

Explorative Analysis of 60 Metagenomes to Unveil the Abundance of Widespread Functions in the Environment and Its Weight on Functional Indices

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Research

Keywords: bacterial and fungal functional richness, metagenome functionality, Diversity, aquatic and terrestrial metagenomes

Posted Date: August 2nd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-50009/v1>

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Title: Explorative analysis of 60 metagenomes to unveil the abundance of widespread functions in the environment and its weight on functional indices

Running title: The buffering effect of widespread functions

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Abstract

Background: The total bacterial and fungal functional richness was recently predicted to be millions of KEGG level 3 functions but due to limited space and resource availability together with constraining environmental conditions, the local functionality only comprises a subset of the total functionality. However, widespread functions whose abundance and redundancy depends on species richness or sequencing depth could bias functional indices. Here, we used 20 randomly chosen extant metagenomes from low to high species richness each from aerial, aquatic and terrestrial ecosystems to quantify the distribution of widespread KEGG level 3 functions and their buffering effect on diversity, redundancy and richness.

Results: A different number of widespread functions were detected in each ecosystem with 1,201 found in all three. Diversity found to be similar in the three ecosystems and redundancy that was significantly lower in aerial metagenomes showed identical patterns when all functions were used or when the widespread functions were removed. However, functional richness changed from being significantly higher in aquatic than in terrestrial and aerial metagenomes when all functions are used to no significant differences when the widespread functions are removed. The variance in diversity and richness was higher without widespread functions, making it possible to compare highly dense environments that would otherwise result in similar values.

Conclusions: Taken together, we describe the widespread functionome in aerial, aquatic and terrestrial metagenomes, and its buffering effect on functional indices that are driven by widespread functions which is why, moving forward, we recommend to remove widespread functions from the analysis of metagenome functionality.

1 **Background**

2 Ecosystem functioning is mediated by biochemical transformations performed by a
3 community of organisms from every domain of life [1]. In every community, multiple organisms
4 from different taxonomic groups can play similar if not identical roles in ecosystem
5 functionality; the so-called functional redundancy [2]. Functions can be statistically inferred
6 based upon homology to experimentally characterized genes and proteins in specific organisms
7 to find orthologs in other organisms present in a given metagenome. This ortholog annotation,
8 among other annotation tools, can be performed in KEGG Orthology (KO) [3,4], which covers a
9 wide range of functional classes (level 1 of KO) comprising cellular processes, environmental
10 information processing, genetic information processing, human diseases, metabolism,
11 organismal system (09150), *brite* hierarchies and functions not included in the annotation of
12 the two databases *pathway* or *brite*. However, the bottleneck of describing metagenome
13 functions is the low number of fully sequenced and annotated genomes as they are mostly
14 limited to those of direct medical or biotechnological interest that have undergone extensive
15 isolation and characterization. Problematically, the vast majority of organisms were not yet
16 studied [5,6] and their annotations are based on their similarity to the genomes of the very few
17 studied model organisms, potentially yielding flawed annotations [7]. Recently, the total
18 functional richness of bacteria [8] and fungi [8,9] was predicted to be millions of KEGG level 3
19 functions but locally, only a subset of the functions will be available [10]. However, widespread
20 functions present in all metagenomes with abundances depending on the local metagenome
21 density could potentially impact functional indices.

22 Here, we used a meta-analysis of 20 randomly chosen extant metagenomes each from
23 aerial, aquatic and terrestrial ecosystems representing the full spectrum of metagenome
24 richness in each ecosystem to quantify the widespread functional richness. We aimed to analyze the
25 impact of removing widespread functions on the commonly used functional indices of diversity
26 (as Shannon diversity), redundancy and richness. We hypothesize that (i) functionality in
27 aquatic and terrestrial ecosystems will be higher than in aerial metagenomes with generally low
28 biomass [11]. Functionality in both bacteria [8] and fungi [8,9] will be dominated by widespread
29 functions, making up roughly 60% of the entire functionality which is why we further

30 hypothesize that (ii) removing the widespread funciome will result in different estimates of
31 functional indices, potentially resulting in varying differences among the three ecosystems.

