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1 **Multi-omics analyses from a single sample: Prior metabolite extraction does**
2 **not alter the 16S rRNA-based characterization of prokaryotic community**
3 **in a diversity of sample types**

4
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13

14 **Abstract**

15 Massive sequencing of the 16S rRNA gene has become a standard first step to describe and
16 compare microbial communities from various samples. Parallel analysis of high numbers of
17 samples makes it relevant to the statistical testing of the influence of natural or experimental
18 factors and variables. However, these descriptions fail to document changes in community or
19 ecosystem functioning. Non-targeted metabolomics are a suitable tool to bridge this gap, yet
20 extractions protocols are different. In this study, prokaryotic community compositions are
21 documented by 16S rRNA sequencing after direct DNA extraction, or after metabolites
22 extraction followed by DNA extraction. Results obtained using the V3-V4 region on non-
23 axenic cultures of cyanobacteria, lake water column, biofilm, gut of wild and lab-reared fish,
24 indicate that prior extraction of metabolites does not influence the obtained image of
25 prokaryotic communities. This validates sequential extraction of metabolites followed by
26 DNA as a way to combine 16S rRNA sequencing with metabolome characterization from a
27 single sample. This approach has the potential to complement community structure
28 characterization with a proxy of their functioning, without the uncertainties associated with
29 the use of separate samples.

30

31 **Introduction**

32 Omics approaches allow analyzing genomes, transcripts, lipids, proteins, and
33 metabolites to an unprecedented level of depth. Each is informing cellular processes and

34 molecular responses at particular time and size scales, making them highly complementary.
35 The next big step forward is thus to combine them into multi-omics approaches. Correlating
36 multi-layered datasets comes with major technical, statistical and conceptual challenges
37 (Krassowski et al., 2020; Tarazona et al., 2021). One basic challenge not to be neglected,
38 particularly relevant to microbiologists, is the fact that these approaches are often destructive,
39 and thus cannot be routinely performed from a single small sample and require parallel
40 extraction on split samples (*e.g.* (Clerissi et al., 2023)).

41 One of the most commonly applied approaches in microbial ecology is the
42 characterization of microbial community compositions using high-throughput sequencing of
43 ribosomal RNA-encoding genes from extracted DNA (Alivisatos et al., 2015; Dubilier et al.,
44 2015). Despite it is widely used and highly informative, this approach providing a description
45 of « who is there » is often criticized for yielding limited information about « what is going
46 on » (Louca et al., 2018). Quick and easy ways to address the functioning of microbial
47 systems with a desirable level of replication are wanting. In this context, non-targeted
48 metabolomics has emerged as a fast, convenient and sensitive approach for fingerprinting the
49 functioning of various biological systems (Colas et al., 2020; Le Manach et al., 2019; Sogin et
50 al., 2019; Zhang et al., 2016). Indeed, metabolome composition of an organism or community
51 can be used as a proxy of its phenotype, as it is a function of organisms (or holobionts)
52 genotype and its interaction with biotic and abiotic factors. Metabolome composition can vary
53 quickly, within seconds, in response to stimuli, stress, changes in the environment or overall
54 health status including age, sex, disease and treatments (Khemtong et al., 2015; Rauckhorst et
55 al., 2022). Metabolomics datasets are usually difficult to interpret on their own, in particular
56 owing to the lack of proper databases that could link peaks identified in mass spectrometry to
57 particular metabolites, even more so in the microbial world (Gertsman and Barshop, 2018).
58 However, these are particularly relevant when combined in multi-omics approaches, for they
59 can provide a quick measure of functional variations that can be linked to another omics, as
60 for example recently performed for transcriptome analysis (Madaj et al., 2023).

61 Once metabolites are extracted from a sample, for example with classical methanol
62 extraction procedure (Prasad Maharjan and Ferenci, 2003), other types of molecules can be
63 recovered from the remaining pellet including proteins, lipid membranes and DNA (Roume et
64 al., 2013; Valledor et al., 2014). However, the effect of prior methanol extraction of
65 metabolites on the datasets obtained when analyzing these other molecules has rarely been
66 investigated so far. Interestingly, a recent study has shown that RNAseq dataset quality was
67 only marginally affected by prior metabolite extraction with 80% methanol in mice model,

68 suggesting that single sample combination of metabolomics and transcriptomics was tractable
69 in this case (Madaj et al., 2023).

