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1 **Nitrogen to phosphorus ratio shapes bacterial community involved in cellulose**
2 **decomposition and copper contamination alters their stoichiometric demand**

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7 **Abstract**

8 All living organisms have theoretically an optimal stoichiometric nitrogen:phosphorus
9 (N:P) ratio below and beyond which their growth is affected but data remain scarce for
10 microbial decomposers. Here, we evaluated optimal N:P ratios of microbial communities
11 involved in cellulose decomposition and assessed their stability when exposed to copper
12 Cu(II). We hypothesized that (1) cellulose decomposition is maximized for an optimal N:P
13 ratio, (2) copper exposure reduces cellulose decomposition and (3) increases microbial
14 optimal N:P ratio, (4) N:P ratio and copper modify the structure of microbial decomposer
15 communities. We measured cellulose disc decomposition by a natural inoculum in
16 microcosms exposed to a gradient of N:P ratios at three copper concentrations (0, 1 and 15
17 μM). Bacteria were most probably the main decomposers. Without copper, cellulose
18 decomposition was maximized at an N:P molar ratio of 4.7. Contrary to expectations, at high
19 copper concentration, the optimal N:P ratio (2.8) and the range of N:P ratios allowing
20 decomposition were significantly reduced and accompanied by a reduction of bacterial
21 diversity. Copper contamination led to the development of tolerant taxa probably less efficient
22 in decomposing cellulose. Our results shed new lights on the understanding of multiple
23 stressor effects on microbial decomposition in an increasingly stoichiometrically imbalanced
24 world.

25 **Key words:**

26 N:P ratio, metal contamination, ecological stoichiometry, nutrient immobilization, nutrient
27 mineralization, bacterial diversity, microbial decomposer

28 **1. Introduction**

29 Decomposition is the biological process that leads to mass reduction and transformation of
30 dead organic matter (Gessner *et al.* 2010). It is a ubiquitous process and a fundamental link in
31 the biogeochemical cycles of elements and central for ecosystem functioning. Plant litter
32 decomposition is a complex process controlled simultaneously by intrinsic factors,
33 environmental factors and abundance and diversity of biological actors (Gessner *et al.* 2010;
34 Bani *et al.* 2018). Some heterotrophic microorganisms, including both fungi and bacteria,
35 form the functional group of microbial decomposers which are the main actors of
36 decomposition (Gessner, Chauvet and Dobson 1999). Among the environmental factors
37 likely to impact the plant litter decomposition process, nutrient availability in the surrounding
38 environment of the litter plays a crucial role (Güsewell and Verhoeven 2006; Güsewell and
39 Gessner 2009; Woodward *et al.* 2012). Plant litter is generally nutrient depleted (Moore *et al.*
40 2004), and microbial decomposers must balance their nutrient requirements through the
41 consumption of mineral elements coming from their environment (a process called nutrient
42 immobilization: Harte and Kinzig 1993; Daufresne and Loreau 2001). When these
43 requirements are satisfied, these organisms are able to efficiently decompose plant litter,
44 converting it into their biomass. At the same time, they also release inorganic carbon through
45 their respiration and nutrients through their exoenzymatic activities, these elements being
46 considered as recycled back into ecosystems (inorganic carbon and nutrient releases
47 correspond to the mineralization process: Harte and Kinzig 1993). Several reviews clearly
48 confirmed the stimulating effects of nutrient enrichment on decomposition rates (Ge *et al.*
49 2013; Ferreira *et al.* 2015b; Zhang *et al.* 2018), suggesting the idea that a balanced nutrient
50 availability which allows the microorganisms to optimally fulfil their nutrient demand should
51 be beneficial for maximizing their decomposition activity. A precise knowledge of the
52 elemental requirement of microbial decomposers is thus an essential prerequisite for

53 predicting the speed and intensity of litter decomposition as well as nutrient and carbon
54 releases.

55 In the ecological stoichiometry framework, the imbalance between consumer
56 requirements and elements availability is often referred to as “stoichiometric constraints”
57 (Sterner and Elser 2002). Among all the necessary chemical elements that ensure living
58 organism growth, nitrogen (N) and phosphorus (P) are generally the most commonly
59 considered. N is one of the main components of amino acids (proteins), and P is, among
60 others, a fundamental component of nucleic acids (DNA and RNA). Litter decomposition is
61 frequently controlled by N and P availability in the medium (Woodward *et al.* 2012). For
62 microbial decomposers, the overall litter and environmental N:P ratio may therefore be a
63 potential indicator for determining whether the N or P might be limiting for microorganisms
64 growth and activity, at least during the early stage of decomposition when carbon availability
65 is not limiting (Tessier and Raynal 2003).

66 While the specific optimal elemental ratios have long been studied in plants (e.g. Tilman
67 1985) and animals (e.g. Frost *et al.* 2006), data are far less available for microorganisms.
68 Using natural microbial inoculum, Güsewell and Gessner (2009) shown that cellulose and
69 plant litter decomposition were maximized at intermediate available N:P ratios, these ratios
70 corresponding to optimal N:P ratios for the decomposition process at the community level.
71 Changes in the available N:P ratios also resulted in altered balance between microbial
72 decomposers, as shown by changes in the bacteria to fungi ratio along their N:P gradient.
73 From a stoichiometric viewpoint, such changes in community structure could result from
74 competitive exclusion and the selection of species based on their optimal nutrient
75 requirements along the N:P availability gradient (Danger *et al.* 2008). Thus, based on
76 microbial nutrient requirements, the relative N to P availability should, at least partly,
77 determine community structure and composition. Concerning the decomposition process,

78 these species replacements are expected to maximize nutrient uptake and reduce variations in
79 litter decomposition efficiency (Fanin *et al.* 2013).

80 Giving the importance of microbial optimal nutrient ratios for the decomposition process
81 and community structures, understanding the response of these optimal ratios to
82 environmental stressors might be of particular interest. If multiple environmental stressors
83 impact microbial diversity, plant litter decomposition may be affected (Niyogi *et al.* 2009;
84 Gessner *et al.* 2010; Ferreira *et al.* 2015a; Tolkkinen *et al.* 2015). Both nutrient availability
85 (Falkowski *et al.* 2000) and ratios (Elser *et al.* 2009; Peñuelas *et al.* 2013) can be problematic
86 for ecosystem functioning due to changes in nutrient balance. Metal contamination is another
87 major recurrent stressor. Metals have long been shown to reduce plant litter decomposition
88 rates in lands (Berg *et al.* 1991) and in streams (Duarte *et al.* 2008). The number of litter-
89 colonizing fungus decreases in presence of high metal concentrations (Solé *et al.* 2008).
90 Microbial activity is lower in metal polluted environments (Niyogi *et al.* 2009). Copper (Cu)
91 represents a classical model of metallic contaminant. This metal is still actively used in
92 agriculture, resulting in its accumulation in soils and leaching into surface water ecosystems.
93 Cu has been largely studied and is known to alter microbial community structures (Wakelin *et*
94 *al.* 2010; Keiblinger *et al.* 2018) and affect litter decomposition (Fernández *et al.* 2015).

95 In this study, using Cu as a model of contaminant, we investigated its impact on the
96 optimal elemental N:P ratios for cellulose decomposition by microbial decomposers. We
97 hypothesized that (H1) cellulose decomposition rate reaches its maximum at an optimal N:P
98 ratio; (H2) high Cu concentration reduces the overall cellulose decomposition rate; (H3) due
99 to increased N-demand for detoxification and reduced microbial growth, the optimal N:P ratio
100 increases under higher Cu contamination, and (H4) both N:P ratio and the presence of Cu
101 interactively affect the composition of microbial decomposer communities. Since natural
102 plant litter nutrient content is often variable (Cornwell *et al.* 2008), pure cellulose,

103 representing a part of plant cell walls, was used as a model carbon substrate to control N and
104 P quantities, as successfully used in the past (Hopkins *et al.* 1990; Chew, Obbard and
105 Stanforth 2001; Güsewell and Gessner 2009). To test our hypotheses, we quantified the
106 decomposition of cellulose discs by a natural inoculum of microbial decomposers in
107 microcosms under laboratory-controlled conditions. Decomposition was followed along a
108 gradient of available N:P ratios in liquid culture media and under three concentrations of
109 dissolved Cu: 0 μM , 1 μM and 15 μM . After 79 days of incubation, we measured the mass
110 loss of the cellulose discs, the quantities of N and P immobilized on the discs by microbial
111 decomposers and we analysed the abundance and diversity of the microbial decomposers.

112 **2. Material and methods**

113 *2.1. Microbial inoculum preparation*

114 Leaves (mainly beech) were collected in 2015, by using suspended filets around trees (1 m
115 above ground) in the forest near the Maix stream in the Vosges Mountains (N53.9143°
116 E27.5791°). In early 2018, eight litterbags (500 μm mesh size) each containing 5 g of leaves
117 (dry weight) were submerged in the same stream to allow bacterial and fungal colonization.
118 The bags were retrieved after 14 days along with samples of stream water. Leaf litter
119 contained in the bags were gathered. Microbial inoculum was prepared by putting 20 g (fresh
120 weight) of leaf litter in 5 L Erlenmeyer flasks with 1 L of fresh stream water, incubated under
121 gentle agitation and aerobic condition for 48 hours at 18 °C. After the incubation, water was
122 separated from large litter debris by passing through a sieve (1 mm mesh size) and was left at
123 4 °C to decant for 2–3 hours before collecting the supernatant. This microbial inoculum was
124 stored for less than 24 hours at 4°C prior its use for the experiment.