32 **Methods**

33 *Data collection and the estimation of functional diversity, redundancy and richness*

34 The publicly available data was downloaded from the integrated microbial genomes &
35 metagenomes (IMG/M) from the Joint Genome Institute (JGI) under
36 <https://img.jgi.doe.gov/cgi-bin/m/main.cgi>. For each of the three ecosystems aerial, aquatic
37 and terrestrial, the gene counts of KEGG level 3 functions (as “with KO” in the column “Protein
38 coding genes” under “Metagenome Statistics” once a genome was selected) were retrieved
39 from 20 randomly chosen metagenomes representing a full spectrum that covers the whole
40 range of microbial density (indicated by metagenome depth, gene count and number of rRNA
41 copies) on February 14th 2020. The distribution of functions among the 20 samples in the three
42 ecosystems was plotted to define the threshold between rare and widespread funciome
43 (**Figure 1**). Admittedly, the threshold of core functions found in at least 16 of 20 samples was
44 subjectively set as it aligned well with the distribution of functions in aquatic and terrestrial
45 ecosystems. In favor of our threshold of the core funciome were previously determined
46 thresholds of core microbiomes from 16S and ITS2 data that ranged between 30 and 100% in
47 corals [12] as parallel of core distributions. For aerial metagenomes, there did not seem to exist
48 a clear threshold of core functions indicated by an increase in abundance in aquatic and
49 terrestrial metagenomes, but we aimed to equally analyze the three ecosystems to better
50 compare resulting functional indices. Many of these genes are likely to be housekeeping genes
51 required by almost every organism to maintain basic cellular function. As shown before [8,9],
52 the data structure suggests the presence of more than just rare and widespread functions.
53 Indeed, aquatic metagenomes showed a local maximum of functions in 9 of 20 samples, but
54 here only the core functions were considered. Functional diversity was determined using the
55 Shannon diversity index H according to **Equation 1**, where p_i is the relative abundance of the
56 KEGG level 3 function. Noteworthy, other diversity measures like the Jost indices [13] could be
57 easier to interpret and yield different results but the Shannon index remains the most popular

58 in microbial ecology. Functional redundancy was calculated as the average, for all functions in
59 each sample, of how often one KEGG level 3 function was present. Functional richness was
60 determined as the number of different KEGG level 3 functions in each sample.

61 Eq. 1: $H = -\sum_{i=1}^k p_i \log(p_i)$

62 *Modelling of the relationship of functional indices to metagenome depth*

63 First, the relationship between diversity, redundancy and richness to sequencing depth of the
64 metagenome as metagenome depth was fitted to a linear model. While redundancy showed a
65 significant correlation of the linear regression to metagenome depth (Adj. $R^2 = 0.8967$, P-value
66 $< 2.2e^{-16}$), the correlation of diversity to metagenome depth was not significant (Adj. $R^2 =$
67 0.0042 , P-value = 0.268) and both the significance and coefficient of determination were
68 weaker for functional richness (Adj. $R^2 = 0.3797$, P-value = $9.589e^{-8}$), with similar results for all
69 functions or when the widespread functions were removed (data shown includes the removal).
70 For diversity and richness, the data was fitted to a saturated (**Equation 2**) and an unsaturated
71 model (**Equation 3**) with the critical point estimated by the term $3A_f$ as previously described
72 [14]. The fit of the models was compared by analysis of variance (ANOVA) and Akaike
73 Information Criterion (AIC) [15] with a penalty per parameter set to k equals two.

74 Eq. 2: $Functional\ richness = \frac{f_{max} * [Species\ richness]}{A_f + [Species\ richness]}$

75 Eq. 3: $Function\ richness = \frac{f_{max} * [Species\ richness]}{A_f + [Species\ richness]} + k * [Species\ richness]$

76 Here, f_{max} is the maximum functional richness, A_f the accretion rate of functions with an
77 increasing number of species and k the constant of the additive term.

