germany), after a preliminary step where 15 g of soil were mixed with 20 ml of  
153 phosphate buffer for 15 minutes as described in (Taberlet, Coissac, Pompanon, Brochmann, & Willerslev,  
154 2012); and with a final elution in 150 µl. We also included one extraction negative control per treatment.

155 Environmental DNA of bacteria, fungi and eukaryotes was amplified using primers designed for  
156 markers Bact02 (Taberlet et al., 2018), Fung02 (Epp et al., 2012; Taberlet et al., 2018) and Euka02  
157 (Guardiola et al., 2015), respectively. Bact02 and Fung02 amplify fragments of about 220-250 bp, while  
158 Euka02 generally amplifies fragments <150 bp. The three markers are well suited for metabarcoding  
159 analyses, as all have a very low number of mismatches in the priming region across target organisms, and  
160 the relatively short length of amplified fragments allows their use with potentially degraded DNA (Taberlet  
161 et al., 2018). To allow bioinformatic discrimination of PCR replicates after sequencing, eight-nucleotide long  
162 tags were added on the 5' end of both forward and reverse primers, so that each PCR replicate was  
163 represented by a unique combination of forward and reverse tags. Tags had at least five nucleotide  
164 differences among them (Coissac, 2012). Samples were randomized on a 96-well plate, along with the five  
165 extraction controls, eight bioinformatic blanks, six PCR negative controls and two PCR positive controls. PCR  
166 positive controls were included to check for potential cross-contaminations and to monitor amplification  
167 and sequencing performances. The positive control was a 1:10 dilution of the ZymoBIOMICS™ Microbial  
168 Community DNA Standard II (Zymo Research, USA) constituted of genomic DNA of eight bacterial and two  
169 fungal strains (i.e., *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica*, *Lactobacillus*  
170 *fermentum*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis*,  
171 *Saccharomyces cerevisiae*, *Cryptococcus neoformans*) at known concentrations.

172 In order to avoid over-amplification of template DNA and to limit chimera formation, we determined the  
173 optimal number of amplification cycles and DNA extract dilution using qPCR. The qPCR assay was  
174 conducted on 48 randomly selected samples, using 1 µl of 1:1000 diluted SYBR® Green I nucleic acid gel  
175 stain (Invitrogen™, USA), with a real-time PCR thermal cycler set to standard mode. qPCR was performed  
176 for both 1:10 diluted and undiluted template eDNA.

177 For Bact02 and Fung02, PCR reactions were performed on 1:10 diluted template DNA, using 32 and 44  
178 cycles respectively. For Euka02, we performed 34 cycles on undiluted DNA. All PCR reactions consisted of  
179 10 µl of AmpliTaq Gold 360 Master Mix 2X (Applied Biosystems™, Foster City, CA, USA), 2 µl of primers mix  
180 at initial concentration of 5 µM of each primer, 0.16 µl of Bovine Serum Albumin (corresponding to 3.2 µg;  
181 Roche Diagnostic, Basel, Switzerland) and 2 µl of DNA extract, for a final volume of 20 µl. The PCR profiles  
182 had an initial step of 10 min at 95°C, followed by several cycles of a 30 s denaturation at 94°C, a 30 s  
183 annealing at 53°C (Bact02), 56°C (Fung02) or 45°C (Euka02), and a 90 s elongation for Bact02 and Fung02,  
184 or a 60 s elongation for Euka02 at 72°C, followed by a final elongation at 72°C for 7 minutes. The  
185 amplification was performed in 384-well plates, with four replicates for each sample. After amplification,  
186 PCR products of the same marker were pooled together in equal volumes and a 5-µl aliquot of the pooled  
187 amplicons was visualized by high-resolution capillary electrophoresis (QIAxcel Advanced System, QIAGEN,  
188 GERMANY) to verify the expected fragments length and to monitor primer dimers. Pooled amplicons were  
189 purified using the MinElute PCR Purification Kit (QIAGEN, GERMANY) following the manufacturer's  
190 protocol. Six subsamples of the pool of amplicons were purified separately for each marker, and then  
191 combined again before being sent for library preparation and sequencing to Fasteris (SA, Geneva,  
192 Switzerland). One library was prepared per marker using the MetaFast protocol (Taberlet et al., 2018) and  
193 then sequenced using the MiSeq (Fung02 and Bact02) or HiSeq 2500 (Euka02) platforms (Illumina, San  
194 Diego, CA, USA) with a paired-end approach (2 × 250 bp for Fung02 and Bact02, and 2 × 150 bp for Euka02).