125 The initial number of fungal spores and bacteria was determined on aliquots of the
126 inoculum. Fungal spores were counted under an optical microscope, inoculum was fixed with
127 formaldehyde (2%) and stained with Trypan blue (0.05%) before being filtered (Millipore

128 MF™ 5.0 µm SMWP02500). Bacteria were counted through DAPI (1 µg mL⁻¹) staining after
129 filtration (Whatman Nucleopore Track-Etch membrane 25 mm, 0.2 µm). Seven images were
130 taken using an epifluorescence microscope (Nikon C-HGFIE equipped with a DS-Qi1Hc
131 CCD camera) and were analysed using a NIS-Elements BR software (Nikon). Although we
132 planned to have a mixed inoculum, the microcosms were largely inoculated with bacteria (c.a.
133 9.6×10^7 bacteria per microcosm) and no fungal spores but with a few mycelia fragments (see
134 discussion).

135 2.2. *Microcosm preparation*

136 The microcosms were prepared under sterile condition, following the model from a
137 previous study (Güsewell and Gessner 2009). In a plastic Petri dish (Ø 90 mm) containing
138 50 g of river sand and covered by a nylon mesh (250 µm porosity), five cellulose discs of Ø
139 22 mm were placed (Fig.S1). The river sand was sieved (< 2 mm), washed, dried and burned
140 at 450 °C for over four hours to sterilize and remove all traces of organic material. The
141 cellulose discs were cut out from cellulose paper filter (Whatman ashless 110 mm, GE),
142 sterilized and were used as the only carbon source for microorganisms. The total cellulose dry
143 mass introduced into each microcosm was 181.5 ± 5.7 mg (36.3 ± 1.2 mg per disc).

144 Each microcosm was filled with 18.5 mL of a sterilized COMBO mineral medium
145 containing 36.8 mg L^{-1} CaCl₂·2H₂O, 37.0 mg L^{-1} MgSO₄·7H₂O, 12.6 mg L^{-1} NaHCO₃,
146 28.4 mg L^{-1} Na₂SiO₃·9H₂O and 24.0 mg L^{-1} H₃BO₃ (Kilham *et al.* 1998) Nitrogen (N),
147 phosphorous (P) and copper (Cu) were also included but with different concentrations to
148 obtain desired N:P ratios and Cu contamination levels.

149 Seven N:P ratios (molar) were created by varying the amount of both N and P in each
150 medium using NH₄NO₃ for N, and a mixture of KH₂PO₄ and K₂HPO₄ (3.85/6.15 v/v, for pH
151 7) for P. The seven N:P ratios were 1.2, 3.7, 11, 33, 100, 300 and 898 with a global supply
152 level of 0.75 [geometric mean of N and P mass in mg for all N:P ratios, i.e. in a microcosm,

153 $(m_N \times m_P)^{0.5} = 0.75$, see Güsewell and Gessner 2009]. Three Cu contamination levels (0, 1
154 and 15 μM) were tested by adding $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ to each medium. The initial concentrations
155 of N, P and Cu were verified. N and P reached at least 86.4 % of their nominal values, which
156 made the practical N:P ratios vary between 93.9 % and 97.5 % of the theoretical values.
157 Measured copper concentrations were $0.69 \pm 0.06 \mu\text{M}$ and $13.33 \pm 0.83 \mu\text{M}$ for the media
158 containing 1 and 15 μM respectively.

159 A total of 21 conditions (seven N:P ratios \times three [Cu]) were tested. Each condition was
160 replicated in five microcosms. Additionally, controls without inoculum were included.

161 The inoculation was done by dispensing 300 μl of freshly prepared and well-homogenized
162 inoculum directly onto each cellulose disc (1.5 mL per microcosm). The microcosms were
163 then placed in dark boxes and kept in an incubator at 18 °C. A water tray was placed in each
164 box to maintain humidity and avoid media evaporation. Humidity of each microcosm was
165 monitored every two weeks during the incubation period. If necessary, water levels were
166 adjusted with MilliQ ultrapure sterile water. Since we were interested in early stages of
167 cellulose decomposition, we initially planned to stop the experiment when ca. 35-50% of
168 detritus mass loss was reached. At day 79, some of the cellulose discs were becoming almost
169 transparent suggesting an advanced stage of decomposition, thus we decided to stop the
170 experiment. Each cellulose disc was collected and conserved individually. To take into
171 account potential heterogeneity between discs in each microcosm, the purpose of each disc
172 was decided randomly. Two discs were freeze-dried and used for cellulose mass loss
173 calculation, one of them was then used for total N and P quantification and the other one was
174 kept for ergosterol extraction. A third disc was used for DNA extraction, and the last two
175 discs were stored at -20 °C as backup.

176 2.3. *Mass loss, ergosterol and N P content*

177 Cellulose mass loss was determined by weighting the two freeze-dried discs to the nearest
178 0.1 mg. Subsequently, one of the discs was used to analyse total N and P content (70 samples,
179 seven N:P ratios × four replicates for [Cu] 0 μM, and seven N:P ratios × three replicates for
180 [Cu] 1 μM and 15 μM). Those discs were reduced into fine powder using Ø 3 mm, clean,
181 steel grinding balls and a bead beater (Mixer Mill, Retsch MM301). Between 1.5 and 2.0 mg
182 of powder was put into tin capsules (Säntis Analytical SA7698110) and used to analyse the
183 total N quantity by an elementary analyser (Carlo Erba NA 2100). The total P quantity was
184 analysed by first digesting 2.0 to 2.5 mg of the powder in 10 mL of NaOH (0.1 M) and
185 Na₂S₂O₈ (0.125 M) for two hours at 10⁵ Pa (1 bar) 120 °C. After cooling, P was measured on
186 the digested solutions using molybdate blue colorimetric method at 880 nm (ISO 6878:2004).
187 The second freeze-dried disc was used to determine ergosterol content (estimation of fungal
188 biomass). During inoculum preparation and incubation, absence of fungal spores and
189 development has been noted, Real-Time qPCR of fungal 18S rRNA genes also resulted low
190 copy numbers (see below). To confirm this result, only one replicate per condition (21
191 samples) was extracted and analysed for ergosterol, confirming the extremely low abundance
192 of active fungi. Ergosterol extraction and HPLC analyse followed a standard protocol
193 (Gessner 2005). Briefly, the cellulose disc was placed into a KOH/methanol (8 g L⁻¹) solution
194 overnight at 4 °C, then heated at 80 °C during 30 min for extraction. The extracts were loaded
195 into SPE cartridges (Oasis HLB 30 μm Extraction Cartridge). Ergosterol was eluted in 1.4 mL
196 isopropanol and stored at -20 °C until HPLC analyses. Extractions were carried out in a series
197 of 12 with 11 samples and a positive control with known concentration of HPLC grade
198 ergosterol (>95 %, Sigma Life Science). HPLC analyses were assured by Chromaster Hitachi
199 (Pump 5110, Autosampler 5210, UV Detector 5410), using LiChrospher RP18 column

200 (LiChroCART 250-4 HPLC cartridge, Merck), equipped with a column thermostat set at 33
201 °C (Jetstream 2 plus).

202 2.4. Molecular analyses (DNA extraction, qPCR, NG-sequencing, OTU analyses)

203 Total genomic DNA was extracted from one disc of each microcosm (65 samples, seven
204 N:P ratios × three [Cu] × three replicates plus two replicates of the initial inoculum), using the
205 PowerSoil DNA Isolation Kit (MO BIO), and following the manufacturer protocol. Between
206 3 and 7 ng μL^{-1} of total DNA (measured with a Shimadzu spectrophotometer equipped with a
207 Traycell tank) were recovered from each cellulose disc.

208 Real-Time qPCR targeting the 16S and 18S rRNA genes (bacteria and fungi, respectively)
209 were performed as described in Cébron *et al.* (2008) and Thion *et al.* (2012). The number of
210 16S and 18S rRNA gene copies was used to estimate bacterial and fungal relative
211 abundances. The primer pairs used were 968F (5'-GAACGCGAAGAACCCTTAC-3') and
212 1401R (5'-CGGTGTGTACAAGACCC-3') for bacterial 16S rDNA (Nübel *et al.* 1996) and
213 Fung5F (5'-GTAAAAGTCCTGGTTCCCC-3') and FF390R (5'-
214 CGATAACGAACGAGACCT-3') for fungal 18S rDNA (Smit *et al.* 1999; Vainio and
215 Hantula 2000). The amplification mix was prepared in 20 μL with: 10 μL 2× iQ SyberGreen
216 Supermix (Bio-Rad); 0.8 μL of each primer (10 μM); 0.4 μL of 3% bovine serum albumin
217 (BSA); 0.2 μL of dimethyl sulfoxide (DMSO); 0.08 μL of T4 bacteriophage gene-32 product
218 (QBiogene); 6.72 μL of DNA-free distilled water and 1 μL of template DNA or distilled
219 water (negative control) or ten-time dilutions of standard plasmids from 10^8 to 10^1 copies μL^{-1} .
220 The amplification programmes used for Real-Time qPCR were as follows: 95 °C for 5
221 min, 40 cycles with 20 s at 95 °C, 20 s at the primers specific annealing temperature (56 °C
222 and 50 °C for 16S and 18S rDNA, respectively), 30 s at 72 °C and 5 s measurement of
223 SYBR[®] Green I signal at 82 °C or 80 °C (for 16S and 18S rDNA, respectively), followed by a
224 melting curve analysis from 50 °C to 95 °C.