70 For microbiologists, being able to produce various omics data from a single sample is
71 particularly relevant. Indeed, microbial habitats are heterogeneous at a micrometer scale, for
72 example in the multiple layers of a biofilm, in micro niches in soils, or in different regions of
73 a host's gut (Nunan, 2017; Shaffer et al., 2022). Splitting samples prior to applying different
74 omics approaches to distinct subsamples thus potentially hampers our ability to apply
75 correlation algorithms on results from the obtained datasets if samples are heterogenous.
76 Single sample characterization of metabolites and prokaryotic community compositions could
77 offer a quick and efficient way to correlate changes observed in the composition of
78 communities and metabolome, linking community structure to functional fingerprinting.
79 Besides this evident complementarity, both methods are time- and cost-effective, allowing
80 comparison of numerous samples, and thus replication levels that allow proper hypothesis
81 testing (Singh et al., 2019). Various recent studies have emphasized the potential of
82 combining these approaches, one remarkable example identifying taxa and metabolites
83 specific to different environments in a large scale study of earth microbiome (Shaffer et al.,
84 2022). Although most of these studies did split samples in aliquots dedicated to each of the
85 methods (Nothias et al., 2023; Shaffer et al., 2022), a few have performed combined
86 extractions of metabolites and DNA including by ourselves (Foucault et al., 2022; Gallet et
87 al., 2023). However, the absence of potential bias introduced by this method using internal
88 control still remains to be demonstrated.

89 In order to investigate whether it is possible to use a single sample to perform both
90 metabolomic and prokaryotic community analyses without affecting results from the latter,
91 the aim of this study is to test whether prior metabolite extraction modifies the recovered
92 image of community compositions. For this, we analyzed community compositions using a
93 standard 16S rRNA high-throughput sequencing approach (V3-V4 region) on two types of
94 extractions: classical DNA extraction using a kit, or standard methanol metabolite extraction
95 followed by the same classical DNA extraction performed on the pellet. Communities
96 structure and compositions were compared after the two types of extraction on different types
97 of samples that are commonly analyzed, some coming from lab settings (non-axenic
98 cyanobacterial strains cultures and gut of lab-reared fish) and some from the environment
99 (lake water, river biofilm, and gut of a wild fish). To our knowledge, this study is the first
100 attempt to test whether single sample metabolite followed by DNA extraction introduces a
101 bias in the obtained image of prokaryotic communities.

102

103 **Material and methods**

104

105 *Sample preparation*

106 Four clonal non-axenic cyanobacterial strains of *Microcystis aeruginosa* were
107 obtained from the culture collection of cyanobacteria at the Museum national d'Histoire
108 naturelle (Hamlaoui et al., 2022). Strains PMC-728.11, PMC-807.12, PMC-810.12 and PMC-
109 826.12 were cultured in BG-11 medium at 25°C in 250-mL Erlenmeyer vessels, with a photon
110 flux density of 12 mmol·m⁻²·s⁻¹ and a 12:12-h light/dark cycle and was lyophilized (24h at -
111 50°C). Gut of six medaka fish (*Oryzias latipes*) from the Museum national d'Histoire
112 naturelle rearing facility, and from one wild specimen of *Perca fluviatilis* from the Jablines
113 lake (48°55 03.3 N 2°43 31.3 E) were dissected, flash-frozen in liquid nitrogen, pooled and
114 ground to power using mortar and pestle, and lyophilized (24h at -50°C). Three distinct water
115 sample were produced by filtering 500 mL of water from three lakes near Paris, namely
116 Champs-sur-Marne (filter 1, 48°51 47.6 N 2°35 51.6 E), Cergy (filter 2, 49°01 29.5 N 2°02
117 47.0 E) and Jablines (filter 3, 48°54 48.3 N 2°43 24.8 E) on a 47 mm diameter, 0.22 µm pore
118 sized PES filter (IT4IP, Belgium). Filters were flash frozen in liquid nitrogen. Mature river
119 biofilms were collected after four weeks of natural colonization on glass slides (5 x 10 cm) on
120 the *Gave de Pau* (43°24 14.2 N 0°37 30.4 W) water (Colas et al., 2023), then lyophilized prior
121 to analysis. After homogenization, the different PMC strains biomasses, ground guts of the
122 two fish species, and biofilms were split into six replicate subsamples by dividing the
123 lyophilizates, three to be used for regular DNA extraction, and three for combined metabolite
124 and DNA extraction. Filters from each of the three lakes were split in two halves, one for each
125 extraction protocol.