195

196 **Bioinformatic treatment**

197 The bioinformatic treatment of sequence data was performed using the OBITools software suite (Boyer et  
198 al., 2016). First, forward and reverse reads were assembled using the *illumina-paired-end* program, keeping  
199 only sequences with an alignment score higher than 40. Aligned sequences were assigned to the  
200 corresponding PCR replicate using the program *ngsfilter*, by allowing two and zero mismatches on primers  
201 and tags, respectively. After sequence dereplication using *obiuniq*, bad-quality sequences (i.e. containing  
202 “N”), sequences whose length fell outside the expected size interval (below 45 bp for Bact02, below 68 bp  
203 Fung02 and below 36 bp for Euka02) and singletons were filtered out. The *obiclean* program was run to  
204 detect potential PCR or sequencing errors with the *-r* option set at 0.5: in a PCR reaction, sequences are  
205 tagged as “heads” when they are at least twice as abundant as other related sequences differing by one  
206 base. Only the sequences tagged as “heads” in at least one PCR were kept.

207 Taxonomic assignment was conducted using the *ecotag* program based on a reference database  
208 constructed from EMBL (version 136) by running the *ecoPCR* program (Ficetola et al., 2010). More  
209 specifically, *ecoPCR* carried out an *in silico* PCR with the primer pair used for the experiment and allowing  
210 three mismatches per primer. The obtained reference databases were further curated by keeping only the  
211 sequences assigned at least at the family level.

212 Further data filtering was performed in R version 3.6.1 (R Core Team, 2018) to remove spurious  
213 sequences that can bias ecological conclusions drawn from DNA metabarcoding data (Calderón-Sanou et  
214 al., 2020). More specifically, we discarded all MOTUs with best identity <85% (Fung02, Bact02) or <80%  
215 (Euka02). These MOTUs were indeed rare (31%, 1.7% and 7.3% of reads for Fung02, Bact02 and Euka02,  
216 respectively) and their effect on the NMDS was marginal, as observed in other studies (e.g. Botnen et al.,  
217 2018). Furthermore, we removed MOTUS with less than five occurrences in the overall dataset, detected in  
218 more than one extraction or PCR negative control (Zinger, Bonin, et al., 2019a), or that were detected in  
219 less than two PCR replicates of the same sample, as they often represent false positives (Ficetola et al.,  
220 2015).

221

222 **Statistical analyses**

223 For all taxonomic groups, we used Generalized Linear Mixed Models (GLMMs) to test if the different  
224 treatments lead to differences in the observed MOTU richness. In GLMMs, the number of MOTUs per  
225 sample was calculated and used as a dependent effect, the five treatments were used as predictors, and  
226 sample identity was used as a random factor. The model was performed with the generalized poisson  
227 distribution error using the R package *glmmTMB* (Brooks et al., 2017), in order to consider overdispersion  
228 (Consul & Famoye, 1992). If GLMM detected significant differences among treatments, we used treatment  
229 contrasts to test if each treatment led to communities significantly different from those unraveled by the  
230 “control” condition. Treatment contrasts are standard non-orthogonal contrasts, in which each category  
231 (treatment) is compared to a user-defined reference category, and are appropriate to compare multiple  
232 treatments against one single control category (in this case, immediate extraction; (Field, Miles, & Field,  
233 2015). The uncorrected number of MOTUs tends to overestimate the actual taxonomic richness (Calderón-  
234 Sanou et al., 2020). Therefore, we repeated this analysis twice: considering all the observed MOTUs, and  
235 considering only MOTUs with frequency  $\geq 1\%$  in each sample (hereafter referred to as "common MOTUs").

236 Subsequently, we used multivariate analyses to assess the variation of bacteria, fungi and  
237 eukaryotic communities across habitats and treatments. Before running multivariate analyses, we  
238 calculated the proportion of reads of each MOTU in each sample. Relative abundance values were then  
239 transformed using the Box-Cox transformation, which simultaneously solves the double-zero problem and  
240 improves the multivariate normality of data (Legendre & Borcard, 2018).