225 For bacterial 16S rRNA gene amplicon-sequencing, the primer pair used for the first-step
226 PCR were 341F (5'-CCTACGGGAGGCAGCAG-3'; Muyzer, de Waal and Uitterlinden
227 1993) and 787R (5'-GGACTACNVGGGTWTCTAAT-3'; Caporaso *et al.* 2011), each
228 attached to an adaptor for a second PCR. The amplification mix was prepared for 50 μ L final
229 volume with: 10 μ L 5 \times HF Buffer; 1 μ L of dNTP (10 mM); 0.25 μ L of dimethyl sulfoxide
230 (DMSO); 0.1 μ L of T4 bacteriophage gene32 product (QBiogene); 1.5 μ L of MgCl₂ (50
231 mM); 1 μ L of each primer (10 μ M); 33.05 μ L of DNA free distilled water; 0.1 μ L of Phusion
232 polymerase (Thermo Scientific) and 2 μ L of DNA samples (or distilled water for negative
233 control). 30 cycles of amplification at 56°C annealing temperature were performed. These
234 first-step PCR products containing unpurified DNA were then sent to Microsynth (Next
235 Generation Sequencing Department, Microsynth AG, Switzerland) for the second-step PCR
236 and MiSeq 2 \times 250 bp paired-end sequencing (Illumina).

237 A total of 4 443 671 paired-end reads from the 65 samples (accessible on SRA-NCBI
238 database under BioProject accession number PRJNA820766) were obtained. Paired-end
239 sequences were processed for quality filtering and clustered into operational taxonomic unit
240 (OTU) at 97% using Mothur (v1.44.3; Schloss *et al.* 2009) and following MiSeqSOP (first
241 access date: 14 December 2020; (Kozich *et al.* 2013)). Taxonomy was assigned using SILVA
242 ssu132 database. Singletons and sequences not affiliated to bacteria were removed. The final
243 dataset comprised 1 896 248 sequences ranging from 4576 to 81 348 sequences per sample.
244 Finally, the dataset was rarefied to the lowest number of sequences *per* sample (i.e. 4576
245 reads/sample, see Fig.S2 for rarefaction curves) to compare the diversity of 2219 OTUs
246 (including data from the initial inoculum). By doing so, we had sufficient sequencing depth
247 and statistical power to compare between conditions although some OTUs had been excluded.
248 Alpha diversity was expressed by calculating Chao1 richness and Shannon diversity indices
249 (Hill *et al.* 2003) using Mothur.

250 2.5. Statistical Analyses

251 All modelling and statistical analyses of our results were carried out with R software
252 (v4.1.0; R Core Team 2022). The N:P ratios were log transformed prior to all analyses.

253 We assume the cellulose mass loss caused by microbial decomposers follows a biphasic
254 tendency (symmetric “bell-shape” curve) alongside the log transformed gradient of N:P ratios.
255 For this reason, the cellulose disc mass loss data was fitted using a Gaussian type model with
256 four parameters:

$$y = (h - d) \exp\left(-2 \left(\frac{x - e}{w - e}\right)^2\right) + d$$

257 Where y is the cellulose disc mass loss in percentages, x is the log-transformed value of
258 N:P ratio, h is the maximum y value which is the height of the curve peak (i.e. the maximal
259 mass loss), d is the mass loss for very low and very high N:P ratios (i.e. the minimal mass loss
260 which is considered to be constant regardless of the N:P ratio), w (>0) is a shape parameter
261 which controls the width of the “bell”, it is the N:P ratio below which the mass loss reaches
262 97.5 % of d . It can also be seen as the highest N:P ratio beyond which the additional mass loss
263 over d is negligible. Finally, e (>0) is the value of x which corresponds to the maximum y
264 value, it is the log of the N:P ratios where the maximal mass loss can be observed. (Fig.1a
265 adapted from Larras *et al.* 2018)

266 Two-way analysis of variance (ANOVA) was used to evaluate the effect of N:P ratio,
267 [Cu] and their interaction on cellulose mass loss, and immobilized N and P quantities. In case
268 of a p -value lower than 0.05, Tukey HSD *Post-Hoc* tests were performed to determine which
269 treatments differ significantly from others. When ANOVA was inapplicable, nonparametric
270 Kruskal-Wallis test followed by Wilcoxon-Mann Whitney *Post-Hoc* test was applied.
271 Levene’s test was applied to verify homogeneity of variance between conditions. The linear
272 correlations between cellulose mass loss and OTU numbers, Chao1 or Shannon indices of all

273 three Cu contamination levels were obtained by calculating the Pearson's correlation
274 coefficient. Levene's test was from "car" R package (v3.0-13; Fox and Weisberg 2019), other
275 tests were from the "stats" package (v4.2.0; R Core Team 2022).

276 The beta diversity based on Bray-Curtis dissimilarity was represented using non-metric
277 multidimensional scaling (NMDS) analysis with two dimensions using the "metaMDS"
278 function of the "vegan" R package (v2.5-7; Oksanen *et al.* 2020). The differences in
279 community composition between each level of [Cu] were tested by permutational multivariate
280 analysis of variance (PerMANOVA) with 999 permutations using the "adonis2" function.

281 In an attempt to identify bacterial taxa that stand out the most under contrasted treatments,
282 indicator species were analysed. The indicator values (IndVal, <1) of OTUs were calculated
283 using the "multipatt" function of the "indicspecies" package (v1.7.9; de Cáceres, Legendre
284 and Moretti 2010). We used the group-equalized IndVal (IndVal.g) index to find OTUs that
285 were proportionally more likely to be present at a group. By limiting the index between 0.7
286 and 1 as done previously by other studies (Demircan *et al.* 2018), we can focus on the most
287 relevant OTUs. The higher the value, the more likely an OTU was abundant in that group.
288 Based on result similarities of bacterial diversities and cellulose decomposition at different
289 N:P ratios and [Cu] (see results section), we have decided to merge these results into four
290 groups: 0 and 1 μM of [Cu] were grouped together as "low Cu (CL)" and the group of 15 μM
291 as "high Cu (CH)", we omitted all the samples of N:P ratio of 33 as it is an intermediate ratio
292 and then grouped the three lower ratios together and the three higher ratios together (as "RL"
293 and "RH", respectively), thus forming four groups: CLRL, CLRH, CHRL and CHRH. The
294 indicator species analysis was performed with 9999 permutations on the rarefied dataset of
295 1603 OTUs (excluding data from the initial inoculum).

296 **3. Results**

297 *3.1. Cellulose disc mass loss – Optimal N:P ratio*

298 Cellulose discs lost up to 30 % of their initial dry mass. Mass losses depended on [Cu]
299 (ANOVA, $F = 62.365$, $Pr < 0.001$) and supplied N:P ratio ($F = 108.317$, $Pr < 0.001$), with a
300 significant interaction ($F = 3.652$, $Pr < 0.001$). Fig.1b shows the mean values of empirical
301 mass losses (points) and the statistical models (curves). For all three Cu contamination levels,
302 the maximal mass loss occurred at the supply ratio of 3.7 and the minimal mass loss occurred
303 at ratios greater than 100. For lower [Cu] (0 and 1 μM of Cu), the minimum mass loss was
304 about 10 % while it was as low as about 2 % for the group of 15 μM .

305 Values of each parameter and their confidence intervals (CI, 95 %) were extracted from
306 models. Optimal N:P ratios (parameter e , Fig.1c) decreased with increasing [Cu]. The CI
307 showed that the optimal N:P ratios for 0 and 15 μM of Cu (4.7 and 2.8, respectively) differed
308 significantly from each other. Minimal mass losses (parameter d , Fig.1d) showed no
309 difference between 0 and 1 μM of Cu (8.0 % and 8.3 %, respectively) but both were
310 significantly different from that of 15 μM (1.5 %). On the other hand, the maximal mass
311 losses (parameter h , Fig.1e) between all three groups were not significantly different from
312 each other (28.6 %, 25.3 % and 23.9 % for 0, 1 μM and 15 μM of Cu, respectively), the value
313 tended to decrease with increasing [Cu]. Although the maximal effective N:P ratio (parameter
314 w , Fig.1f, N:P ratio beyond which the mass loss is minimal and stays constant) values were
315 not significantly different among the three Cu contamination levels, they were reduced from
316 102.4 to 33.6 between 0 μM to 15 μM of Cu.