126

127 *Metabolite extraction*

128 Prior mechanical extraction (GLH850 OMNI; 25 000 r. min⁻¹; 30s) was performed on
129 all sample types except PMC strains. In all sample types, sonication (Sonics Vibra-Cell VCX
130 13; 60% amplitude; 30s) was then performed on weighted biomass or filter samples
131 suspended in cold extraction solvent, consisting of 75-25% UHPLC methanol-water, 1 mL
132 per 100 mg of tissue or per 10 mg of lyophilized culture, on ice (Colas et al., 2020). After
133 centrifugation (10 min; 4°C; 15,300 g), remaining pellets were dried and used for subsequent
134 DNA extraction.

135

136 *DNA extraction*

137 DNA was extracted from the pellets obtained after metabolites extraction using the
138 QIAGEN POWERLYZER POWERSOIL DNA extraction kit with a prior FastPrep 5G beat
139 beater disruption (DNA Matrix; 5×30s; 8m.s⁻¹) following manufacturer's instructions
140 (Foucault et al., 2022; Gallet et al., 2023). An extraction-blank control sample was also
141 performed. Direct DNA extractions were also performed on the other three replicates of each
142 sample type. In this case, DNA extraction was conducted using the same prior Bead-beating
143 step, DNA extraction kit and protocol as above (but without prior extraction of metabolites).

144

145 *Sequencing and analysis of the V3-V4 region of prokaryotic 16S rRNA*

146 The V3-V4 region of the 16S rRNA encoding gene was amplified using primers 341F
147 (5'-CCTACGGGNGGCWGCAG -3') and 806R (5'-GGACTACVSGGGTATCTAAT-3')
148 (Parada et al., 2016) and sequenced on an Illumina MiSeq 250×2 bp platform (GenoToul,
149 Toulouse, France). Reads were deposited into the Sequence Read Archive (SRA) database
150 (accession number PRJNA995525 (samples SRR25302593 - SRR25302641); Table S1).

151 Sequence analysis was performed using the QIIME2-2022.8 pipeline (Bolyen et al., 2019).
152 Forward and reverse reads were trimmed at 230 and 225 bp, respectively. Amplicon Sequence
153 Variants (ASVs) were obtained with the DADA2 plugin (default parameters) and affiliated
154 with the SILVA 138-99 database. Diversity metrics were computed in RStudio (v4.1.3) with
155 the R package phyloseq (v1.38.0) (McMurdie and Holmes, 2013). Statistical analyses were
156 performed using R packages vegan (v2.6-4) (Oksanen et al., 2022) and pairwiseAdonis (v0.4)
157 (Arbizu, 2022). All values are displayed as median ± standard deviation unless otherwise
158 indicated.

159

160 **Results and discussion**

161

162 *Non-significant impact of extraction method on diversity levels*

163 Direct DNA extraction as well as sequential extraction of metabolites and DNA
164 yielded similar PCR profiles with the primers used, and comparable numbers of reads in the
165 Illumina sequencing (supplementary table 1). ASV richness, evenness and Shannon index
166 obtained using both extraction procedures were not significantly different for a given sample
167 type (Figure 1A-C, Kruskal-Wallis (KW) $p > 0.05$, supplementary tables 1 and 2). On the
168 other hand, major differences were observed among sample types. Lowest richness was
169 observed in non-axenic cyanobacterial strains, dominated by the *Microcystis aeruginosa*

