Phylogenetic and functional diversity have contrasting effects on the ecological functioning of bacterial communities

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Abstract

Biodiversity can have a positive impact on ecosystem functions because greater numbers of species, and their associated genetic and functional differences, can contribute at different times and contexts to overall ecosystem functioning. Quantifying the relative contributions of microbial species to ecosystem functioning is challenging, because many species are believed to be functionally redundant, or their function is not known but inferred by their phylogeny. Here, we used synthetic bacterial communities to test the independent effects of two aspects of biodiversity, phylogenetic and functional diversity, on ecosystem functioning, expecting that greater diversity of each would be associated with greater ecosystem function. Moreover, we tested whether the phylogenetic and functional diversities interact in our system and if that interaction results in the amplification of diversity positive effects on ecosystem functions. To study these effects, we used bacterial isolates to construct communities with different diversity traits and employed exoenzyme activities (EEAs) as proxies of bacterial functioning. Overall, EEAs increased with increasing phylogenetic diversity but decreased with functional diversity. Communities with phylogenetically dissimilar species exhibited more moderate, but mostly positive, changes in their EEAs. Furthermore, we observed that the effects of phylogenetic and functional diversity differed between EEAs investigated: EEAs on carbohydrates were positively correlated with phylogenetic diversity and negatively correlated with functional diversity, while EEAs on fatty acids typically showed no correlation at all. Furthermore, EEAs of phylogenetically similar groups were strongly affected by the taxonomy of the isolates and their growth strategies. We concluded that phylogenetic and functional diversity have contrasting effects, which furthermore depended on the ecosystem function under examination – diversity effects were depended on the substrates' biochemical nature and the molecular mechanisms related to each substrate's degradation. This highlights the importance of investigating individual aspects of biodiversity but also their interactions as the variations

within the biological mechanisms and processes that underscore them and their influence on community ecosystem functioning can inform ecological models and improve our understanding of what we consider diverse or redundant.

Keywords

ecosystem functions; phylogenetic diversity; functional diversity; biodiversity; bacteria; synthetic communities; exoenzymes

Introduction

More diverse ecosystems are typically more productive (Lehman and Tilman 2000, Byrnes et al. 2014, Maron et al. 2018), perform more functions (Hector and Bagchi 2007, Maron et al. 2018), are more resistant to disturbances (Ives and Carpenter 2007, Gross et al. 2014) and have populations that are more stable (Lehman and Tilman 2000). Bacteria activities are inextricable from ecosystem functions (Finlay et al. 1997) and recent research has highlighted that microbial diversity contributes to the functioning of ecosystems, challenging earlier ideas that microbes might have a high degree of functional redundancy (Yin et al. 2000, Louca et al. 2018, Taubert et al. 2019). While doubts have been raised, whether metrics such as species richness are informative enough for contemporary biodiversity research (Mokany et al. 2008, Thompson et al. 2015, Hillebrand et al. 2018), phylogeny and genetic relatedness allow for an increased representation of species traits in biodiversity indices and algorithms (Webb et al. 2002, Faith 2002, Anderson et al. 2004, Chase 2005, Srivastava and Vellend 2005). Phylogenetic information is also used to infer functional traits (Martiny et al. 2013). Increasing phylogenetic diversity, in general, improves ecosystem functioning due to complementarity and niche partitioning effects (Balvanera et al. 2006, Duffy 2009, Cardinale 2011, Jousset et al. 2011, Cardinale et al. 2012, Hooper et al. 2012, Naeem et al. 2012, Turnbull et al. 2013). Phylogenetic diversity is easy to quantify by standard sequencing methods, and can capture microorganism relatedness, growth rates (Morrissey et al. 2016), and species interactions via ecological networks (Srivastava et al. 2012). However, not all functional traits have a strong phylogenetic signal, as many ecologically relevant traits are spread across many phylogenetic clades (Srivastava et al. 2012, Martiny et al. 2013, Goberna and Verdú 2016). A direct assessment of functional diversity hence allows a better prediction of community ecosystem functions (Mokany et al. 2008, Salles et al. 2009, Krause et al. 2014). Functional diversity, however, is more difficult to define and quantify (Mouchet et al. 2010, Cadotte et al. 2011, Steudel et al.

2016). Functional diversity requires the measurement of functional trait differences of organisms to estimate functional diversity (Petchey and Gaston 2002, 2006, Naeem et al. 2009). Ways that microbial functional traits can be determined include functional gene-targeting microarrays and metabolic activity assays (Litchman and Klausmeier 2008, Naeem et al. 2009, Paula et al. 2014, Wang et al. 2017). The latter can also be used as a measure for community functioning itself along with other ecologically important community functions, such as nutrient cycling, organic matter decomposition and CO_2 production, which were shown to be positively correlated with functional diversity (Rivett et al. 2016, Evans et al. 2017). Furthermore, phylogenetic and functional diversity are related (Srivastava et al. 2012, Letten et al. 2014, Thompson et al. 2015) and dependent on each other (Loreau 2010a, 2010b). A critical feature that underscores the connections between the two biodiversity aspects is that while they have distinct underlying mechanisms, they are connected by evolutionary processes. Phylogenetic diversity emerges due to the genetic divergence of species, yet a lot of their functions are conserved. However, functional redundancy can encompass situations such as alternative adaptations from convergent evolution or differences in metabolic rates and variability in enzyme temperature optima and substrate specificity due to selection or drift. Differences in these underlying mechanisms can potentially influence how each biodiversity aspect influences community ecosystem functions and how the two interact. Identifying the effects of phylogenetic and functional diversity interaction is, therefore, crucial for the further development of microbial ecological theory (Krause et al. 2014, Shade et al. 2018).

Such biodiversity aspects and their effects on microbial ecosystem functions can be studied with different approaches, each with its own challenges. Natural microbial communities are highly taxonomically and functionally diverse (López-García and Moreira 2008) and exist in complex interaction networks (Ben-Jacob 2009, Faust and Raes 2012, Zuñiga et al. 2017). Therefore, while useful when relating experimental results back to the real world, it makes it

difficult to identify and disentangle specific biodiversity effects influencing ecosystem functioning. In contrast, monoculture approaches offer a simple, controlled study environment but do not allow the investigation of interspecies interactions, which can radically affect their ecosystem functioning (Gause 1934, Hardin 1960, Lawrence et al. 2012, Dolinšek et al. 2016, Levine et al. 2017). Synthetic communities allow us to include interactions from natural communities while keeping the experimental simplicity advantage of monocultures (De Roy et al. 2014, Fredrickson 2015, Chodkowski and Shade 2017). The ability to manipulate the degree of diversity in the synthetic communities makes them suitable models to study biodiversity influences on community ecosystem functions (Rainey et al. 2000, Bell et al. 2005, Großkopf and Soyer 2014).