317 *3.2. Immobilized N and P quantities on cellulose discs*

318 The quantity of immobilized N was independent of [Cu] (ANOVA, $F = 1.301$, $Pr =$
319 0.28158), but varied with provided N:P ratio ($F = 12.477$, $Pr < 0.001$) and showed a
320 significant interaction ($F = 3.308$, $Pr = 0.00147$) (Fig.2a). The variation was non-linear and
321 the difference ($\Delta = \text{Max.} - \text{Min.}$) was greater for samples without Cu (0.24 mmol g^{-1})

322 compared to the one with 1 μM and 15 μM of Cu (0.11 mmol g^{-1} and 0.13 mmol g^{-1} ,
323 respectively). Since the global average of immobilized N was around 0.17 mmol g^{-1} , the
324 percentage of provided N that has been immobilized decreased significantly with increasing
325 N:P ratio (Kruskal-Wallis, $X^2 = 62.998$, $p < 0.001$) (Fig.2b). The quantity of immobilized P
326 was lower at 15 μM [Cu] ($X^2 = 8.5283$, $p = 0.01406$) and decreased with increasing N:P ratio
327 ($X^2 = 38.221$, $p < 0.001$) (Fig.2c). As a result, the percentage of provided P that has been
328 immobilized increased significantly with increasing N:P ratio ($X^2 = 44.306$, $p < 0.001$) and
329 lower with [Cu] ($X^2 = 6.8641$, $p = 0.03232$) (Fig.2d). As a proxy of the microbial N:P ratio,
330 the ratio of immobilized N:P ranged from 12 to 125, with a 10-fold increase with increasing
331 of the provided N:P ratio and up to 3-fold at the higher [Cu]. The condition with 15 μM of Cu
332 showed a greater intra-replicate variation and higher immobilized N:P ratios compared to the
333 0 and 1 μM of Cu (Fig.2e).

334 3.3. Identification of the cellulose decomposition actors

335 The relative abundance of bacteria and fungi was estimated through qPCR quantification
336 of 16S and 18S rRNA genes. The relative proportion of bacteria to fungi (Fig.3) was not
337 changed by N:P ratios ($X^2 = 1.8853$, $p = 0.9299$) but significantly decreased 10 folds with
338 increasing [Cu] ($X^2 = 42.229$, $p < 0.001$). Abundance of bacterial 16S was about 10^3 to 10^5
339 times higher than that of fungi. The 16S rRNA gene copies stayed relatively constant (10^6 to
340 10^7 copies μL^{-1}) among the provided N:P ratios ($X^2 = 12.733$, $p = 0.04748$) and [Cu] ($X^2 =$
341 3.943 , $p = 0.1392$). The number of 18S rRNA gene copies was not influenced by N:P ratios
342 ($X^2 = 1.2234$, $p = 0.9757$), but increased with [Cu] from 10^2 to 10^4 copies μL^{-1} ($X^2 = 46.624$, p
343 < 0.001) (See Fig.S3). The ergosterol assays also confirm the low abundance of fungi since
344 ergosterol was detected only on 2 out of 21 samples analysed (0.08 and 0.20 $\mu\text{g ml}^{-1}$
345 compared to at least 4.15 $\mu\text{g ml}^{-1}$ for positive controls, which correspond to 7.1 and 26.4 $\mu\text{g g}^{-1}$
346 1 of cellulose disc). All these results indicated that cellulose discs were mainly colonized by

347 bacterial communities confirming our visual observations made during incubations (no fungal
348 mycelium development on cellulose disc surface).

349 3.4. Bacterial community diversity

350 Since bacteria were dominant, only bacterial community diversity was assessed through
351 16S rDNA sequencing. Alpha-diversity indices are shown in Fig.S4. The Chao1 index
352 indicated that the mean bacterial species richness decreased with increasing N:P ratio ($\chi^2 =$
353 14.126, $p = 0.04898$) and with increasing [Cu] ($\chi^2 = 48.128$, $p < 0.001$). The Shannon index
354 showed that the bacterial diversity stayed relatively constant for all tested [Cu] ($F = 5.079$, Pr
355 < 0.001). For bacterial communities exposed to 0 and 1 μM [Cu], the diversity index values
356 were lower for high N:P ratio indicating the emergence of very abundant taxa. Higher
357 variability in Shannon diversity indices was observed with increasing [Cu] (Levene's Test, F
358 $= 6.4451$, $Pr < 0.001$) indicating a higher intra-replicate heterogeneity.

359 Linear correlations between cellulose mass loss and OTU numbers, Chao1 richness or
360 Shannon diversity indices of all three Cu contamination levels are shown in Fig.4. For 0 and 1
361 μM of Cu, cellulose mass loss was positively correlated with the OTU number, Chao1 index
362 and Shannon index. For 15 μM Cu, similar correlation was found between cellulose mass loss
363 and Chao1 index, but no correlation was found with OTU number and Shannon index. The
364 variation in richness was minimal compared to that of the mass loss.

365 The bacterial taxonomic diversity for each condition is shown in Fig.5 as the relative
366 abundance of the five major phyla and the major *Proteobacteria* class (i.e.
367 *Gammaproteobacteria*) representing more than 10% of the sequences. The relative abundance
368 of each phylum was significantly different between N:P ratios and between [Cu]. The
369 bacterial communities from conditions with 0 and 1 μM of [Cu] were dominated by either
370 *Gammaproteobacteria* at lower N:P ratio, by *Bacteroidetes* at higher N:P ratio or by
371 *Chloroflexi* regardless of the N:P ratio. In the microcosms with the higher [Cu] (15 μM), the

372 bacterial community was dominated either by *Bacteroidetes* at lower N:P ratio, or by
373 *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* at higher N:P ratio. *Actinobacteria* and
374 *Firmicutes* were stimulated in condition with 15 μ M of Cu (Wilcoxon-Mann Whitney test, p
375 <0.001 for both phyla). The relative proportion of *Chloroflexi* decreased at the higher [Cu] (p
376 $< 2e-16$).

377 Beta diversity of the communities developing in all the tested conditions was shown
378 through NMDS analyses (Fig.6). Samples were clearly separated (first dimension, NMDS1)
379 based on the [Cu] (Fig.6a). A higher variability among samples exposed to 15 μ M of Cu was
380 observed compared to 0 and 1 μ M of Cu. A shift in the community composition was
381 highlighted according to the N:P ratios mainly for samples exposed at higher [Cu] (separation
382 on the second dimension NMDS2; Fig.6b). PerMANOVA analyses confirmed the significant
383 difference of the community composition between each of the three [Cu] ($F = 10.795$, $p =$
384 0.001), while the difference was lower but also significant between each of the seven N:P
385 ratios ($F = 1.365$, $p = 0.024$).

386 The results of indicator species analysis with indicator values are shown in Fig.S5. The
387 reasons behind our decision of merging the conditions into four groups are, firstly bacterial
388 diversities with 0 and 1 μ M of [Cu] were similar compared to that of 15 μ M of [Cu], and
389 secondly cellulose decompositions at N:P ratios lower than 33 at different [Cu] varied
390 compared to that at ratios higher than 33 which stayed constant. For the group CLRL (low
391 copper, low ratios), there were 11 indicators of which four *Bacteroidetes*, three
392 *Gammaproteobacteria* and four *Verrucomicrobia*. For the group CLRH (low copper, high
393 ratios), there were two indicators of which one *Bacteroidetes* and one *Gammaproteobacteria*.
394 For the group CHRL (high copper, low ratios), there were nine indicators of which seven
395 *Bacteroidetes*, one *Gammaproteobacteria* and one *Verrucomicrobia*. For the group CHRH
396 (high copper, high ratios), there were ten indicators of which one *Bacteroidetes*, four

397 *Gammaproteobacteria*, one *Firmicutes*, and four *Actinobacteria*. The analysis also revealed
398 15 indicator species related to the combination of groups CLRL and CLRH, which is
399 equivalent to the group with low copper (CL). And finally, four indicator species for the
400 groups of CHRL and CHRL, which is the equivalent to the group with high copper (CH). For
401 the group CL, there were five *Bacteroidetes*, three *Gammaproteobacteria*, five *Chloroflexi*
402 and one *Verrucomicrobia*. For the group CH, there were three *Bacteroidetes* and one
403 *Gammaproteobacteria*. More detailed information can be found under Fig.S5.

404 **4. Discussion**

405 *4.1. General observations*

406 Cellulose decomposition was maximized at an optimal N:P ratio with, or without copper
407 contamination. Copper contamination had no significant effect on the maximal decomposition
408 rate around the optimal N:P ratio but had strongly reduced the range of the ratio in which
409 cellulose decomposition can occur. These changes were followed by large shifts in bacterial
410 community compositions. Bacteria sensitivities toward the contaminant and the relative N:P
411 availability in environment can both select particular taxa within a bacterial decomposer
412 community.