241 First, we used Nonmetric MultiDimensional Scaling (NMDS) to describe differences in communities  
242 among the three habitats, and check whether different treatments yield different interpretations of  
243 ecological relationships among samples. NMDS uses an optimization process to find a configuration of  
244 points (samples) in a space with a small number of dimensions, and is suitable for metabarcoding analyses  
245 that aim to reconstruct variation in community composition as well as possible, without preserving any  
246 particular distance measure among objects (Borcard, Gillet, & Legendre, 2011; Chen & Ficetola, 2020; Paliy  
247 & Shankar, 2016). Given its robustness and flexibility, NMDS is often used as the first step to characterize  
248 the similarity of communities in metabarcoding studies (Chen & Ficetola, 2020; Paliy & Shankar, 2016).

249 NMDS was run on the Euclidean distance computed on Box–Cox-chord-transformed data (Legendre &  
250 Borcard, 2018), by building 1,000 ordinations.

251         Second, we used *ProcMod*, a Procrustes-based analysis (Coissac & Gonindard-Melodelima, 2019),  
252 to measure the multivariate correlations between the communities obtained using the different  
253 treatments. *ProcMod* can be used to measure the shared variation between matrices, and is particularly  
254 appropriate to test relationships between datasets obtained through DNA metabarcoding and  
255 metagenomics (Coissac & Gonindard-Melodelima, 2019). Procrustes analyses tend to overfit the data,  
256 therefore we used a modified version of Procrustes correlation that is robust to highly-dimensional data  
257 and allows a correct estimation of the shared variation between data sets (Coissac & Gonindard-  
258 Melodelima, 2019). The Procrustes-based correlation tests were performed using the *corls* function in the R  
259 package *ProcMod*, using 1,000 randomizations to test the mean covariance between random matrices  
260 (Coissac & Gonindard-Melodelima, 2019).

261         Third, we used redundancy analysis (RDA) to measure the amount of variation among communities  
262 that is explained by differences in habitat and treatments (Legendre & Legendre, 2012; Ter Braak, 1986).  
263 With habitat typology and treatment as constraining matrices, we used treatment contrasts to test if each  
264 treatment led to communities significantly different from those unraveled by the control treatment. Thus,  
265 significant treatment contrasts indicate that results between control and experimental treatments differ in  
266 an important way, while non-significant results mean that deviation from ideal conditions is not specifically  
267 pronounced. Significance of RDA and treatment contrasts was tested through 10,000 permutations using  
268 the *vegan* package in R (Borcard et al., 2011; Oksanen et al., 2019).

269         For bacteria only, RDA detected significant differences between the control and some of the  
270 treatments. We thus ran a similarity percentage analysis with the *simper* R function (Clarke, 1993) from  
271 *vegan* to identify the taxa contributing to the overall pairwise treatment difference (Geyer et al., 2014).  
272 Significance was tested using 50,000 permutations. Given the large number of tests performed, the  
273 significance of tests was corrected using the False Discovery Rate (FDR) method with the *fdrtool* package  
274 (Strimmer, 2008). FDR has greater power than traditional approaches (e.g. Bonferroni correction) when

275 performing multiple comparisons (Benjamini & Hochberg, 1995). All statistical analyses were performed in  
276 the R environment.  
277

## 278 RESULTS

279 A total of 6.3, 7.9 and 25.7 million reads were obtained from the Bact02, Fung02 and Euka02 libraries,  
280 respectively. After read assembly, quality filtering, spurious sequence and contaminant removal, 481,411;  
281 2,511,721 and 13,232,441 good-quality sequences remained, consisted of 660 (Bact02), 1,075 (Fung02) and  
282 3,611 (Euka02) unique sequences (i.e. MOTUs).