170 ASVs, which is congruent with recent reports indicating that these strains harbor a
171 phycosphere that consists of a limited number of co-isolated or attached bacteria (Fu et al.,
172 2020; Louati et al., 2015; Pascault et al., 2021; Seymour et al., 2017). Low richness was also
173 observed in the medaka fish, congruent with recent reports (Duval et al., 2022) and the
174 reported impact of domestication which usually leads to lower diversity compared to wild
175 relatives (Alessandri et al., 2019; Hird, 2017). This is indirectly illustrated by the higher
176 diversity level observed in the wild specimen of *Perca fluviatilis* collected from a lake
177 compared to the medaka *Oryzias latipes* (Figure 1A-C; KW $p < 0.05$, Wilcoxon test (WT) $p <$
178 0.05 ; Supplementary tables 1 and 2). Highest diversity levels were observed in the Gave de
179 Pau river's biofilm sample that contain a quite diverse microbial community as previously
180 observed for other biofilms (Romero et al., 2020), as biofilms are reported as hotspots of
181 microbial diversity (Wu et al., 2019). Overall, recovered richness levels are thus congruent
182 with the literature, and unaffected by the extraction method. This suggests that prior
183 extraction of metabolites does not affect alpha diversity metrics in both low- and higher-
184 diversity communities.

185

186 *Extraction method does not affect community composition and comparisons*

187 Comparison based on Jaccard distances indicated that community compositions were
188 highly similar among replicates from a given sample type and extraction method, as well as
189 between the two extraction methods (DNA alone or sequential, Figure 1D; PERMANOVA p
190 > 0.05 ; Supplementary table 2). Replicates from both extraction methods for each sample type
191 formed tight clusters. The only exception was one of the three samples from *P. fluviatilis* for
192 which DNA was extracted directly. It appeared outside of the cluster formed by the two others
193 and the three on which sequential extraction was performed (Figure 1D). Clusters
194 corresponding to each sample type were separated from each other except for clusters PMC-
195 810.12 and PMC-826.12 which were overlapping. Because Jaccard distance is based on ASVs
196 presence and absence, these results indicate that the two extraction methods yielded many
197 identical ASVs, and thus a comparable image of the prokaryotic community. Bray-Curtis
198 similarity plot, which also accounts for the abundance of these shared ASVs, yielded an
199 overall similar picture (Figure 2A). One difference was that PMC-728.11 clustered with
200 PMC-810.12 and PMC-826.12, suggesting a high abundance of the ASVs shared with the
201 latter two communities.

202 With the exception of the aforementioned *P. fluviatilis* sample, Bray-Curtis pairwise
203 dissimilarities computed between couples of samples within a given sample type based on

204 direct DNA extraction and sequential extractions were highly similar and correlated (Figure
205 2B; spearman 0.62, ANOVA R^2 0.76, $p < 0.01$; Supplementary table 2), suggesting that the
206 extraction method had a very limited influence on inter-sample comparison metrics, and thus
207 on our ability to properly compare community compositions. Compositions from individual
208 replicates confirmed that they were very similar within a given sample type, regardless of the
209 extraction method applied (Figure 2C). Various expected taxa typical of each sample type
210 were identified, including for example abundant Firmicutes in fish samples (Llewellyn et al.,
211 2014), Cyanobacteria, Proteobacteria, Bacteroidota and Verrucomicrobiota in peri-urban lakes
212 (Louati et al., 2015), and the dominance of cyanobacteria in *Microcystis aeruginosa* strains as
213 well as in the phototrophic biofilm. A similar picture was obtained at the genus level
214 (Supplementary figure 1).

215

216 In conclusion, we found no evidence that prior extraction of metabolites had any
217 influence on the result of prokaryotic community characterization using the V3-V4 region of
218 the 16S rRNA-encoding gene and Illumina sequencing. This held true in a variety of sample
219 types including low (strains) as well as high diversity communities (biofilm). These results
220 indicate that metabolomic and 16S rRNA-based community characterization can be
221 performed from a single sample, offering a unique opportunity to supplement the description
222 of prokaryotic communities with a fingerprint of microbiome functioning. Single sample
223 multi-omics are particularly relevant to microbiology because of the heterogeneity of most
224 microbial habitats, the small scale at which we need to work, and potential cost- or biology-
225 related limited sample availability. In the future, it will be important to confirm with
226 additional sample types, 16S rRNA regions (Bukin et al., 2019), and other metabolite
227 extraction mixes, but most importantly to test which additional types of omics could be
228 performed from a single sample.