413 Despite the use of a natural microbial inoculum, fungal spores were not present in our
414 inoculum, and fungi hardly developed in our microcosms. Some possible explanations might
415 be that we have collected the leaf bags at a late stage of decomposition. During inoculum
416 preparation, partially decomposed leaves were reduced into small pieces instead of staying
417 intact as we usually get while inducing fungal spores. This observation is consistent with the
418 common assumption that bacteria only become abundant at a later stage on small leaf litter
419 fragments (Sinsabaugh and Findlay 1995). The decantation step we applied in the inoculum to
420 limit the risk of introducing leaf litter fragments in our microcosms might also have
421 participated in the absence of fungal spores. Finally, aquatic hyphomycetes that were

422 expected to be present in our inoculum generally require very high levels of oxygen to
423 maintain their activity (Medeiros, Pascoal and Graça 2009) and our microcosms, leading to
424 the development of a few anaerobic taxa (see section 4.2), were certainly not perfectly
425 adapted for ensuring their development. As a result, bacteria were largely dominant under all
426 tested conditions, and we ultimately decided to focus on bacterial communities for taxonomic
427 analysis.

428 *4.2. Stoichiometric demand of microbial decomposers for cellulose decomposition*

429 In our study, cellulose decomposition was largely controlled by ratios of dissolved
430 inorganic N and P in the medium. As predicted by our first hypothesis (H1), the
431 decomposition rate reached its maximum at a dissolved N:P ratio of 4.7. At this particular N:P
432 ratio, N and P availabilities were certainly co-limiting microbial activity allowing an optimal
433 decomposition of the carbon substrate. The occurrence of such a bell-shaped response of
434 cellulose decomposition had already been observed by Güsewell and Gessner (2009), by
435 using a natural microbial inoculum containing both bacteria and fungi. At the same nutrient
436 supply level, they have found an optimal mass N:P ratio for cellulose decomposition of 15 (33
437 molar ratio), which is higher than the optimal N:P ratio found in our study. Meanwhile, the
438 same authors also found that bacterial development was maximized at lower environmental
439 N:P ratios than for fungal development. In our study, microbial communities were largely
440 dominated by bacteria, and the low optimal N:P ratio we observed might be explained by this
441 dominance. Bacteria and fungi have different nutrient requirements, yet both are found to be
442 flexible. This flexibility is the result of differences in cellular contents and processes between
443 bacteria and fungi (Godwin and Cotner 2018; Camenzind *et al.* 2020a, 2020b). The
444 differences in nutrient requirements between bacteria and fungi can directly affect their
445 competitive abilities and dominance on different substrates (Danger, Gessner and Bärlocher
446 2016).

447 In the absence of Cu contaminant, significant cellulose mass losses were observed
448 throughout the whole N:P ratio range tested, even when the medium was extremely depleted
449 in P (high N:P ratio). Mass loss was always above 8 %. This result suggests that even though
450 the decomposition rate was reduced outside the optimal N:P ratio of the given microbial
451 decomposer community, this process was never fully stopped. Nevertheless, it was not
452 possible to follow decomposition kinetics using a single sampling date. The next step will be
453 to verify if stoichiometric imbalances would mostly slow down the decomposition process or
454 if this process would eventually stop due to nutrient unavailability.

455 Although the average quantity of N immobilized on cellulose disc stayed at a relatively
456 constant level around 0.17 mmol g⁻¹ of dry cellulose disc, N immobilization was not linear
457 along the gradient of N:P ratios tested. The quantity of N immobilized was maximized at the
458 optimal N:P ratio for cellulose decomposition and minimized at the N:P ratio where the
459 minimal decomposition rate occurred. The portion of N immobilized increased when the ratio
460 was low. This result agrees with that of Güsewell and Gessner (2009), showing that
461 decomposers were more efficient at immobilizing N when it was the most limiting element.
462 The quantity of P immobilized followed a similar profile as cellulose decomposition rate, i.e.
463 a bell-shaped response, P immobilization was maximized at an N:P ratio of 3.7. The
464 proportion of total P immobilized increased when N:P ratios were high, suggesting the
465 decomposers immobilize P more efficiently when it is the limiting element. This observation,
466 however, contrasts with the previous observations of Güsewell and Gessner (2009). A
467 possible explanation could be related to the very low abundance of fungi in our study, since
468 fungi are known to be able to store large amounts of extra P as polyphosphates when P is not
469 limiting (Beever and Burns 1981).

470 Interestingly, bacterial species richness dropped significantly for the highest N:P ratio,
471 where cellulose decomposition rate slowed down to its minimum. As already observed in

472 previous studies (e.g. Leflaive *et al.* 2008; Lemmel *et al.* 2019), our results evidenced positive
473 correlations between the intensity of microbial functions and the diversity: cellulose mass
474 losses were significantly and positively related to bacteria OTU numbers, Chao1 richness and
475 Shannon diversity indices. This suggests that only a few bacterial taxa are capable of
476 surviving in environment with highly imbalanced N and P, and these species are probably less
477 efficient in decomposing cellulose under high N:P ratios. When looking more specifically to
478 the taxa present along the N:P gradient, we found that the relative abundance of
479 *Gammaproteobacteria* was strongly related to cellulose decomposition efficiency, these being
480 more abundant around the optimal N:P ratio. Bacteria from this class probably carried out
481 most of the decomposition in our study. Even if we cannot exclude the possibilities that
482 certain bacteria with low abundances can also be very actively involved in decomposition.
483 Although *Gammaproteobacteria* are still present at higher N:P ratios, their abundance
484 decreased in favour of *Chloroflexi* and *Bacteroidetes* which became dominant. Among
485 *Chloroflexi*, *Anaerolineae* was the dominant class. Although the ecological role of the
486 *Anaerolineae* members remains unclear, these bacteria have been shown to exhibit
487 cellulolytic activities (Hug *et al.* 2013; Xia *et al.* 2016). The detection of these strict anaerobic
488 bacteria suggests that despite the absence of total water immersion, parts of the cellulose discs
489 could have ended up in anaerobic conditions. For the highest N:P ratio, *Bacteroidetes*
490 represented up to 75 % of total abundance. *Verrucomicrobia* was shown to be less abundant
491 yet consistently present throughout all the N:P ratios. A recent study has argued a possible
492 under estimation of its abundance due to methodological biases (Chiang *et al.* 2018). All these
493 findings confirm partially our fourth hypothesis (H4) that N:P ratio strongly contributes to the
494 composition of active bacterial communities. Even though the functional redundancy among
495 bacterial decomposers could ensure basal cellulose decomposition, the activities of these
496 different active taxa at different N:P ratios were not equally efficient.

497 4.3. *Stoichiometric demand for cellulose decomposition in the presence of copper*

498 In the presence of Cu in the microcosms, the overall cellulose decomposition remained
499 unchanged in the presence of copper when N:P ratios were close to microbial stoichiometric
500 optimal requirements, whatever the copper concentration tested. For the highest Cu
501 concentration (15 μ M of Cu), cellulose mass loss was significantly reduced when deviating
502 from this optimal ratio. This observation partly confirms our second hypothesis that high Cu
503 concentration would reduce cellulose decomposition rates (H2) and is consistent with
504 previous studies investigating copper effects on leaf litter decomposition (Roussel, Chauvet
505 and Bonzom 2008; Ferreira *et al.* 2016) and alter the structure of bacterial decomposer
506 community (Duarte *et al.* 2008). This result might be due to direct Cu toxicity which has long
507 been evidenced for both bacteria and fungi (e.g. Dupont, Grass and Rensing 2011).

508 Contrary to our expectation (see hypothesis H3), the optimal N:P ratio for maximal
509 cellulose decomposition rate decreased with raising Cu concentration. Cu at low
510 concentrations is known to be an essential microelement (Cervantes and Gutierrez-Corona
511 1994), metal-transporting-proteins which regulate multiple metal homoeostasis (including Cu)
512 are naturally present on bacterial membranes to regulate intracellular metal concentrations.
513 Cu(II) ion appears to be less toxic than Cu(I), as we introduced Cu(II) from CuSO₄, bacteria
514 were exempt from synthesizing Cu(I)-detoxification-proteins (Ladomersky and Petris 2015),
515 which would have required more N. Cu(II) export, on the other hand, does not require
516 synthesizing additional transporters, but mainly relies on existing ATP-dependent proteins to
517 actively relocate Cu outside the cell membrane (Ma, Jacobsen and Giedroc 2009). A stronger
518 increase in energetic requirements (and thus in P-rich molecules, such as ATP) than in the
519 production of N-rich detoxification proteins might be a plausible explanation of why the
520 optimal N:P ratio for decomposition had decreased.

521 Our results also highlighted that the minimal cellulose mass losses under high Cu
522 contamination (close to 0 %) were significantly lower than those of control and low Cu
523 contamination treatment (which always remained above 8%, whatever the N:P ratio tested). In
524 other words, rather than keeping a slow but non-zero rate of decomposition, high Cu
525 contamination arising concomitantly with N:P ratios higher than 33 had led to a total stop of
526 cellulose decomposition. As pointed out by Peñuelas *et al.* (2013), anthropic input of N into
527 the biosphere is excessively higher than that of P, creating an increasingly imbalanced
528 environmental N:P ratio, This N:P imbalance alone had already affected the life histories of
529 microorganisms and other life forms (Elser *et al.* 2009). In case of high Cu contamination, it
530 could take the decomposition process to halt and effectively stop carbon turnover. Our
531 findings suggest that human-induced global nutrient imbalances might lead to exacerbated
532 responses of microbial processes in presence of contaminants.