283

### 284 Differences in MOTU richness among treatments

285 Generalized Linear Mixed Models allowed identifying shifts in the richness of observed MOTUs. When we  
286 considered all the detected MOTUs, GLMM detected significant differences in MOTUs richness among  
287 treatments for all the markers considered (Bact02:  $\chi_4 = 38.9$ ,  $P < 0.001$ ; Fung02:  $\chi_4 = 18.2$ ,  $P = 0.001$ ;  
288 Euka02:  $\chi_4 = 21.7$ ,  $P < 0.001$ ; Fig. 2). Compared to the control, contrasts showed small but significant  
289 changes in MOTUs richness under the 4°C treatment (Bact02:  $z = 2.54$ ,  $P = 0.010$ ; Fung02:  $z = -2.17$ ,  $P =$   
290  $0.029$ ; Euka02:  $z = 2.65$ ,  $P = 0.008$ ), the silicagel treatment (Bact02:  $z = -2.93$ ,  $P = 0.003$ ; Fung02:  $z = -3.99$ ,  $P$   
291  $< 0.001$ ; Euka02:  $z = 3.92$ ,  $P < 0.001$ ), and the silicagel+6h treatment (Bact02:  $z = -3.74$ ; Fung02:  $z = -4.02$ ;  
292 Euka02:  $z = 4.18$ ; all  $P < 0.001$ ). The 6h treatment caused a small but significant decrease in MOTUs richness  
293 for fungi ( $z = -2.42$ ;  $P = 0.015$ ), but not for bacteria and eukaryotes ( $P = 0.456$ ,  $P = 0.283$ , respectively; for all  
294 contrasts: Table S1).

295 Nevertheless, when we repeated analyses by excluding MOTUs with a frequency  $< 1\%$ , differences  
296 in richness were much smaller, and were only significant for bacteria and fungi (Bact02:  $\chi_4 = 9.69$ ,  $P = 0.045$ ;  
297 Fung02:  $\chi_4 = 14.1$ ,  $P = 0.006$ ; Euka02:  $\chi_4 = 2.22$ ,  $P = 0.693$ ; Fig. 2). Compared to the control, MOTUs richness  
298 decreases for Bact02 under the 4°C treatment ( $z = -2.91$ ;  $P = 0.003$ ) and increases for Fung02 under the two  
299 silicagel treatments ( $z = 2.77$ ;  $P = 0.005$ ;  $z = 1.75$ ;  $P = 0.080$ ; respectively), while no significant effect was  
300 detected for Euka02 under any of the treatments (all  $P > 0.170$ ; for all contrasts: Table S1).

301 Habitat caused a significant effect in MOTUs richness only for Fung02 both before and after  
302 removing rare MOTUs (before:  $\chi_1 = 11.8$ ,  $P < 0.001$ ; after:  $\chi_1 = 20.5$ ,  $P < 0.001$ ).

303

304 **Ecological similarity of communities among treatments**

305 Nonmetric MultiDimensional Scaling showed a stress value of 0.13 for Bact02, 0.14 for Fung02 and 0.12 for  
306 Euka02. For each of the three markers, the NMDS plots obtained for the five sample preservation  
307 treatments were extremely similar, and the ecological differences among the three habitats were clearly  
308 identified by all the preservation treatments (Fig. 3).

309 The multivariate correlation between the communities obtained with the five treatments was  
310 always very strong (Procrustes-modified correlation: for all comparisons between “control” and treatments  
311  $r \geq 0.84$ ,  $P < 0.0001$ ; Fig. 4) indicating, for all markers, that most of the variation of retrieved communities  
312 was shared across all the treatments. Procrustes correlations were particularly high for Fung02 and Euka02  
313 (all  $r \geq 0.9$ ), and between the control and the treatments 6h and 4°C (all  $r \geq 0.93$ ; Fig. 4).

314

315 **Differences between the obtained communities**

316 Redundancy analysis allowed us to measure the amount of variation explained by differences among  
317 habitats and by treatments. Overall, 33%, 24%, and 33% of variability was explained by differences in  
318 habitat for bacteria, fungi, and eukaryotes, respectively. The community differences among habitats were  
319 strongly significant for the three taxonomic groups (permutation test: all  $P \leq 0.001$ ). Differences among  
320 treatments were much weaker, and explained 9%, 2% and 2% of variation only for bacteria, fungi and  
321 eukaryotes, respectively. Differences between treatments were significant for bacteria (permutation test:  $P$   
322  $< 0.0001$ ), but not for fungi and eukaryotes (both  $P = 1$ ).