Therefore, to study the effects of phylogenetic and functional diversity, we used bacterial groundwater isolates to form simplified four-member communities with different phylogenetic and functional dissimilarity, allowing us to test whether and how these two aspects of biodiversity interact as they shape community ecosystem functions. Bacterial functioning, related to nutrient cycling and organic matter decomposition, depends on the accessibility and uptake rate of energy sources. Exoenzymes are one of the main mechanisms that bacteria can use to access exogenous, complex carbon and energy sources and can be produced constantly or on-demand. As such, exoenzymes can be considered “public goods” that can be shared among different species (Drescher et al. 2014, Smith and Schuster 2019). Bacterial species exhibit differences in what sources they can access, and species using the same complex source can also differ in their efficiency. We used catabolic exoenzyme activities (EEAs) assays to identify and measure differences in degradation rates of different complex sources between bacterial isolates and, as an extend, determine functional diversity for the community construction. Additionally, EEAs were used as a measure of functioning in the communities. Measuring EEAs allowed us to examine how differences in the phylogenetic and functional

diversity between the species can affect community functions. We hypothesised that 1) phylogenetic diversity and 2) functional diversity increase community EEAs. Additionally, we expected that 3) the phylogenetic and functional diversity traits will interact positively, amplifying the effect on community EEAs when both have higher diversity.

125 # Material and Methods

126 ## Selection of bacterial isolates and culture setups

127 The twenty-seven bacterial strains used in this study (Table S1) were isolated from pristine
128 groundwater (Geesink et al. 2018), obtained from the Hainich Critical Zone Exploratory located
129 in Thuringia, Germany (Küsel et al. 2016). The isolates were selected from a pool of more than
130 340 groundwater isolates as representatives of the most abundant taxonomic families within the
131 total groundwater bacterial population, based on 16S rRNA gene MiSeq amplicon sequencing
132 data (Schwab et al. 2017, Kumar et al. 2017), throughout the taxonomic classes of
133 *Actinobacteria*, *Bacilli*, *Flavobacteriia*, *Sphingobacteria*, *Alpha*- and *Gammaproteobacteria*.
134 The isolates were maintained on S2P medium agar (Geesink et al. 2018). Before each of the
135 experimental assays, isolates were cultured aerobically in 20 ml of liquid S2P medium, in cap-
136 perforated 50 ml Falcon tubes, horizontally shaken (125 rpm) at room temperature, for 48 h to
137 early stationary phase. Growth in the liquid cultures was being determined via optical density
138 measurements at a wavelength of 600 nm (Synergy™ H4 Hybrid Multi-Mode Microplate
139 Reader, BioTek, Germany).

140 ## Molecular analysis and taxonomic classification of bacterial isolates

141 DNA from bacterial isolates was extracted with GenElute™ Bacterial Genomic DNA Kit
142 (NA2110-1KT, Sigma-Aldrich, Germany). Sterile inoculation loops were used to gather 1 µl-
143 worth of biomass from the agar medium cultures, for DNA extraction. For the 16S rRNA gene
144 amplification, the primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) (Turner et al. 1999)
145 and 1492R (5’-GGT TAC CTT GTT ACG ACT T-3’) (Turner et al. 1999) were used. PCR was
146 performed with a PEQSTAR thermocycler (PEQLAB, Germany) using the following cycling
147 conditions: initial denaturation at 95 ºC for 10 min, 30 cycles of 95 ºC (denaturation), 55 ºC

148 (primer annealing) and 72 ºC (elongation) for 30 sec, 45 sec and 60 sec, respectively, followed
149 by 10 min at 72 ºC (final elongation). DNA purification and sequencing of the 1484bp-length
150 PCR products was carried out by Macrogen (Amsterdam, Netherlands) with the 27F (5'-AGA
151 GTT TGA TCM TGG CTC AG-3') primer.
152 The sequences were assigned to their closest reference relative using BLASTn (Zhang et al.
153 2000) (v2.8.0+) and cross-referenced with SILVA Incremental Aligner (SINA) (Pruesse et al.
154 2012) (v1.2.11). A rooted maximum-likelihood phylogenetic tree for the 16S rRNA gene
155 taxonomic classification of the organisms was constructed with MEGA X (Kumar et al. 2018)
156 (v10.1.5).

157 Exoenzyme activity assays (EEAs)

158 EEAs for each bacterial isolate were performed using eleven substrates (Table S2) (Sigma-
159 Aldrich, Germany), each carrying a fluorescent moiety allowing spectrophotometric
160 measurements of activity using a microplate reader (Synergy™ H4 Hybrid Multi-Mode
161 Microplate Reader). The sensor was set to detect fluorescence at wavelengths 360/9 nm for
162 excitation and 450/9 nm for emission.
163 Prior to the EEA assays, the optical density of the bacterial cultures was determined
164 spectrophotometrically (600 nm). For the EEA assays, a modified version of the protocols from
165 Frossard *et al.* (Frossard et al. 2012) and Rivett *et al.* (Rivett et al. 2016) was used here, as
166 follows: a black 96-well plate setup, with 100 µl HEPES buffer at 100mM stock concentration
167 in each well, was inoculated with 100 µl of bacterial culture and to each test well 50 µl of
168 substrate solution at 40 µM final concentration was added. Negative controls consisted of wells
169 containing 100 µl of sterile S2P liquid medium and wells with just 100 µl sterile MilliQ water
170 (Merck Millipore, Germany). Background fluorescence, measured in negative controls with
171 either substrate solution or MilliQ water, was subtracted from the respective assay plates.

172 Measurement of the assay plates begun immediately after the substrate addition and continued
173 for 90 min, at 2.5 min measurement intervals.

174 Determining phylogenetic and functional diversity of isolates

175 To examine effects of diversity on the function of synthetic bacterial communities, the 16S
176 rRNA gene taxonomy and EEAs of the twenty-seven bacterial isolates were used as the basis
177 of the community traits; phylogenetic and functional diversity. We formed four trait-groups,
178 each consisting of five isolates, being either phylogenetically and functionally similar (Psim-
179 Fsim), phylogenetically similar but functionally dissimilar (Psim-Fdis), phylogenetically
180 dissimilar and functionally similar (Pdis-Fsim) as well as phylogenetically and functionally
181 similar (Pdis-Fdis). In total, the isolates cover ten taxonomic families of five classes (Figure
182 S1). In the Psim groups, isolates were affiliated with the same genus; Psim-Fsim held
183 *Flavobacteria* isolates and Psim-Fdis group held *Pseudomonas* isolates. Pdis groups were
184 formed so that all members of a group were related to different families. Hierarchical clustering
185 based on Euclidean distance was used to quantise functioning of the total EEAs for each isolate
186 (Figure 1) and categorise them as functionally similar or dissimilar. The clustering analysis was
187 performed in R (v. 3.6.1) (R Core Team 2019) using the core dist() and hclust() functions. Fsim
188 groups were formed from isolates of the same cluster, Fdis groups were formed so that each
189 member of the group belonged to a different functional cluster (Figure 1). Principal component
190 analysis (PCA) based on the functional traits of the isolates confirmed the higher functional
191 dissimilarities between isolates of Psim-Fdis and Pdis-Fdis compared to group Psim-Fsim and
192 Pdis-Fsim (Figure S2).