229

230 **Data availability**

231 R scripts are available on Github upon publication (<https://github.com/PierreFoucault/>). All
232 microbial communities' samples sequencing reads were deposited into the NCBI Sequence
233 Read Archive (SRA) database (accession number PRJNA995525 (samples SRR25302593 -
234 SRR25302641); Supplementary Table 1).

235

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238 Kandiah Santhirakumar who managed the medaka fish maintenance.

239

240 **Author’s contributions**

241 BM and AG designed the sequential extraction protocol. SD and BM designed the study. CD,
242 MG, AG, SC and PF performed the extractions and produced the DNA datasets. PF and SD
243 analyzed the results. SD drafted the manuscript, all authors contributed and agreed with the
244 latest version.

245

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251

252 **Ethics**

253 Experimental procedures were carried out in accordance with European legislation on animal
254 experimentation (European Union Directive 2010/63/EU) and were approved for ethical
255 contentment by an independent ethical council (CEEA Cuvier n°68) and authorized by the
256 French government under reference number APAFiS#19316-2019032913284201 v1. Fish
257 were anesthetized in 0.1% tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO)
258 buffered with 0.1% NaHCO₃ prior to sacrifice.

259

260 **Supplementary table 1:** sample IDs, SRA accession numbers, primer pairs used, raw and
261 filtered (quality check, merging, chimera removal and taxonomy filtered) paired-end reads
262 counts, metadata, Shannon, richness and evenness indices.

263

264 **Supplementary table 2:** Alpha and Beta diversity statistical test summaries.

265

266 **Supplementary figure 1:** prokaryotic community composition at the genus level in each
267 individual sample. Only the 20 most abundant genera are displayed.