78 **Results**

79 *The abundance of widespread functions in environmental metagenomes*

80 The distribution of KEGG genes over 20 samples showed the highest number of genes in
81 a single sample, totalizing 24.8% in aerial metagenomes as opposed to 10.9% in aquatic and
82 13.2% in terrestrial metagenomes (**Figure 1a**). Conversely but only visible in aquatic and
83 terrestrial metagenomes, an increase in KEGG functions was seen when at least 16 of 20

84 samples (80%) were considered, comprising 9.6% of all KEGG functions in aerial, 24.9% in
85 aquatic and 25.0% in terrestrial metagenomes. Noteworthy, another maximum of KEGG
86 functions was visible between 45-60% of the samples, especially in aquatic ecosystems. In total,
87 aerial, aquatic and terrestrial ecosystems shared 1,201 widespread functions (**Figure 1b**).
88 Particularly, the widespread KEGG genes found in all ecosystems were affiliated with metabolic
89 pathways (427), biosynthesis of secondary metabolites (217), biosynthesis of antibiotics (154),
90 microbial metabolism in diverse environments (89) and carbon metabolism (89) as the five
91 most abundant pathways. On the level of modules, the widespread KEGG genes included the
92 citrate cycle (16), NADH:quinone oxidoreductase in prokaryotes (15), reductive citrate cycle
93 (14), 2-oxoglutarate → oxaloacetate (13) and glycolysis (12) as the top five most abundant
94 modules (a detailed list of the widespread functions can be found in the supplementary
95 information, **Table S1**). Only 6 widespread functions were solely found in aerial ecosystems as
96 opposed to 1,021 in aquatic and 279 in terrestrial metagenomes. Aerial and aquatic ecosystems
97 shared 10, aerial and terrestrial metagenomes shared 22, and aquatic and terrestrial
98 ecosystems shared 1,954 widespread functions.

99 *The relationship of functional indices and metagenome depth*

100 When the functional indices of all functions were compared to the functions remaining
101 after the removal of widespread functions, statistically similar functional diversities across the
102 three metagenomes were observed (Tukey HSD, P-value > 0.05) [16] (**Figure 2**). However, the
103 removal of widespread functions resulted in generally lower values and a higher variation
104 within each ecosystem. Similarly, the pattern of significantly (P-value < 0.05, HSD-test) lower
105 functional redundancy in aerial metagenomes was found whether all functions were used or if
106 the widespread functions were removed. However, even though the values were generally
107 lower, they also showed lower variation. Lastly, functional richness was significantly (Tukey
108 HSD, P-value < 0.05) higher in aquatic metagenomes compared to terrestrial and aerial
109 ecosystems. With the removal of widespread functions, the three ecosystems showed similar
110 functional richness, which was generally lower and had a higher variation. Functional diversity
111 of all functions showed an asymptote that tends to towards 7.6, indicated by the better fit to
112 the saturated model (**Figure 3a**) while functional redundancy showed a significant positive

113 linear correlation to metagenome depth (**Figure 3b**). Otherwise, the correlation between
114 functional richness and metagenome depth was best described by an unsaturated model
115 (**Figure 3c**). Admittedly, the difference in AIC of the saturated and unsaturated model was lower
116 (0.7%) for richness as compared to diversity (AIC-difference = 1.7%), which could mean that the
117 evenness changed. The relationships were of identical nature when the widespread functions
118 were removed but a higher variation in both functional diversity (**Figure 4a**) and richness
119 (**Figure 4c**) resulted in higher AIC values and less significant parameters with higher standard
120 errors. On the contrary, functional redundancy showed a higher coefficient of determination
121 with the removal of widespread functions albeit with a much slighter slope and overall lower
122 values (**Figure 4b**). Noteworthy, due to the wide range of metagenome depths that are
123 dominated by low depth metagenomes near or equal to zero in both redundancy and richness,
124 both slopes and correlations of the variables are likely skewed towards the high depth
125 metagenomes. However, the important aspect of the correlations are the higher variations of
126 functional diversity and functional richness and a reduction of functional redundancy.