323 For bacteria, contrasts did not detect significant differences between control and the 6h or 4°C  
324 treatments. Differences between control and the two silicagel treatments were significant but explained a  
325 limited amount of variation (for both treatments,  $\approx 3\%$  of variation explained;  $P < 0.0001$ ; Table 1). We thus  
326 used similarity percentage analysis to identify the MOTUs significantly contributing to these differences.  
327 Only one single MOTU showed a significant contribution ( $P = 0.03$  after FDR correction) to the differences  
328 between control and silicagel treatment; this MOTU (belonging to the Bacteroidetes phylum) showed a



329 very limited frequency under the silicagel treatment (Fig. S1). After FDR correction, no MOTU showed a  
330 significant contribution to the differences between control and the silicagel+6h treatment.  
331

## 332 **DISCUSSION**

333 Monitoring soil biodiversity with eDNA metabarcoding over large geographical and taxonomic scales and  
334 sometimes in remote places **is increasingly** important in ecological research. Understanding how  
335 preservation conditions affect estimates of taxonomic richness and community composition is essential to  
336 ensure sound conclusions. Our study shows that soil metabarcoding results are surprisingly robust to  
337 preservation conditions, as we observed limited differences in community structure and diversity estimates  
338 when samples were preserved using different strategies. However, some taxonomic groups and diversity  
339 components are more sensitive than others to certain preservation conditions. This allowed **us to develop**  
340 **guidelines** for preservation depending on the aims of monitoring programs and on focal taxa.

341 The aim of this study was comparing realistic approaches to soil preservation against an ideal  
342 situation. Immediate extraction was our reference approach, as it avoids both DNA degradation (i.e.  
343 potential under-representation of certain taxa) and continued growth of certain taxonomic groups (i.e.  
344 potential over-representation of other taxa). Unfortunately, immediate extraction is only possible if  
345 sampling occurs nearby facilities, or when a mobile eDNA laboratory is available (e.g. Zinger, Taberlet, et al.  
346 2019b), and logistical constraints often hamper its application in remote areas. We selected preservation  
347 conditions among the most achievable, cost-effective and frequent practices to sampling soil for eDNA  
348 studies (Dickie et al., 2018), **although additional storage methods (e.g. liquid nitrogen, dry ice, RNA later)**  
349 **are available.**

350

### 351 **Influence of preservation methods on richness estimates**

352 Preservation methods generated some small but significant differences in MOTUs richness compared to  
353 what is observed in the “control”, with some contrasting effects across taxa. When considering all the  
354 MOTUs, none of the preservation conditions yielded estimates of alpha-diversity identical to the “control”.  
355 For instance, just six hours at room temperature caused a significant decrease of MOTUs richness in fungi.  
356 It has been shown that estimates of alpha-diversity using metabarcoding are extremely sensitive to  
357 methodological choices (Calderón-Sanou et al., 2020). Our study underlines that even preservation for a

358 very short time can affect the detection of rare MOTUs and highlights the sensitivity of fungi to  
359 preservation at room temperature (Delavaux, Bever, Karppinen, & Bainard, 2020). MOTUs richness of all  
360 the taxa was also affected by preservation at 4°C, which caused a slight increase of MOTUs richness for  
361 bacteria and eukaryotes, and a slight decrease for fungi. The effect of temperature and time storage in  
362 fungal and bacterial growth **has already been proven** (see e.g. Orchard et al., 2017; Pettersson & Bååth,  
363 2003). Despite this, in addition to temperature, we can expect that other parameters such as initial soil  
364 moisture and pH influence bacterial growth (Bååth & Arnebrant, 1994; Drenovsky, Vo, Graham, & Scow,  
365 2004; Fernández-Calviño & Bååth, 2010; Kaiser et al., 2016) with a combined effect. Finally, drought affects  
366 the richness of microbial communities in soil ecosystems with differential effects across taxa depending on  
367 their ecology (Evans, Wallenstein, & Burke, 2014; Meisner, Jacquiod, Snoek, Ten Hooven, & van der Putten,  
368 2018; Ochoa-Hueso et al., 2018), and three weeks of preservation with silica gel generally reduced the  
369 observed MOTUs richness in bacteria and fungi, while it increased the richness of eukaryotes.

370 However, our study also shows that specific caution is mostly necessary when rare MOTUs are of  
371 interest. The exclusion of rare **and uncommon** MOTUs strongly reduced differences between optimal  
372 conditions and different preservation. The **remaining effects were much weaker for bacteria and fungi,**  
373 **while disappeared for eukaryotes (Fig. 2), and can be due to the heterogeneous growth across taxonomic**  
374 **groups, or to differential DNA degradation under different preservation conditions.** This suggests that the  
375 effect of preservation approach on taxonomic richness mostly occurs on rare species, as already suggested  
376 for microbial communities (Meisner et al., 2018). Several authors have shown that eDNA metabarcoding  
377 does not represent the best tool for the detection of rare MOTUs, as some rare MOTUs remain undetected,  
378 while many sequences detected at rare frequency are **artifacts** (Brown et al., 2015). Estimates of  $\alpha$ -diversity  
379 should therefore **always be taken** with caution, and indices that underweight rare MOTUs (e.g. Shannon or  
380 Simpson diversity) can provide more robust estimates (Brown et al., 2015; Calderón-Sanou et al., 2020;  
381 Bálint et al., 2016).