193 Bacterial community setups and experimental design

194 Synthetic communities were designed to contain bacterial species according to their functional
195 and phylogenetic diversity, based on the four trait groups; Psim-Fsim, Psim-Fdis, Pdis-Fsim,

196 Pdis-Fdis. From the initial twenty-seven isolates tested, a total of seventeen isolates were used
197 for designing communities. Within each trait group, five distinct communities were formed by
198 omitting one isolate out of the five, per community, allowing us to control whether within-
199 group community EEA variations were due to the activity of a specific community member.
200 This resulted in twenty communities, five for each trait group, identified as M1 to M5; we
201 confirmed the phylogenetic dissimilarity between the isolates via estimates of average
202 evolutionary divergence over sequence pairs within groups and their functional dissimilarity
203 based on the Euclidean distance matrix of the EEAs (Table S3). The phylogenetic and
204 functional dissimilarity values for each community showed a distinct differentiation of the
205 communities from each of the four groups, confirming that we successfully separated the
206 bacterial isolates and communities into the four intended groups (Figure 2).
207 Cultures of each isolate were grown in liquid medium as described above. Optical density (600
208 nm) was used to calculate the required final volume for each isolate, so that all members of
209 each community were to be introduced at similar biomass levels. The communities were
210 established in cultures by mixing a predetermined amount of liquid isolate culture for a total
211 volume of 2.5 ml. The community cultures were incubated in deep 96-well plates (2.5 ml
212 volume), at room temperature and sealed with rayon film seals (391-1262, VWR, Germany) to
213 allow gas exchange. The EEAs of the microcosms were assessed after 0 h, 6 h and 24 h of
214 incubation. Additionally, at the 0 h and 24 h marks, a 200 µl culture sample was pelleted by
215 centrifugation (17.000 × g, 5 min) and stored at -80 °C, for subsequent molecular analysis.
216 Data from EEA assays were transformed into a proportional change of EEA with the following
217 steps (visualised in Figure S3): The measured EEA of the isolates within each community group
218 was used to calculate expected EEA for each of the group's communities. This expected EEA
219 was compared to the measured EEA of the experimental communities and the result was
220 represented as percent proportional changes of EEA. The proportional changes were used for
221 subsequent analyses and visualisation of the data.

222 ## Molecular and diversity analysis of synthetic communities

223 Community DNA was extracted as described above. DNA concentration was measured
224 spectrophotometrically (NanoDrop 1000, PEQLAB, Germany) and samples were stored at -20
225 °C.
226 For Illumina MiSeq amplicon sequencing of 16S rRNA genes, PCR was performed using the
227 primer set Bakt_341F (5'-CCT ACG GGN GGC WGC AG-3') (Herlemann et al. 2011) and
228 Bakt_805R (5'-GAC TAC HVG GGT ATC TAA TCC-3') (Herlemann et al. 2011) targeting
229 the V3-V4 hypervariable region of the rRNA gene. DNA amplification was carried out with a
230 PEQSTAR 2X thermocycler (PEQLAB, Germany) in the following conditions: initial
231 denaturation at 95 ºC for 15 min, 30 cycles of 94 ºC (denaturation), 55 ºC (primer annealing)
232 and 72 ºC (elongation) at 45 sec for each step, followed by 10 min at 72 ºC (final elongation).
233 The PCR DNA products were purified using NucleoSpin Gel and PCR Clean-up (Ref. 740609,
234 Macherey-Nagel, Germany) following the manufacturer's protocol. The final step, DNA
235 recovery from the columns, was performed twice for a total volume of 60 µl purified PCR
236 product samples.
237 MiSeq-generated sequence data was analysed using mothur (Schloss et al. 2009) (v.1.39.1;
238 mothur.org) along with the mother MiSeq SOP developed by Kozich *et al*. (Kozich et al. 2013)
239 (accessed November 19th 2018). Paired-end reads were combined and sequences below 400 bp
240 or above 429 bp as well as sequences with ambiguous bases or homopolymer runs above 8 bp
241 were removed. Sequences were aligned to the SILVA reference database release SSU 132
242 (Quast et al. 2012) and clustered when differed in four or fewer bases. Chimeric sequences were
243 removed with uchime (Edgar 2016) using the GOLD database (Mukherjee et al. 2019) as a
244 reference. OTUs were binned from sequences with a 3% identity cut-off and were classified
245 using the aforementioned SILVA database. Raw data has been deposited in the European

Nucleotide Archive under the study with accession number PRJEB34832 (samples ERR3588683 - ERR3588722).

Statistical analyses of EEAs and diversity treatment effects

We evaluated significance for the phylogenetic and functional diversity treatment effects on EEA using ANOVA of linear regression models, including the interaction of the of these two treatments. For the linear models, EEA data were log2-transformed. Phylogenetic and functional dissimilarity metrics were scaled using z-score standardisation. The significance of EEA changes over 24 h was tested using Student's t-test (2-tailed) and false discovery rate controlled with the Benjamini–Hochberg procedure (McDonald 2014).

Results

EEAs of synthetic communities and comparison to constituent isolates

EEAs changes of the synthetic four-species communities showed sharp decreases, down to 100%, and increases, up to 385%, at 0 h and exhibited strong differences between substrates (Figure 3; Figure S4). The EEA changes for each one substrate within a group, however, were typically consistent (< 50% EEA proportional change from the average) for 33 of the 44 (75%) community-substrate combinations, especially for substrates with ester bonds like 4-MUB acetate (ACE), 4-MUB-butyrate (BUT) and 4-MUB oleate (OLE), while the highest relative increases in EEA were typically observed for substrates with glycosidic bonds like 4-MUB-N-acetyl-β-D-glucosaminide (NAG), 4-MUB-β-D-cellobioside (CB) and 4-MUB-β-D-xylopyranoside (XYL) (see Table S2 for complete list of substrate abbreviations). This indicates that, in most cases, community behaviour was not strongly influenced by the excluded fifth isolate of each group.

When comparing EEA changes between the groups (Figure S4), we observed that Pdis communities had relatively small to no changes in EEA: 76% of community-substrate combinations of group Pdis-Fsim and Pdis-Fdis remained between +50% and -50% EEA proportional change. Contrarily, communities of Psim groups differed greatly in activities compared to their constituent isolates. Group Psim-Fsim exhibited the majority the largest EEA increases (up to 330%) with 27% of the group's 55 community-substrate combinations measuring > +50% EEA proportional change. Group Psim-Fdis communities recorded the greatest amount of negative EEA proportional changes (53% of the group's 55 community-substrate combinations having < -50% EEA proportional change) with the maximum EEA proportional change increase at only 5.4%. These results show that phylogenetically similar

communities exhibited more variable and genus-specific responses than phylogenetically dissimilar communities, indicating towards a stabilizing effect of phylogenetic diversity.

While this seemed to run counter to our first hypothesis, linear regression analyses of both phylogenetic and functional diversity effects on community EEAs offered a clearer picture (Table S4). Averaging across all communities and substrates, we observed significant positive correlation for phylogenetic diversity (0.64 ± 0.12, estimate ± std. error; $F_{3,211} = 18.19$, $p < 0.001$) and significant negative correlation for functional diversity (-0.88 ± 0.12, estimate ± std. error; $F_{3,211} = 43.34$, $p < 0.001$). The interaction of phylogenetic and functional diversity also had a significant positive correlation with EEAs (0.63 ± 0.12, estimate ± std. error; $F_{3,211} = 27.36$, $p < 0.001$). A significant effect from the interaction points towards synergistic effects between the two diversity parameters affecting the EEAs. The model results contrast with our hypothesis which stated that all diversity would increase EEAs. Phylogenetic diversity fits the hypothesis predictions, but functional diversity effects do not. In contrast, the interaction between phylogenetic and functional dissimilarities fit the hypothesis. This signifies that biodiversity effects are more complex than expected.