127 **Discussion**

128 Consistent with our first hypothesis, both functional redundancy and richness were
129 significantly (P-value < 0.05) higher in aquatic and terrestrial metagenomes as compared to
130 aerial metagenomes. Otherwise, functional diversities showed similar values in the three
131 ecosystems. In aerial metagenomes, low redundancy and richness may be a direct consequence
132 of low metagenomic density, as depicted by a sequencing depth several orders of magnitude
133 lower than for their aquatic and terrestrial counterparts. Noteworthy, a sufficient sequencing
134 depth is key to cover all organisms and their functions in each sample and could result in lower
135 functional indices values but with the data at hand, it is hard to ascertain. Even in environments
136 of low functionality, the functional diversity still reaches a similar maximum at a Shannon
137 diversity of 7.6 ± 0.1 . Because diversity is based on both richness and evenness, a higher
138 evenness could counteract a low richness to yield a comparable diversity. This derives from the
139 structure of functions and their distribution in sequenced metagenomes that seemed to be
140 similar in every metagenome if a certain threshold of metagenome density is reached. In our

141 case, the threshold was with a metagenome depth of 100,000,000 bases. In comparison, the
142 size of the fungal genomes of *Aspergillus nidulans* and *Saccharomyces cerevisiae* were reported
143 to be 30,000,000 [17] and 12,100,000 base pairs (from the *Saccharomyces* genome database),
144 respectively. Even though bacterial genomes tend to be an order of magnitude smaller, i.e.
145 4,600,000 base pairs of *Escherichia coli* [18], and other organisms such as viruses have even
146 smaller genomes but can contribute to the functionality, a metagenome of 100,000,000 bases
147 will likely comprise of only a few different organisms. With that in mind, we assume that the
148 maximum potential of functional diversity is reached in every energy or nutrient unlimited
149 environment, particularly in aquatic and terrestrial ecosystems. Both functional redundancy
150 and richness increased with increasing metagenome depth, implying unlimited potential of
151 functional diversity with metagenome depth. Adding more organisms will not only add more
152 rare functions but also increase the chances of the addition of already present widespread (or
153 rare) functions [8,9] that, in turn, will increase both functional redundancy and richness.
154 However, the local metagenome will be limited by space and resource availability as well as
155 constraining environmental conditions which is why unlimited functional redundancy and
156 richness are only theoretically possible. The largest metagenome in our meta-analysis was of
157 6,556,162,052 bases, representing thousands of different organisms inhabiting the marine
158 sediment from White Oak River estuary in North Carolina, and could indicate the maximum
159 capacity of functional diversity.

160 The removal of widespread functions logically resulted in lower values for all indices
161 with higher variation in diversity and richness but lower variation in redundancy. The Shannon
162 index takes richness and evenness into account, which is why removing widespread functions
163 increased evenness more than it reduces richness, resulting in a higher diversity. In line with
164 our second hypothesis, the significant differences in functional richness vanished with the
165 removal of widespread functions, indicating that most functions especially in microbe-rich
166 environments are indeed widespread. Further, similar functional richness of non-widespread
167 functions indicated that the total functionality in aerial, aquatic and terrestrial metagenomes is
168 very similar despite the different microbial density. Otherwise, functional diversity (similar
169 between the three ecosystems) and redundancy (significantly lower in aerial metagenomes)

170 still showed the same trends. Only functional redundancy was able to capture the difference
171 between aerial, aquatic and terrestrial ecosystems after the removal of widespread functions
172 because those were presumably highly redundant functions. However, greater variation in both
173 diversity and richness can be advantageous to compare microbe-rich environments.
174 Particularly, functional diversity ranged from 6.19 to 7.64 after the removal of widespread
175 functions, which represents a two-fold increase in range compared to all functions (7.37-8.10).
176 The scale of sequencing depths between habitats varied greatly but we aimed to depict a
177 representation of each of those habitats that are publicly available rather than normalizing the
178 amount of sequences. However, the outcome could be affected as there is a higher probability
179 of sequencing already abundant genes or functions by chance as opposed to rarer ones.