382

383 **Differences in community structure**

384 If the study interest is in community structure and not in richness estimates, then preservation choices  
385 become even less important. In fact, the similarity of communities obtained through the different  
386 preservation conditions is surprisingly high (see Procrustes correlation coefficients; Fig. 4); the amount of  
387 variation explained by preservation conditions was much lower than the observed differences among  
388 habitats (see redundancy analysis), and multivariate analyses consistently allowed to detect community  
389 differences among habitats (Fig. 3). In other words, metabarcoding is able to identify the ecological  
390 differences among sites, independently of the preservation approach. Even though metabarcoding analyses  
391 are sensitive to methodological choices, estimates of relationships between diversity and the environment  
392 are often robust (Calderón-Sanou et al., 2020; Ji et al., 2013), and this is a very good news if we want to  
393 apply these approaches to broad-scale monitoring programs, aiming at assessing the effects of  
394 environmental changes.

395 Bacteria were the only taxon for which we detected significant differences between the “control”  
396 and the preservation conditions, with ≈3% of variability explained by differences between the “control” and  
397 the desiccation treatments. The observed differences most likely refer to some taxa that are affected by  
398 the dry conditions and could lead to an overrepresentation of some taxa that are more resistant under  
399 these conditions. We expect some taxa to better survive and grow in dry treatments with respect to others,  
400 and this would make their DNA more available in the samples. Differences between the desiccation  
401 treatments and the control were small, and only one out of 660 MOTUs showed a significant variation in  
402 abundance with the control. This MOTU (belonging to the Bacteroidetes phylum of bacteria, see Supporting  
403 Information) was generally abundant in the control and preservation conditions 2 and 3 (average frequency  
404 of reads around 10%) while it drastically decreased under preservation conditions 4 and 5 (Fig. S1). This  
405 agrees with studies showing that different genera belonging to this phylum respond differently to drought  
406 (Meisner et al. 2018). In fact, the Bacteroidetes *Flavobacterium* shows an increase in abundance over dried-  
407 treatments, even though differences after three weeks were not significant (see Fig. S2).

408 The significant differences observed for some taxa and preservation conditions stress the  
409 importance of selecting the preservation method before starting a monitoring program and using it

410 consistently through the whole monitoring, to avoid confusion between the effects of methods and of  
411 environmental changes.

412

### 413 **Conclusions: guidelines for optimizing preservation conditions**

414 Standardized protocols are essential for repeatable and reliable biodiversity monitoring, and our results  
415 allow to propose guidelines to improve and standardize the preservation of soil samples for eDNA  
416 metabarcoding analyses (Fig. 5):

- 417 1) If sampling occurs close to lab facilities, or a mobile lab is available, extracting DNA as soon as possible  
418 is the best approach. Storing samples a few hours at room temperature does not have major impact  
419 on the outcome of analyses, especially if the focus is not on rare MOTUs;
- 420 2) If lab facilities are available after a short-time transportation, storing samples in the fridge (0-4°C) for a  
421 few days is a safe approach as it does not have a significant impact on community composition, and  
422 only moderately affects MOTUs richness. However, this approach can be problematic if the aim is to  
423 estimate MOTUs richness, and particularly the occurrence of rare MOTUs. The feasibility of this  
424 strategy also depends on the number and volume of samples, and to the possibility of maintaining the  
425 cold chain;
- 426 3) If monitoring in remote areas, sample desiccation (e.g. using silica gel) and long-term preservation at  
427 room temperature is a reasonable approach, and it is particularly convenient when working with a  
428 large volume of samples. This approach preserves ecological signal, but can affect the detection of  
429 some taxa, particularly among the rarest ones. Therefore, this approach is suboptimal for monitoring  
430 programs aiming at detecting rare MOTUs.