Modelling the average EEA for all substrates resulted in high unexplained variance (70.36% explained by residuals, adj. $R^2 = 0.29$), potentially due to the high variability in EEAs between the different substrates. When we investigated the datasets for all substrates individually using linear models, we indeed observed broad differences in the relationships of EEAs to the functional and phylogenetic diversity between them (Table S4). For example, CB showed positive significant correlations with phylogenetic diversity (1.02 ± 0.44, estimate ± std. error; $F_{3,16} = 3.83$, $p = 0.03$) and the interaction between phylogenetic and functional diversity (1.30 ± 0.42, estimate ± std. error; $F_{3,16} = 9.44$, $p = 0.01$). Substrates XYL and NAG behaved similarly to CB (Table S4). When combining datasets from these three substrates for the analysis, the resulting model improved substantially from the all-substrate model, with unexplained variance decreasing to 39.53 % and coefficient of correlation increasing to adj. $R^2 = 0.58$. There was a

304 significant effect of phylogenetic diversity (1.20 ± 0.25, estimate ± std. error; $F_{3,56} = 15.26$, $p <$
305 0.001) and functional diversity (-1.78 ± 0.25, estimate ± std. error; $F_{3,56} = 44.98$, $p < 0.001$) and
306 their interaction (1.21 ± 0.24, estimate ± std. error; $F_{3,56} = 25.43$, $p < 0.001$) with an adj.
307 $R^2 = 0.58$. Interestingly, all substrates in these groups are biochemically similar in that the
308 targets for exoenzymes are glycosidic bonds. This might hint towards predictable EEA patterns
309 in biochemically similar substrates. As another substrate containing glycosidic bonds, 4-MUB-
310 β-D-glucopyranoside (bG) showed different correlations with no significant effect from
311 phylogenetic diversity (0.36 ± 0.25, estimate ± std. error; $F_{3,16} = 0.93$, $p = 0.16$), significant
312 effect from functional diversity (-0.75 ± 0.25, estimate ± std. error; $F_{3,16} = 8.09$, $p = 0.01$) and
313 a non-significant effect from phylogenetic and functional diversity interaction (0.43 ± 0.24,
314 estimate ± std. error; $F_{3,16} = 3.13$, $p = 0.1$) with adj. $R^2 = 0.33$. Interestingly, as a peptide
315 analogue substrate, Z-L-Arginine-7-AMC HCL (ARG) exhibited a similar linear regression
316 trend and statistical significances, with a slightly higher adj. R^2 of 0.61, compared to the 3-
317 substrate model (Table S4).
318 Based on these observations, we also looked at substrates containing ester bonds, ACE, BUT
319 and OLE, as another biochemically similar group. None of these substrates showed significant
320 effects in their models (Table S4). A model with all three substrates did not have an improved
321 fit, despite of their biochemical similarities.
322 Therefore, while there are biochemical similarities behind the substrates that are visible in the
323 data (Figure 1 and Figure 3), the similarities do not appear to have a universal ecological role,
324 something that is reflected in the statistical models.

325 Effects of diversity in EEA changes over 24 h

326 We further investigated how the community EEA changed with increasing time after
327 community mixing, by comparing EEA results from time points of 0 h, 6 h and 24 h (Figure 4).
328 Both Fsim groups exhibited only negative trends over the 24 h. The Psim-Fdis group showed

329 both significantly negative and positive trends between EEAs measured over 24 h after mixing.
330 Changes in the Pdis communities' composition within 24 h did not explain the observed activity
331 changes, because all the introduced species appear to persist in the community cultures during
332 24 h post-mixing, with only close to ~1-fold change of relative abundance for most species and
333 a 2-fold change for two species. Thus, the observed changes in EEA over time should not be
334 the result of a shifting community composition supporting our hypothesis, that EEA changes
335 are due to diversity traits effects of the original makeup of the communities (Figure S5).

336 Substrate acclimation in EEA assays

337 Acclimation describes any phenotypic plasticity an organism can undergo to adjust to changes
338 in its environment. Effects of substrate acclimation can be observed directly by measuring the
339 increase of EEA due to potential upregulation of exoenzyme production. We tested the effect
340 of substrate acclimation, by incubating communities for 270 min in the presence of the substrate
341 before monitoring fluorescence for 90 min. We compared measurements for both isolates and
342 communities with and without this additional 270 min of substrate exposure. For various
343 substrates, we observed an increase of EEAs that were exposed to substrates for a longer period.
344 For isolate activities in the 4-MUB-α-D-glucopyranoside (aG) assay, the largest increase
345 (~3.3 times increase) was observed from 757 ± 19 to 1762 ± 54 RFU min^{-1}. For community
346 activities, increases were even larger (~11 times increase) from 139 ± 5 to 1378 ± 84 RFU
347 min^{-1}. On average, the acclimation effect was much more pronounced in communities, resulting
348 in a ~5-fold increase, compared to a ~1-fold increase in isolates (Figure 5). This increase in
349 activity did not match the EEA changes observed for communities after 6 h of mixing without
350 the addition of substrates, and thus the effect was indeed substrate acclimatisation of the
351 bacteria during the longer assays and not an effect of mixing the community cultures. These
352 observations link back to our original hypothesis, where we predict that the diversity traits of
353 the group will enhance the functioning of communities.

Discussion

We examined the current census in biodiversity and ecological research, that higher biodiversity leads to enhanced ecosystem functioning. To do so, we investigated how different aspects of biodiversity, phylogenetic and functional diversity, affect the ecosystem functions of synthetic bacterial communities. We stated that higher diversity would lead to higher EEA of bacterial communities compared to their constituent isolates. Although the group having highest increase in EEAs was Psim-Fsim, the group with the highest decrease in EEAs was Psim-Fdis. On the other hand, both Pdis groups did show a moderate increase in EEAs in total (Figure S4). Thus, our model, teasing apart the treatment effects, shows that overall phylogenetic diversity effects were in line with the hypothesis and correlated with increases in EEAs (Figure 6B). Functional diversity effects, however, were not (Figure 6C), signifying that the effects of diversity in the study were more complex than we previously thought.

The contrasting diversity effects revealed that while substrate biochemical similarity can explain similarities in response to biodiversity effects, it's not necessarily a universal predictor. In addition, the direction of the correlation effects, positive for phylogenetic diversity and negative for functional diversity remained the same for all but one substrate. Rather, the strength of the diversity effect was changing (Figure 6). Such differences can be related to inherent variations in exoenzyme production, maintenance and regulation. Biochemically unrelated substrate groups such as monosaccharide (aG, bG), polysaccharide (CB, XYL, NAG) and ester (ACE, BUT, OLE) had very different responses to the diversity treatments and others like amino acids (ARG) and polysaccharides had almost identical responses. Variations between and within the same substrate biochemical groups could be due to fundamental differences for the class of exoenzymes targeting them. For example, polysaccharides are targeted by hydrolytic exoenzymes that are produced on-demand (Warren 1996, Lynd et al. 2002). In contrast, enzymes involved in the degradation of fatty acids belonging to the ester

379 group of substrates are part of bacterial core metabolism, and thus can be continuously
380 produced. Our results show that greater diversity effects are more important to ecosystem
381 functions that rely on environmental factors, such as the temporal or spatial presence of plant-
382 derived polysaccharides. Hence, not only the diversity of ecosystem functions tested (Huston
383 1997, 2000, Tilman 1997, Loreau 2000) but also their underlying biochemical mechanisms
384 have a great effect on the outcomes of biodiversity research.