180 **Conclusion**

181 Altogether, we unveil the weight of widespread functions in statistical analyses and
182 quantify their abundance. The removal of widespread functions resulted in a higher quality of
183 functional diversity, redundancy and richness, and will make possible the comparison of
184 microbe-rich environments that would otherwise have a similar distribution. Admittedly, our
185 estimates are based on a meta-analysis of 20 randomly chosen metagenomes that represent a
186 snapshot of all possibilities but given the low number of aerial metagenomes (n = 108), it
187 seemed to be the most feasible and unbiased approach. Moving forward, we recommend the
188 removal of widespread functions, i.e. by using the functions provided in **Table S1**, to analyze
189 metagenome functionality as we demonstrated their tremendous impact of functional indices.
190 A core functionome needs to be defined similar to the core microbiome where
191 widespread/generalist functions are done by all or most and rare/specialist functions are done
192 by only a few or in rare circumstances [19]. Other than the similarity-based assessment of
193 functions, the production of knock-ins on a large scale by adding whole contigs and sequencing
194 the mutant inserts of potentially interesting phenotypes represents an interesting approach
195 suitable for unveiling yet unknown functions [20].

196 **Declarations**

197 *Ethics approval and consent to participate*

198 Not applicable

199 *Consent for publication*

200 Not applicable

201 *Availability of data and material*

202 The data is publicly available at the integrated microbial genomes & metagenomes (IMG/M)
203 from the Joint Genome Institute (JGI) under <https://img.jgi.doe.gov/cgi-bin/m/main.cgi>

204 *Competing interests*

205 The authors declare no competing interests.

206 *Funding*

207 This work was supported by the Czech Science Foundation (20-02022Y).

208 *Author's contribution*

209 RS and SPC designed the study. RS analyzed the data. RS and SPC wrote and revised the
210 manuscript. All authors read and approved the final manuscript.