431 An effective application of eDNA metabarcoding to biodiversity monitoring is complex, and protocols of  
432 sample preservation are key methodological choices that have to be taken into account when designing a  
433 metabarcoding-based monitoring. When working in difficult and remote environments researchers are  
434 faced with the trade-offs between a faithful representation of biodiversity, and multiple logistic constraints  
435 in the field. Accurate a-priori planning is often the basis of successful monitoring programs and our

436 guidelines can help researchers and practitioners to identify the best approach to sample preservation,  
437 depending on the studied taxa and research goals.  
438

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445

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616 **DATA ACCESSIBILITY**

617 Raw sequences as well as filtered data are available in the DRYAD Digital Repository  
618 (<https://doi.org/10.5061/dryad.zkh189382>)

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620 **AUTHOR CONTRIBUTIONS**

621 TM, GFF, LG, AB, WT and AG designed the experiment. LG, TM and AG conducted the field work. AG  
622 conducted all molecular analyses and performed the bioinformatic treatment of sequences with the help of  
623 AB. AG, AB and GFF ran statistical analyses. AG, GFF and AB drafted the manuscript. All the authors  
624 contributed substantially to the revision process, and accepted the final version.

625 **ORCID**

626 Alessia Guerrieri: <https://orcid.org/0000-0002-1519-3517>  
627 Aurélie Bonin: <https://orcid.org/0000-0001-7800-8609>  
628 Tamara Münkemüller: <https://orcid.org/0000-0001-9743-1322>  
629 Ludovic Gielly: <https://orcid.org/0000-0001-5164-6512>  
630 Wilfried Thuiller: <https://orcid.org/0000-0002-5388-5274>  
631 Gentile Francesco Ficetola: <https://orcid.org/0000-0003-3414-5155>

632 **TABLES**

633 Table 1. Treatment contrasts assessing differences between the control (immediate extraction) and four  
 634 approaches to soil conservation before eDNA extraction. Each conservation treatment was compared  
 635 against the control in order to determine the percentage of explained variability.

|   | Bact02                    |          | Fung02                    |          | Euka02                    |          |
|---|---------------------------|----------|---------------------------|----------|---------------------------|----------|
|   | Explained variability (%) | <i>P</i> | Explained variability (%) | <i>P</i> | Explained variability (%) | <i>P</i> |
| Treatment 2: room temperature, extraction after 6h;                     | 0.58                      | 0.956    | 0.53                      | 1        | 0.44                      | 0.993    |
| Treatment 3: 4°C, extraction after 3 days;                              | 0.81                      | 0.563    | 0.58                      | 0.976    | 0.54                      | 0.949    |
| Treatment 4: silica gel immediately inserted, extraction after 21 days; | 3.14                      | <0.001   | 1.03                      | 0.518    | 0.63                      | 0.876    |
| Treatment 5: silica gel inserted after 6h, extraction after 21 days;    | 3.16                      | <0.001   | 0.73                      | 0.880    | 0.75                      | 0.725    |

636

637 **FIGURES CAPTIONS**

638 Fig. 1. Experimental sampling design.

639 Fig. 2. MOTUs richness across the different treatments (control: immediate extraction; T2: extraction after  
640 6h at room temperature; T3: extraction after three days at 4°C; T4: immediate preservation in tubes with  
641 silica gel, extraction after 21 days; T5: preservation in tubes with silica gel after 6h at room temperature,  
642 extraction after 21 days) before (left) and after (right) removing MOTUs with frequency < 1% in each  
643 sample.

644 Fig. 3. Plots of non-metric dimensional scaling showing dissimilarities of communities among the three  
645 habitats: broadleaved forest (black); grassland (green); vegetated riverbank (blue). Each plot shows the  
646 results of metabarcoding analysis based on soil samples subjected to five different treatments.

647 Fig. 4. Procrustes correlation between communities obtained from metabarcoding analyses based on soil  
648 samples across environmental conditions subjected to five sample treatments (control: immediate  
649 extraction; RT+6h: extraction after 6h at room temperature; 4°C: extraction after three days at 4°C;  
650 silicagel: immediate preservation in tubes with silica gel, extraction after 21 days; silicagel+6h: preservation  
651 in tubes with silica gel after 6h at room temperature, extraction after 21 days. All correlation coefficients  
652 are highly significant (all  $P < 0.0001$ ).

653 Fig. 5. Guidelines for improving monitoring strategies with eDNA from soil.