533 Copper reduced the richness of the bacteria community at each N:P ratio even at the
534 lowest level test. For the highest copper concentration, the effect of N:P ratios over bacterial
535 richness was minimized, suggesting a higher selective pressure of Cu rather than N:P balances
536 on bacterial communities. Cellulose decomposition blocked in both high N:P and high copper
537 conditions could be partly explained by the elimination, or at least a decrease in abundance of
538 certain cellulolytic taxa that are sensitive to high Cu concentrations, and their replacement
539 (with similar abundance) by Cu-tolerant taxa (Indicator species: *Flavobacterium*,
540 *Nitrosospira*, *Pseudomonas*, *Aeromonas*, *Variovorax*, *Paenibacillus* and *Nocardiaceae*)
541 probably less efficient or even incapable of decomposing cellulose. The positive correlations
542 between cellulose mass losses and OTU numbers, richness and diversity of the bacterial
543 community observed in control and low Cu conditions almost disappeared in the presence of
544 15µM of copper. This means that in the most contaminated conditions, the community
545 richness had little to do with cellulose mass loss, which suggests a shift in the community

546 composition and taxa replacements. As shown that *Bacteroidetes* dominance were replaced by
547 *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* from low to high N:P ratio. Different
548 indicator species were also identified under different situations. All these observations
549 confirm our hypothesis (H4) that the contaminant and the N:P ratio in the environment
550 interactively control microbial community structure, with large consequences on their
551 functions and activities (i.e. cellulose decomposition).

552 **5. Conclusion**

553 Bacteria were the main decomposers in our study. Without copper contamination,
554 cellulose decomposition was maximized at an N:P molar ratio of 4.7. Both N and P
555 immobilization by bacterial decomposers were more efficient when these elements were the
556 limiting ones. Bacterial species diversity and richness dropped at high N:P ratios (when P was
557 limited) but the community as a whole maintained a basal level of cellulose decomposition.

558 Overall, the lowest Cu concentration tested had little to no negative effect over bacterial
559 decomposer communities, their taxonomic compositions, their N and P stoichiometric
560 demands, and their capacity to decompose cellulose. In contrast, at the highest Cu
561 concentration tested, the composition of bacterial communities was drastically reshaped,
562 which had significantly reduced the optimal N:P ratio for cellulose decomposition to 2.8, and
563 negatively affected the global cellulose decomposition efficiency in the most
564 stoichiometrically imbalanced conditions (the highest N:P ratios). In this study, we focused on
565 cellulose decomposition, but other microbial functions could be simultaneously affected and
566 would require further studies. For example, Cu has been shown to inhibit bacterial glucose
567 utilization, or nitrifiers and denitrifiers metabolic activities (Ochoa-Herrera *et al.* 2011). To
568 conclude, the current increased imbalance between N and P availabilities on Earth, which
569 correspond to a major parameter of global change, might lead to unexpected interactions with
570 other stressors, in turn largely impacting microbial functions. Our results shed the light on a

571 potentially important and understudied environmental problem that could have large
572 repercussions on ecosystem functioning worldwide.

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580 **Conflict of Interest**

581 The authors declare no conflict of interest.

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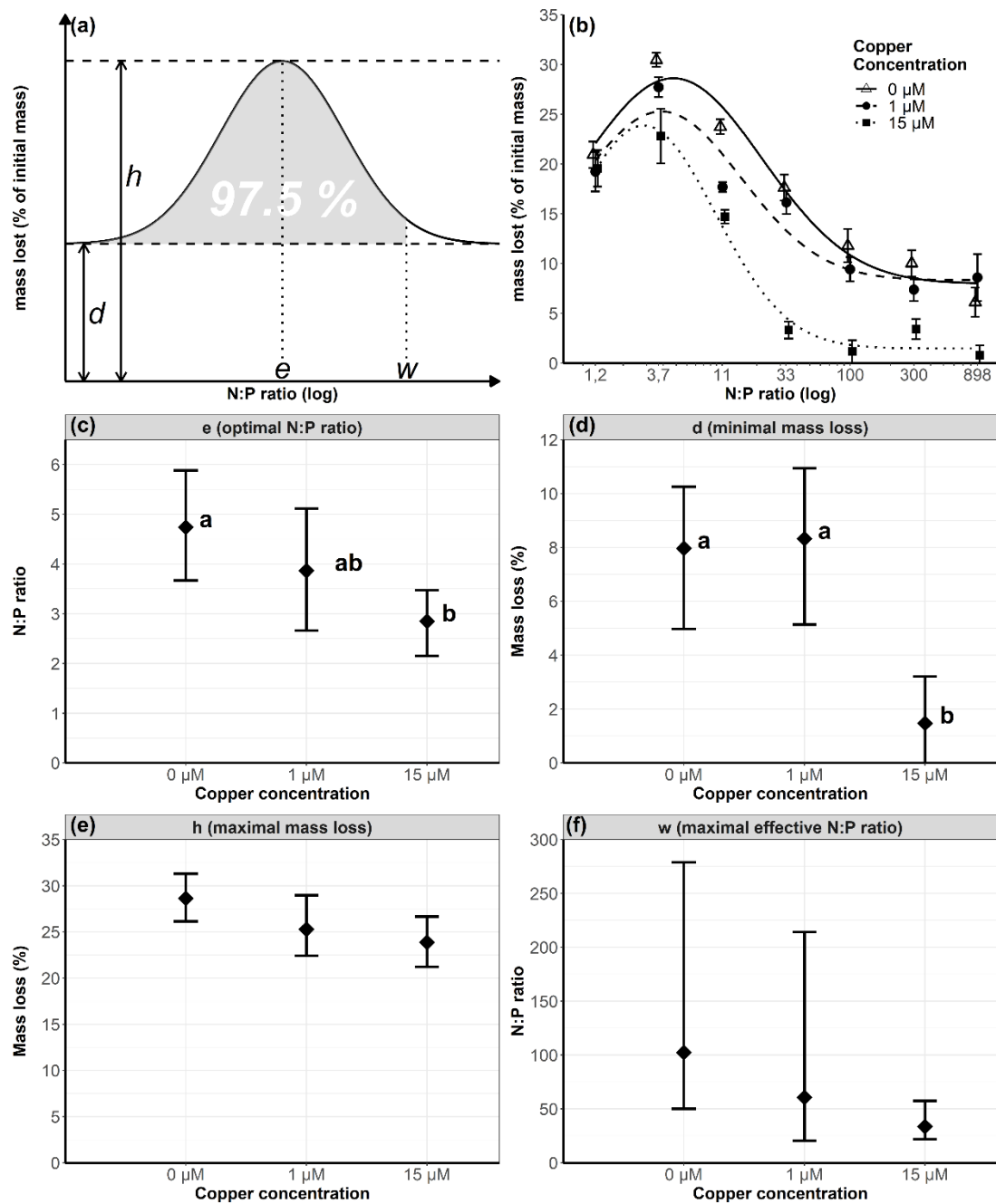
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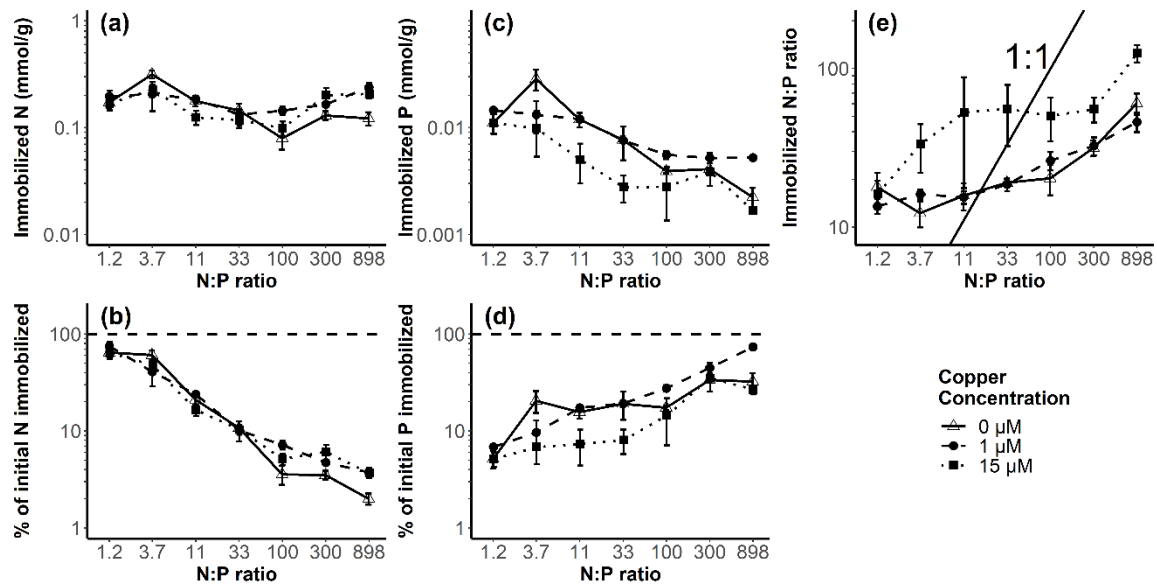
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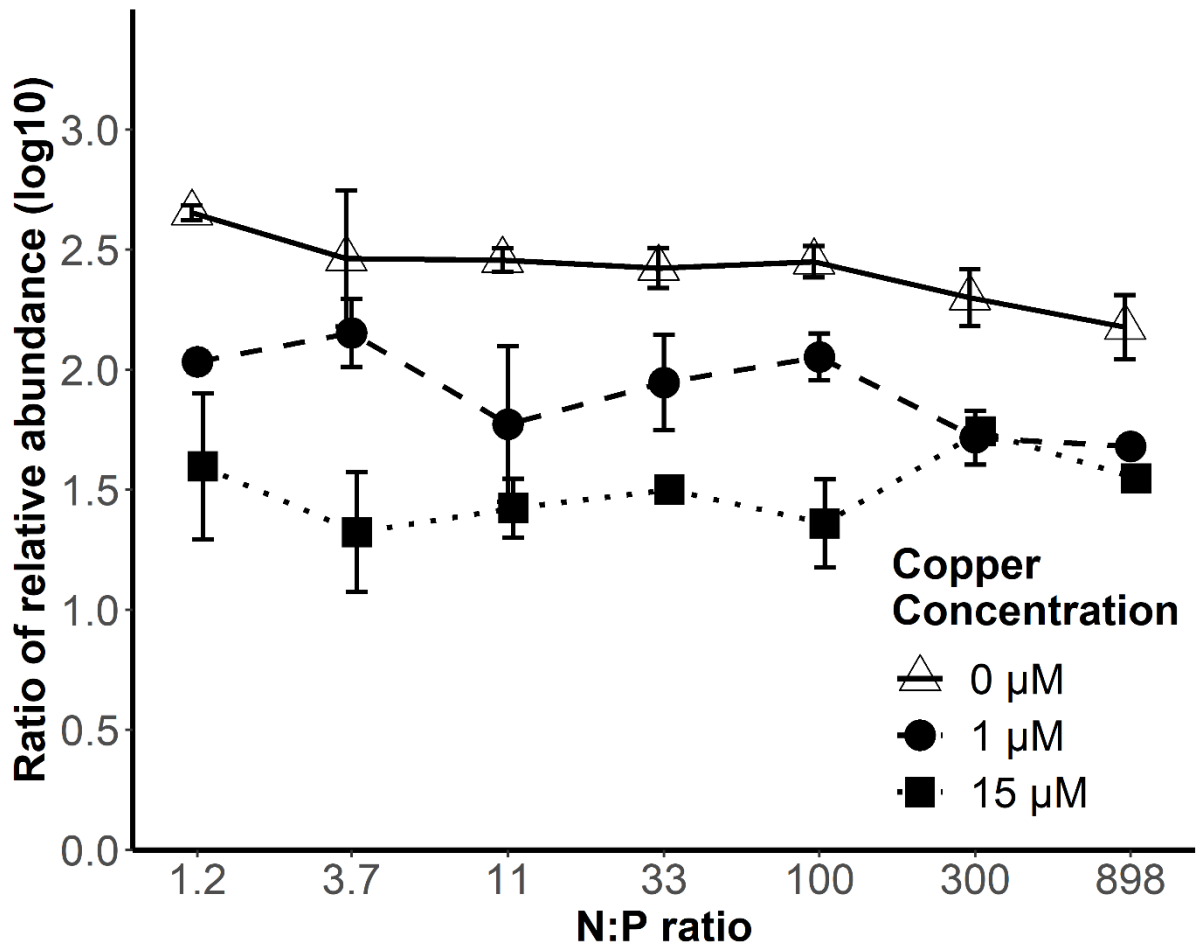