385 To understand the very strong contrasting changes in EEAs of the Psim groups, their genus-
386 specific capacity for co-existence with its related species is crucial. Psim-Fsim communities
387 contained *Flavobacteria* species which are usually non-motile organisms, prefer aquatic
388 environments and have strong differences between species. The genus includes generalist
389 species that can use a wide range of carbohydrates, to more specialist asaccharolytic species
390 that prefer nitrogenous substrates (i.e. amino acids) (Bernardet and Bowman 2006). These
391 physiological characteristics might allow the *Flavobacteria* communities to increase their EEA
392 as different species could be utilising different carbon sources in their growth medium.
393 Communities of these specialist *Flavobacteria* could be facilitating community interactions that
394 translate into increases in their community EEAs. In contrast, the *Pseudomonas* species in
395 Psim-Fdis communities are generally motile-planktonic bacteria, ubiquitous in almost all oxic
396 environments, including soil and water, due to their expansive metabolic repertoire, simple
397 nutritional requirements and rapid growth rates (Moore et al. 2006). However, the reduced
398 capacity of many *Pseudomonas* species to withstand starvation (Moore et al. 2006) might lead
399 them to adapt more selfish nutrient acquisition strategies (Reintjes et al. 2017, 2019, Arnosti et
400 al. 2018). As generalists, *Pseudomonas* species might be more competing, resulting in
401 *Pseudomonas* communities with lower EEAs than their respective isolates. Thus, bacteria with
402 a broad substrate range might not benefit when living in communities of the same genus but of
403 dissimilar functioning. Contrastingly, in the Pdis communities these genus-specific effects in
404 nutrient acquisition strategies can be balanced due to the increased species diversity.

405 Nevertheless, even competing generalists like *Pseudomonas* species will profit from species
406 interactions when incubated for longer time in the presence of certain substrates. When we used
407 the Psim-Fdis communities to run EEA assays for longer time (Figure 5), we identified that the
408 amplifying effect of substrate acclimation on the EEAs is much stronger for the communities
409 than for the constituent isolates on substrate aG. But oversimplifying the experimental design
410 and just looking at one substrate or one group, would have let to an underestimation of the
411 complexity of diversity ecosystem functioning relationships, and explain why we see studies
412 showing opposite effects of biodiversity (Balvanera et al. 2006).
413 Altogether, our results concur with previous studies that focus on separating the different
414 aspects of biodiversity and examining the relationship between biodiversity and ecosystem
415 functioning (Griffiths et al. 2001, Zhang and Zhang 2006, Balvanera et al. 2006, Naeem et al.
416 2009, Jousset et al. 2011, Purschke et al. 2013, Thompson et al. 2015, Gamfeldt and Roger
417 2017). A meta-analysis demonstrated that biodiversity effects depend on the organisation level
418 with positive effects at the community level, positive but weaker effects at the ecosystem level
419 and negative effects at the population level (Balvanera et al. 2006). A modelling approach
420 showed that when considering increasing number of functions, the effect of biodiversity is lost
421 (Gamfeldt and Roger 2017). Experiments done by Griffiths *et al*. (Griffiths et al. 2001)
422 recognised no consistent effect from biodiversity on soil functions. Thus, the need for more
423 research including the effects of the environmental context, like nutrient availability, on the
424 relationship between biodiversity and ecosystem functioning was recognized (Zhang and Zhang
425 2006). Our study fills this gap by focussing not only on phylogenetic and functional diversity
426 of organisms, but also measuring a broad diversity of activities controlled by different
427 underlying mechanisms of enzyme regulations. Such a complex approach is required to tackle
428 the complex dimensions of biodiversity research. We show that biodiversity had a significant
429 impact on most EEAs, but the strength of the effect depended on the context. As such,
430 biodiversity's effects on community ecosystem functioning cannot always be predicted as a

positive correlation between species diversity and enhanced ecosystem function (Aarssen 1997, Tilman 1997, Huston 2000, Carroll et al. 2011). Natural, larger scale experiments struggle with introducing different levels of complexity in their experimental design (Cairns et al. 2018). That is why synthetic communities, as simplified systems, are ideal to study such complex biodiversity effects. Looking forward, we should aim to understand how diversity effects can mechanistically influence ecosystems, synthetic and natural (Fox and Harpole 2008, Naeem et al. 2009, Turnbull et al. 2013), and not take the effects as granted (Shade 2017).

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Author Contributions

KK and SWH secured funding for the project. CX, MT, and KK designed the experiments. CX performed the experiments and carried out all assays and molecular analyses; the data analysis was performed by CX closely supported by MT. Statistical analysis was performed by CX supported by MT and SWH. All co-authors assisted in interpreting the results; CX initiated the manuscript writing, which was finalized with substantial contributions from MT, KK and SWH.

Competing Interests

The authors declare that they have no conflict of interest.

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724 ## Legends for figures and tables

725 **Figure 1: Functional and phylogenetic classification and grouping of groundwater**
726 **isolates.** EEAs are shown for twenty-seven groundwater isolates incubated with eleven
727 fluorescent substrate analogues. Activities are given in relative fluorescence units (RFU) on a
728 logarithmic scale. Empty (white) boxes denote activity outside the detectable range. The left
729 dendrogram shows clusters of isolates with similar function based on Euclidean hierarchical
730 dissimilarity distances. The topmost dendrogram shows hierarchical clustering of substrates
731 that produced similar activity patterns in the assays. The assignment of isolates to the
732 communities of each trait-group is depicted to the right of the heatmap. Double-coloured boxes
733 indicate isolates that were assigned to more than one group. In the legend, the colours indicate
734 the community group and the phylogenetic (P) and functional (F) traits of the communities are
735 indicated as similar (sim) or dissimilar (dis).

736

737 **Figure 2: Individual communities plotted against the phylogenetic and functional**
738 **dissimilarities within and between groups**. Colours indicate the community groups; red for
739 Psim-Fsim group, green for Psim-Fdis group, blue for Pdis-Fsim group and purple for Pdis-
740 Fdis group. Phylogenetic and functional dissimilarity metrics have been z-score standardized.
741 Variation of dissimilarity scores exists within groups, but the groups are cleanly separated from
742 each other.