268

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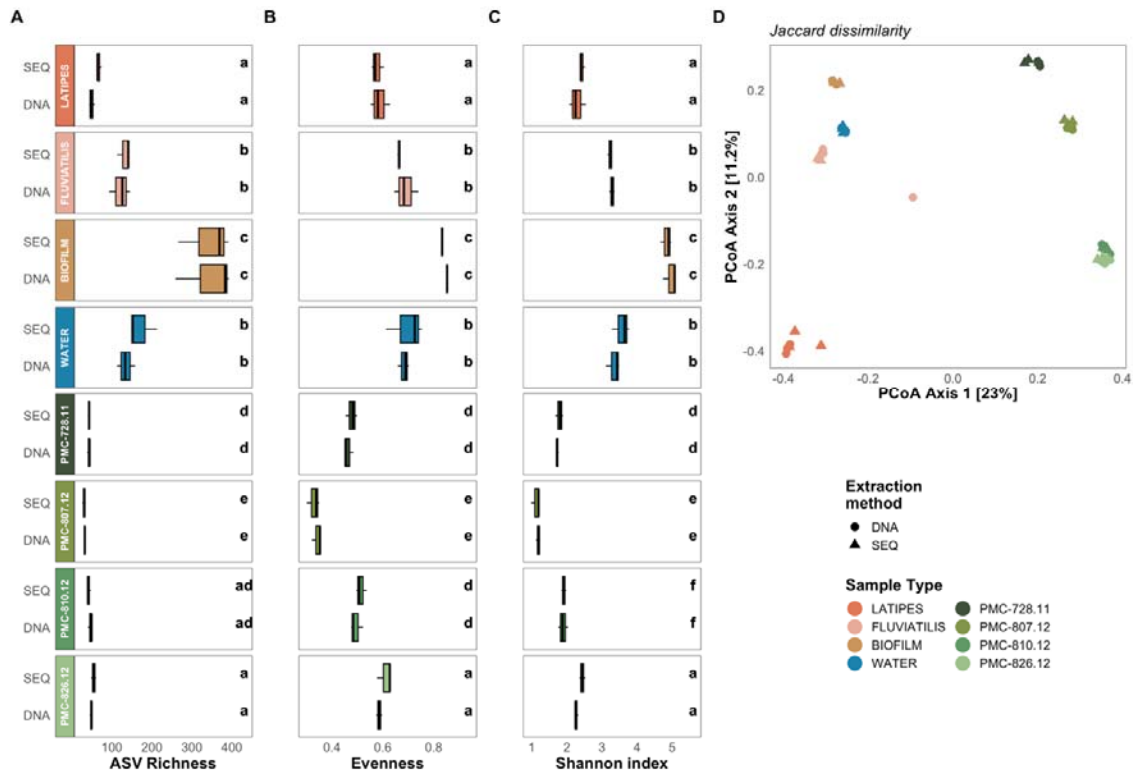
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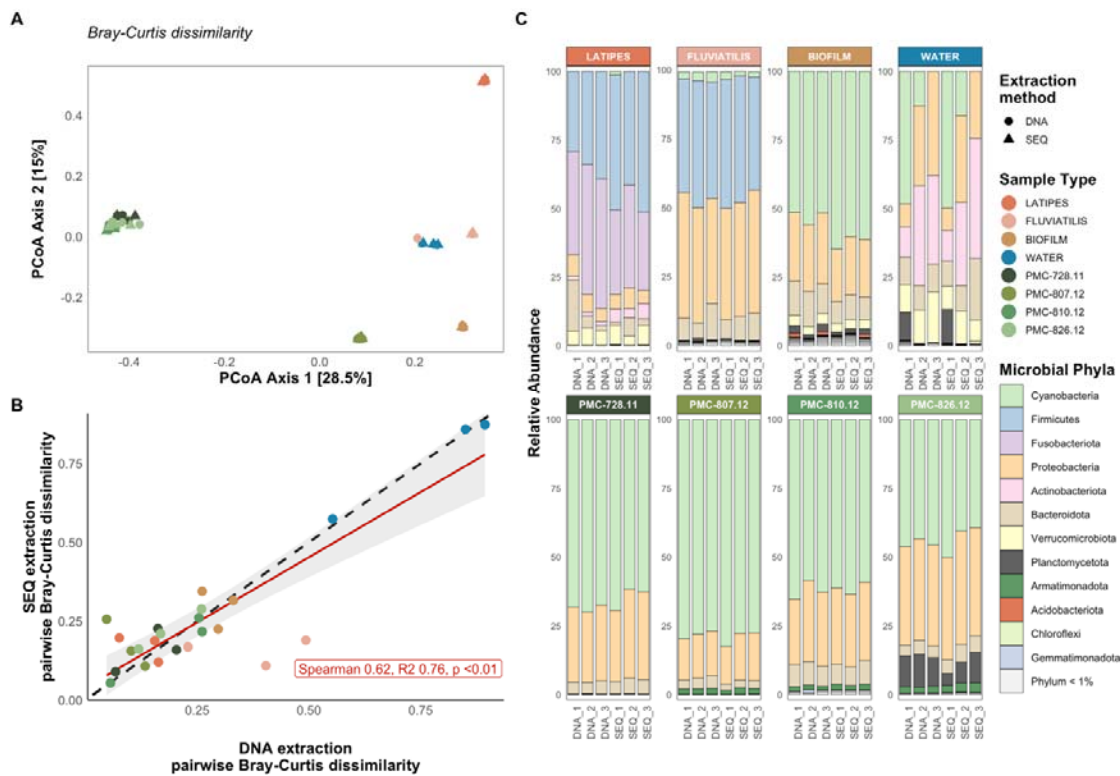
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442 **Figure 1:** ASV richness (A), evenness (B) and Shannon index (C) for both extraction
443 procedures, namely metabolites then DNA (sequential, SEQ) and classical DNA extraction
444 (DNA) in the eight sample types (see methods). Median values and confidence intervals
445 provided are based on three replicates per extraction type and sample type, with lowercase
446 letters (a to e) on histograms indicating significance groups (Kruskall-Wallis test followed by
447 a Wilcoxon post-hoc test). D: principal coordinates analysis of individual samples based on
448 Jaccard distances, displaying explained variance percentage explained by the first two axes.



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 459 **Figure 2:** A: Principal coordinates analysis of individual samples based on Bray-Curtis
 460 distances, displaying explained variance percentage explained by the first two axes. B: Bray-
 461 Curtis pairwise dissimilarities between couples of samples within a given sample type
 462 computed from sequential (SEQ, y-axis) and DNA (DNA, x-axis) extractions. Red line
 463 corresponds to inferred linear relation, dashed black line corresponds to the 1:1 diagonal. C:
 464 prokaryotic community composition at the phylum level in each individual sample.



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