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 2 **Figure 1 – (a) Schematic representation of the Gaussian model.** x -axis is the log
 3 transformed N:P ratios; y -axis is the mass loss. d is the minimal mass loss; h is the
 4 maximal mass loss; e is the optimal N:P ratio for the maximal mass loss; w is the
 5 maximal effective N:P ratio less than which there are 97.5 % of cumulative mass loss
 6 from the level of d (grey area). **(b) Cellulose mass losses.** Cellulose disc mass losses
 7 after 79 days, calculated in percentage of the average initial mass (36.3 mg) minus the
 8 average 3% loss of the controls without inoculum. x -axis for provided N:P ratios is log-
 9 transformed but shows as the molar ratio for reading convenience. Curves show mass
 10 loss calculated by the Gaussian model. **(c, d, e, f) Graphical representation of each**
 11 **parameter estimated by the gaussian model for all three tested copper**
 12 **concentration levels.** The error bars show the 95 % confidence interval (CI) for each
 13 estimation.



14

15 **Figure 2 – N and P immobilized on cellulose disc for three copper concentrations in**
 16 **relation to the provided N:P ratio. (a, c) millimoles of N and P immobilized per gram**
 17 **of final cellulose dry mass; (b, d) percentage of provided N and P immobilized on the**
 18 **disc; (e) immobilized N:P ratio, the 1:1 line shows the immobilized N and P in**
 19 **proportion to the provided ratio. Error bars show the standard deviation of 4 replicates**
 20 **for 0 copper, and 3 replicates for 1 μM and 15 μM copper. x-axis for provided N:P ratios**
 21 **is log-transformed but shows as the molar ratio for reading convenience.**



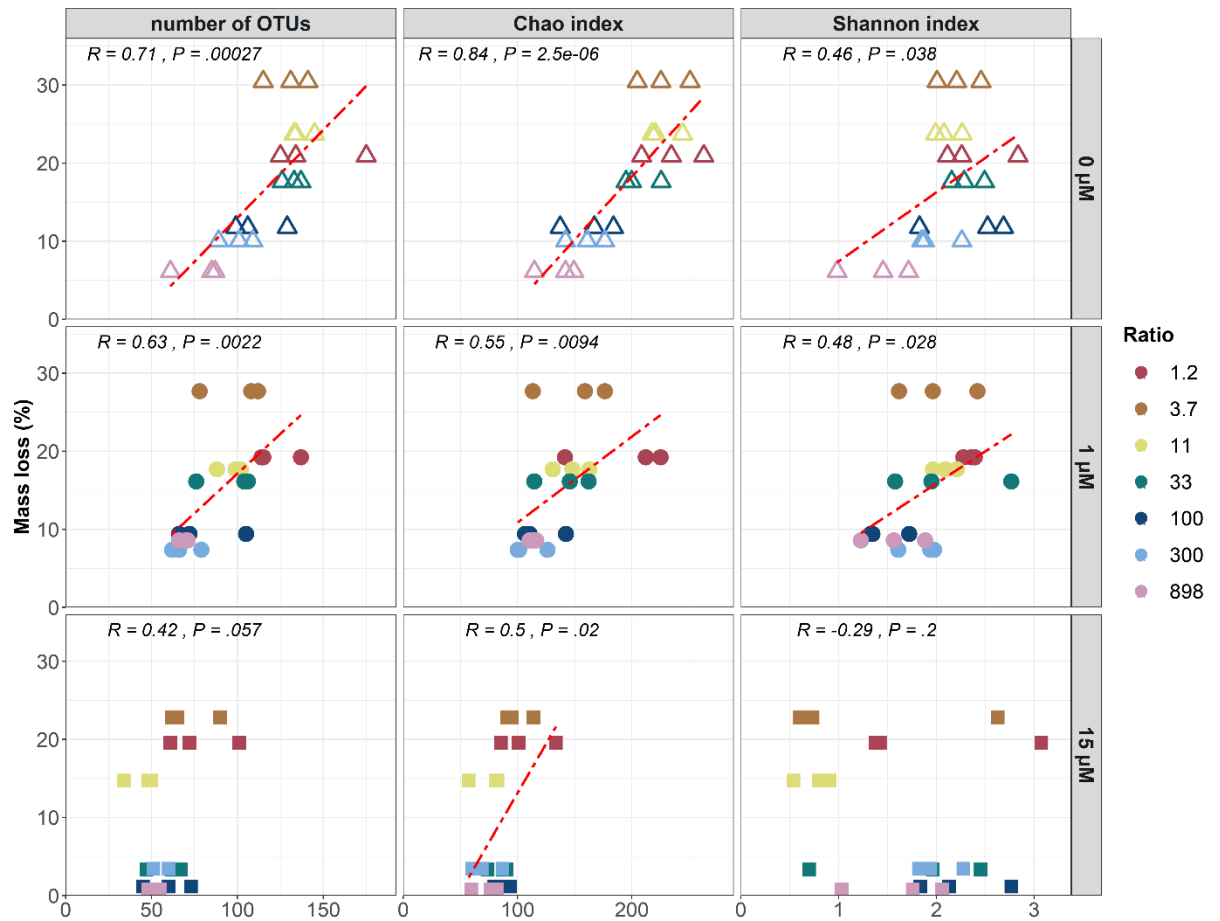
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Figure 3 – Ratio between bacterial 16S and fungal 18S rRNA gene copies. *x-axis* for provided N:P ratios is log-transformed but shows as the molar ratio for reading convenience. For the abundances of 16S and 18S rRNA, see Fig.S3.