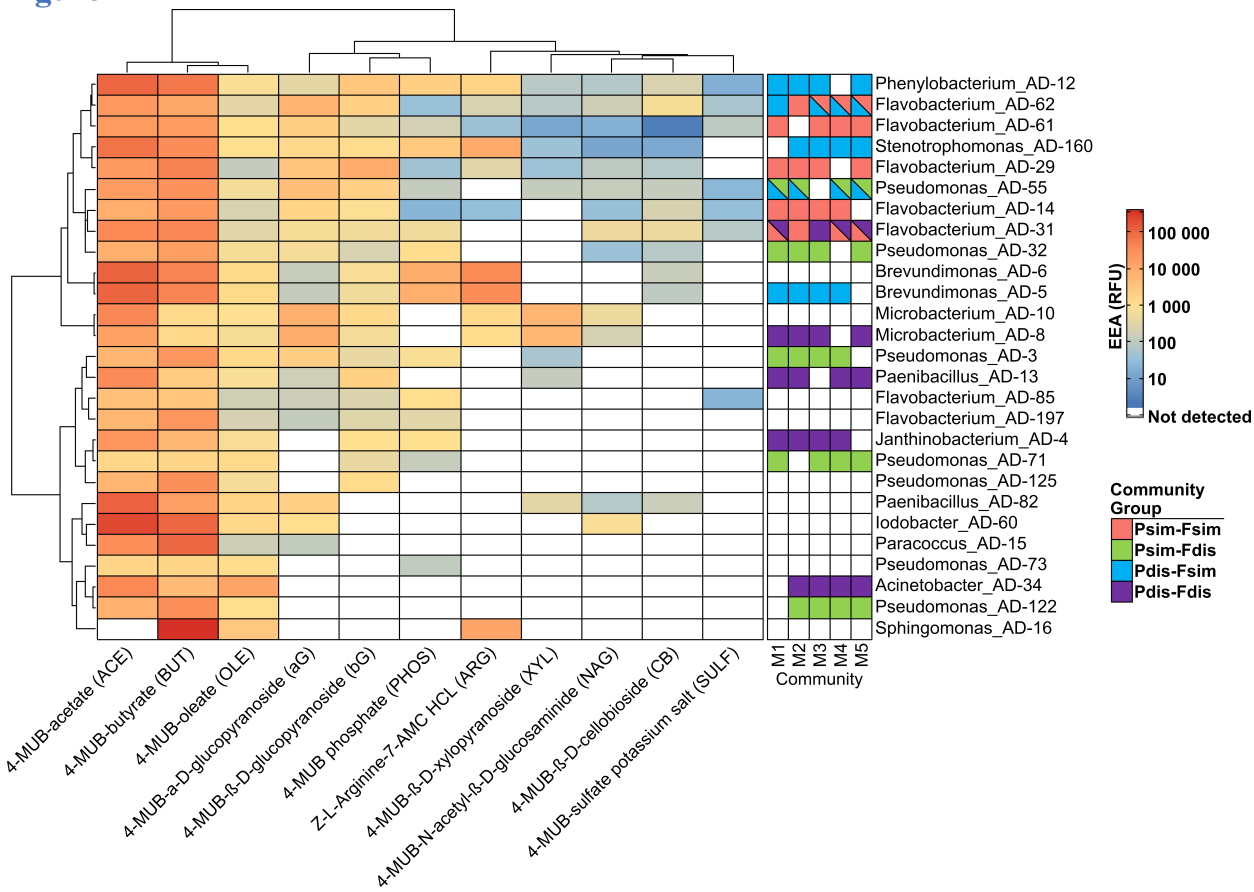
743

744 **Figure 3: Heatmap of community EEA proportional changes**. The heatmap scale depicts
745 proportional changes from -100 to 100%. Communities that exhibited changes over 100% have
746 their percent change value shown in the heatmap matrix. Red colours depict a higher activity
747 of the community; blue colours depict a lower activity of the community than estimated based
748 on the activities of the individual isolates. White boxes show measurements outside the

749 detection limits. On the right, the label for each row indicates the community and the group
750 from where the activities are taken from; red for Psim-Fsim group, green for Psim-Fdis group,
751 blue for Pdis-Fsim group and purple for Pdis-Fdis group communities. Columns indicate the
752 substrates used in the EEA assays. The dendrogram depicts hierarchical clustering of each
753 substrate's activities, showing groups of substrates with similar activity responses.
754
755 **Figure 4: Temporal trends of EEA changes between communities and isolates from 0 h to**
756 **24 h after community mixing**. Rows show community groups and columns show the substrate
757 where the activity change was noted. The average changes between the timepoints are indicated
758 by the colour and size of the arrows and their statistical significance (Student's *t*-test, 2-tailed)
759 showed on top of the arrows. Significance symbols: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$;
760 non-significant (ns) $p > 0.05$.
761
762 **Figure 5: Substrate acclimation effect on isolates and communities.** EEA of Psim-Fdis
763 group isolates and communities on 4-MUB-α-D-glucopyranoside (aG) are shown as an
764 example of the substrate acclimation effect. Data is normalized to EEA measured at 0h after
765 mixing without preincubation with the substrate (green) with a fold-change of 1. EEA is shown
766 after 270 min of preincubation with the substrate (orange) and at 6 h after community mixing
767 without additional preincubation with the substrate (grey). Bars depict average fold-changes in
768 the EEA of isolates and communities and error bars show the standard deviation (n = 5).
769 Community EEA are shown to increase ~3 times more on average when incubated with the
770 substrate.
771
772 **Figure 6: Strength and direction of phylogenetic and functional diversity treatments on**
773 **the EEA of bacterial communities.** (A) The linear regression model slopes for all 11 substrates
774 and the average for all substrates (Table S4) are plotted here as points. In (B) the average for

all substrates, community EEA activity, is positively correlated with phylogenetic diversity ($p < 0.001$). In (C) the average for all substrates, community EEA activity, is negatively correlated with functional diversity ($p < 0.001$). Coloured points in (A) indicate different substrates and the average. Coloured squares in (B) and (C) indicate community groups: Psim-Fsim in red, Psim-Fdis in green, Pdis-Fsim in blue and Pdis-Fdis in purple.

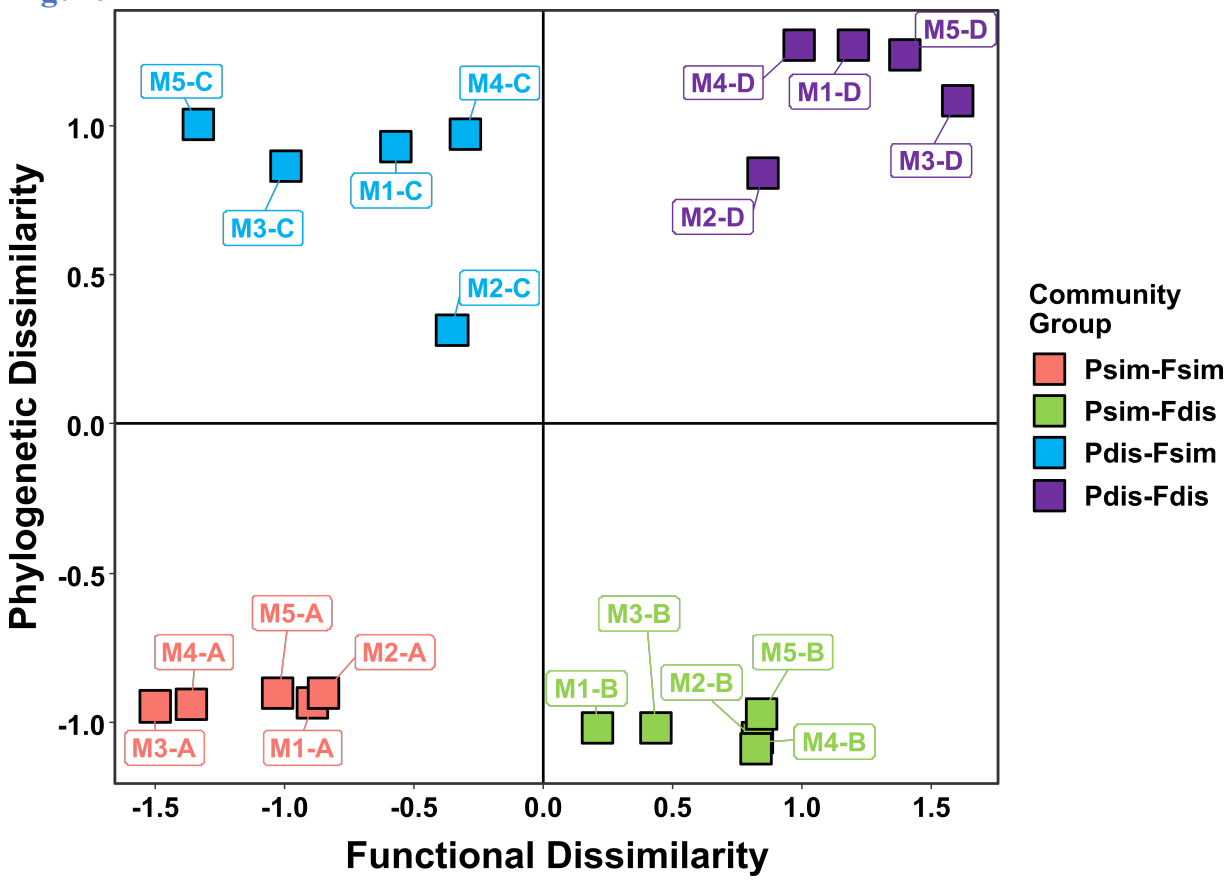
780 **Figure 1**



781

782

783 **Figure 2**



784

785

Figure 3

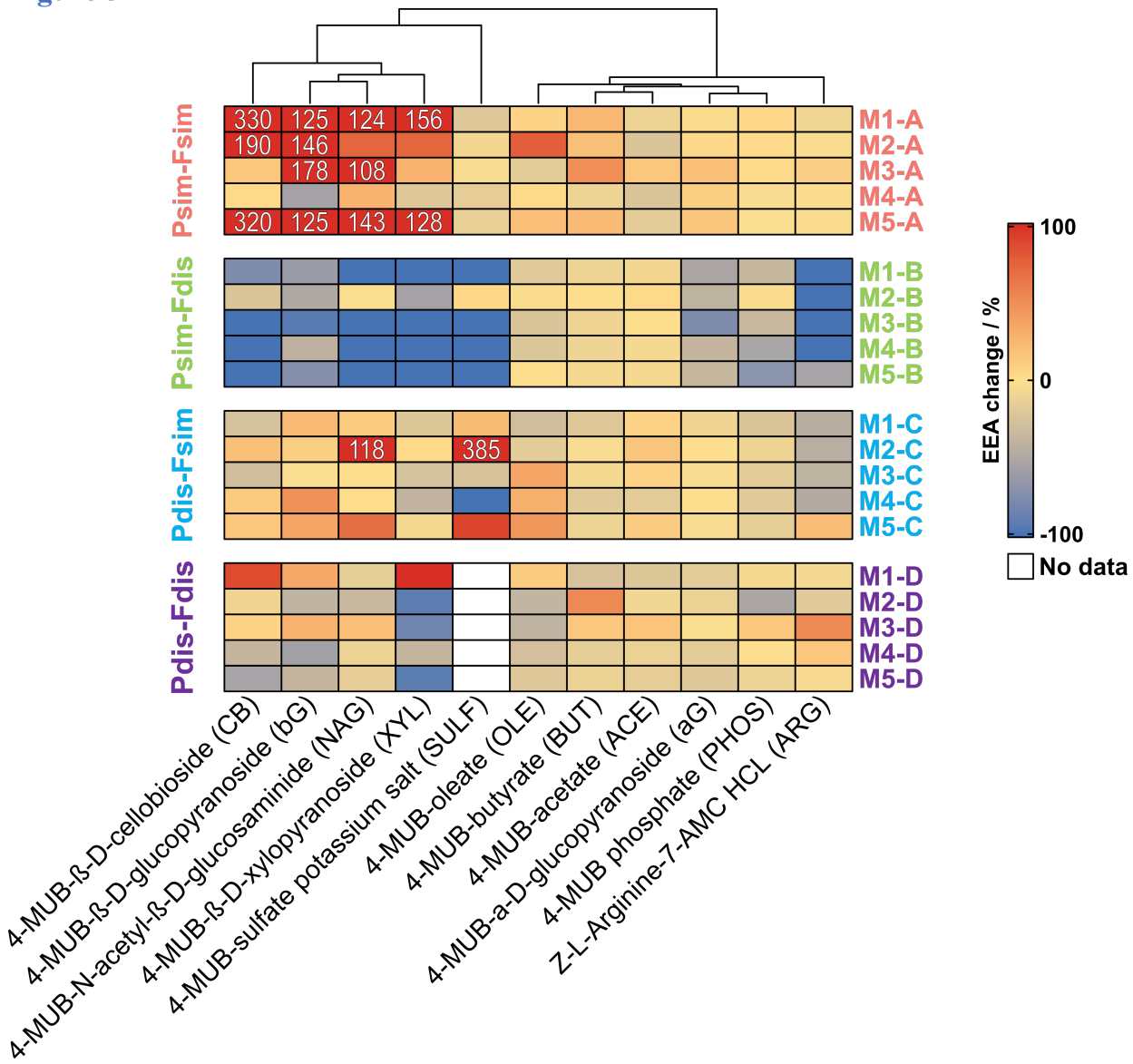

Psim-Fsim
Psim-Fdis
Pdis-Fsim
Pdis-Fdis
330 125 124 156
190 146
178 108
320 125 143 128
118 385
M1-A
M2-A
M3-A
M4-A
M5-A
M1-B
M2-B
M3-B
M4-B
M5-B
M1-C
M2-C
M3-C
M4-C
M5-C
M1-D
M2-D
M3-D
M4-D
M5-D
4-MUB-ß-D-cellobioside (CB)
4-MUB-ß-D-glucopyranoside (bG)
4-MUB-N-acetyl-ß-D-glucosaminide (NAG)
4-MUB-ß-D-xylopyranoside (XYL)
4-MUB-sulfate potassium salt (SULF)
4-MUB-oleate (OLE)
4-MUB-butyrate (BUT)
4-MUB-acetate (ACE)
4-MUB-a-D-glucopyranoside (aG)
4-MUB phosphate (PHOS)
Z-L-Arginine-7-AMC HCL (ARG)
EEA change / %
100
0
-100
No data