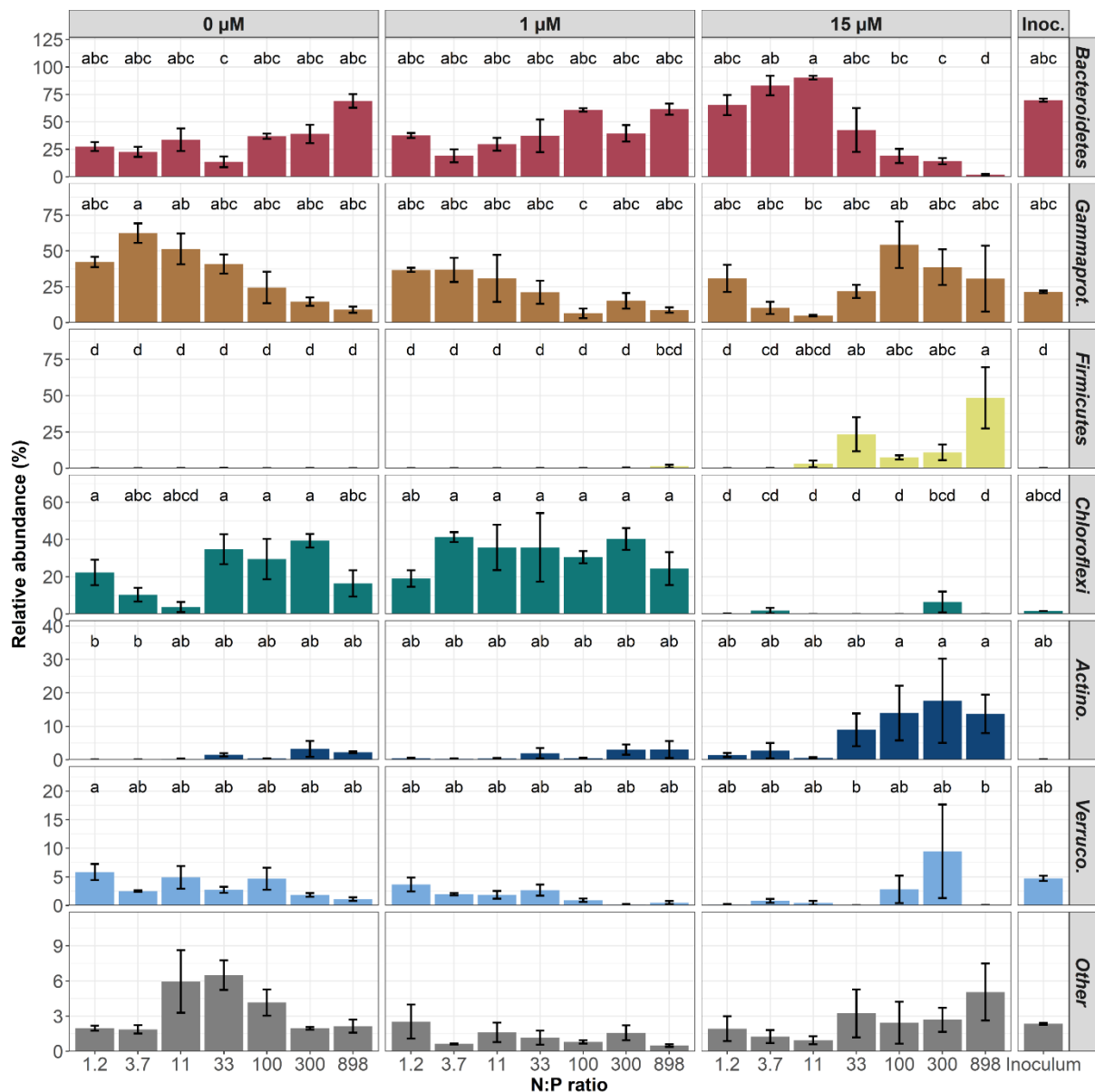


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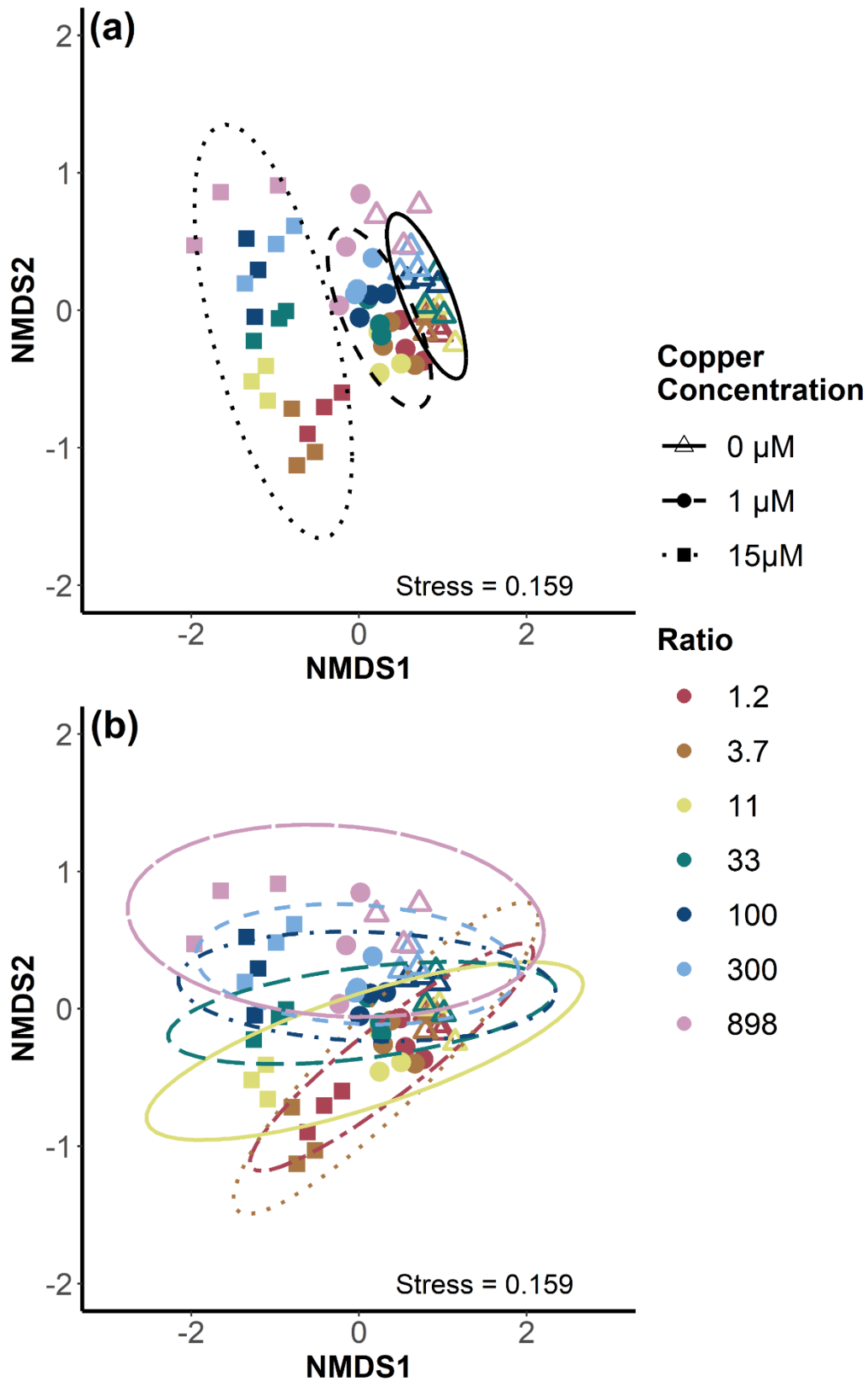
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Figure 4 – Linear correlation between mass losses and numbers of OTUs, Chao or Shannon indexes of three Cu contamination levels.



29

30 **Figure 5 – Bacterial diversity for all three levels of copper contamination and the**
 31 **inoculum.** *Gammaprot.:* *Gammaproteobacteria* (class). The rest are shown at phylum
 32 level. *Actino.:* *Actinobacteria*, *Verruco.:* *Verrucomicrobia*. Values are the mean
 33 abundance of replicates (n =3, for inoculum n = 2). Error bars show the standard
 34 deviation between replicates. Letters show significance groups, there is no significant
 35 differences between relative abundances in “Other”. “Other” groups all OTUs with a
 36 relative abundance less than 10 % in any of the replicates. The phyla in the “Other”
 37 group are: *Armatimonadetes*, *BRC1*, *Clamydiae*, *Cyanobacteria*, *Deinococcus-Thermus*,
 38 *Dependentiae*, *FBP*, *Fibrobacteres*, *Hydrogenedentes*, *Lentisphaerae*, *Nitrospirae*,
 39 *Planctomycetes*, *Spirochaetes*, unclassified bacteria and *Proteobacteria* (none
 40 *Gammaproteobacteria*).



41

42 **Figure 6 – OTU based beta diversity in NMDS using Bray-Curtis dissimilarity**
 43 **index. (a) beta diversity for all tested [Cu]; (b) beta diversity for all tested N:P ratios.**
 44 **Ellipses show the 95 % confidence interval.**