Figure 4

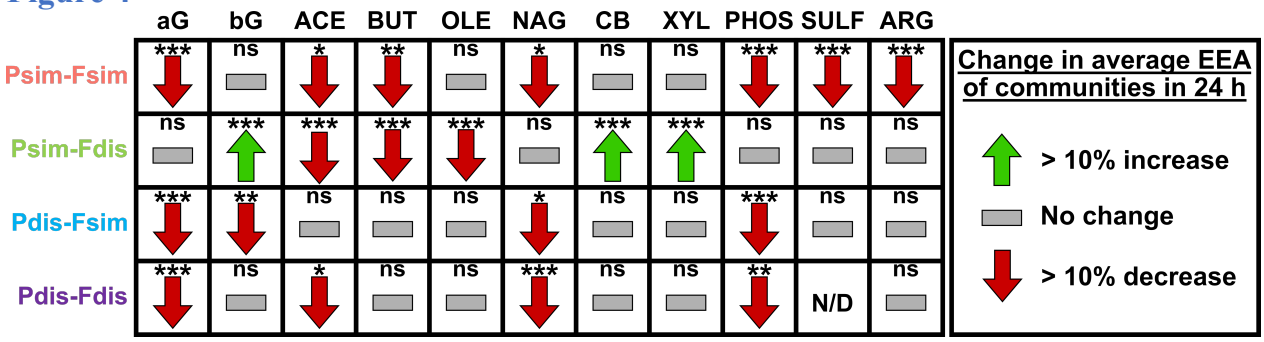

aG bG ACE BUT OLE NAG CB XYL PHOS SULF ARG
Psim-Fsim
Psim-Fdis
Pdis-Fsim
Pdis-Fdis
*** ns * ** ns * ns ns *** *** ***
ns *** *** *** *** ns *** *** ns ns ns
*** ** ns ns ns * ns ns *** ns ns
*** ns * ns ns *** ns ns ** N/D ns
Change in average EEA of communities in 24 h
> 10% increase
No change
> 10% decrease

Figure 5

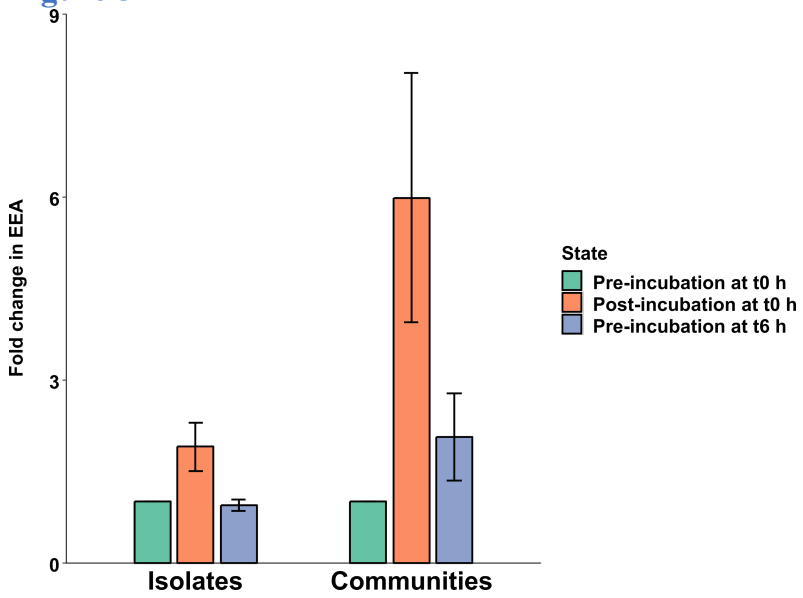
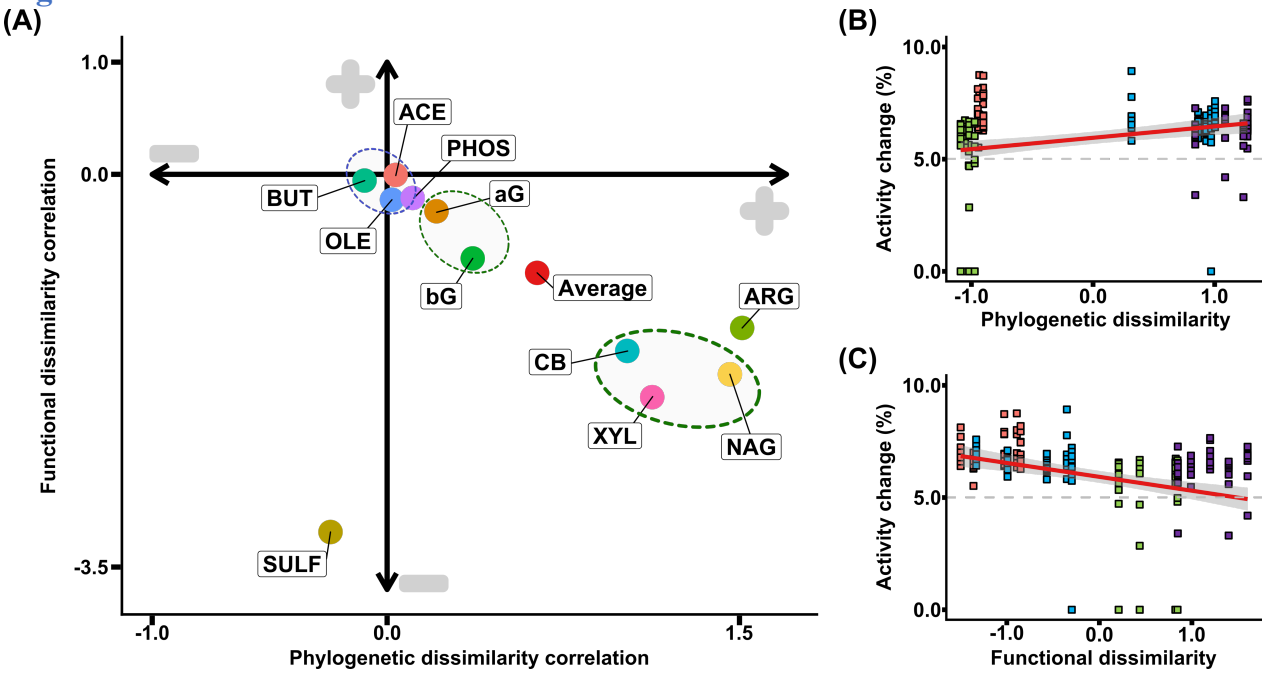

Fold change in EEA
9
6
3
0
Isolates
Communities
State
Pre-incubation at t0 h
Post-incubation at t0 h
Pre-incubation at t6 h

Figure 6


(A)
1.0
0.0
-3.5
Functional dissimilarity correlation
ACE
PHOS
BUT
aG
OLE
bG
Average
ARG
CB
XYL
NAG
SULF
-1.0
0.0
1.5
Phylogenetic dissimilarity correlation
(B)
10.0
5.0
0.0
Activity change (%)
-1.0
0.0
1.0
Phylogenetic dissimilarity
(C)
10.0
5.0
0.0
Activity change (%)
-1.0
0.0
1.0
Functional dissimilarity