211 *Acknowledgements*

212 Not applicable

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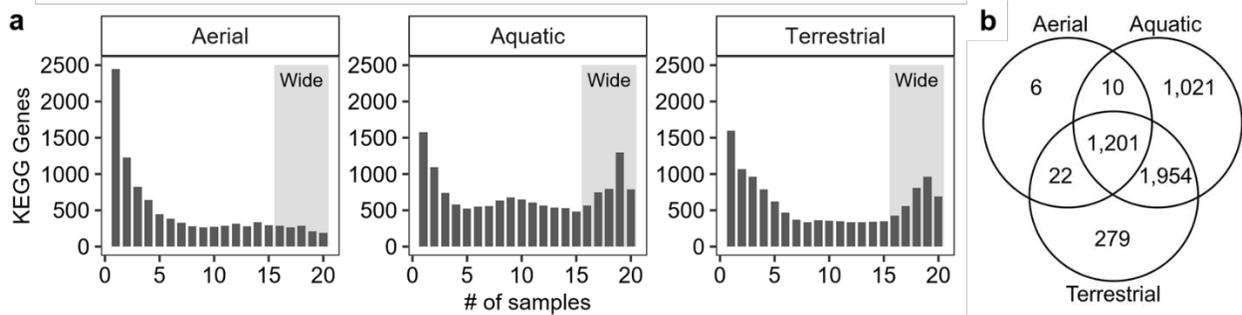
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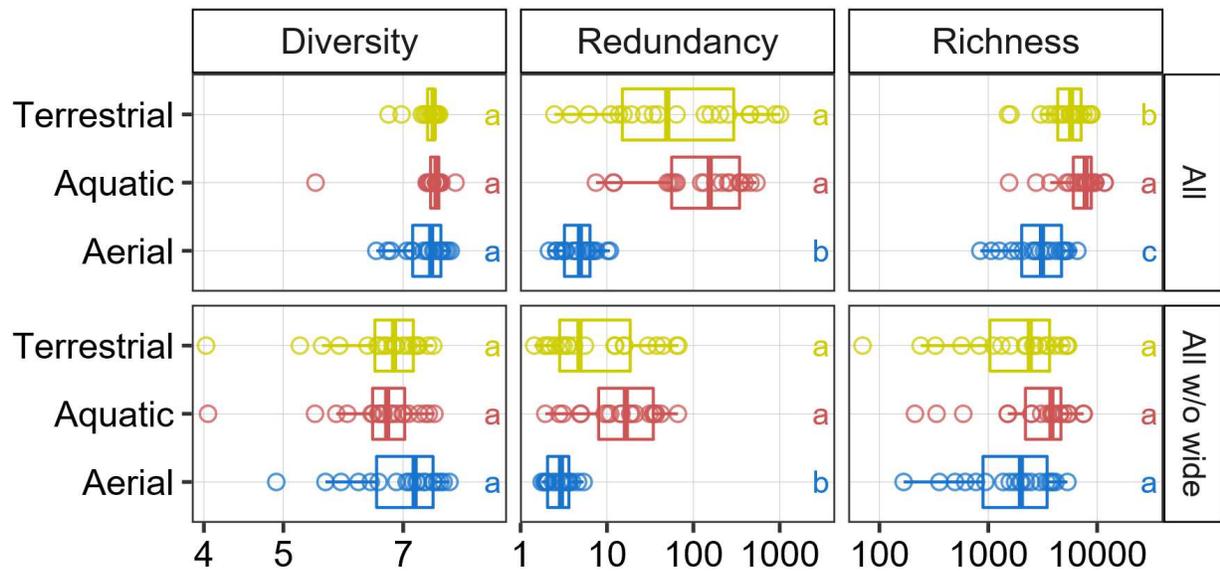
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253

254 **Figure and figure legends**



255
256 **Figure 1:** The number of genes with KEGG level 3 functions found in subsets of the 20 aerial,
257 aquatic and terrestrial metagenomes with their widespread function in at least 16 of 20
258 samples indicated in grey (a) and the share of widespread functions between the three
259 ecosystems (b).



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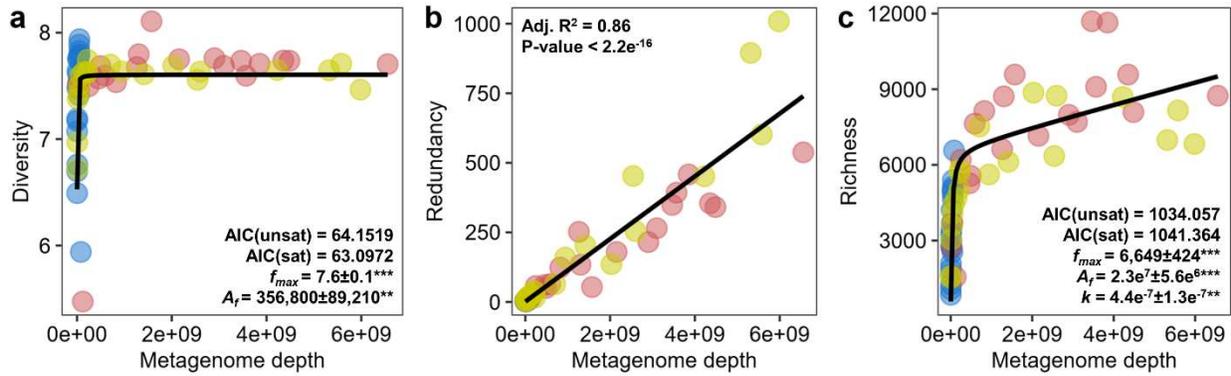
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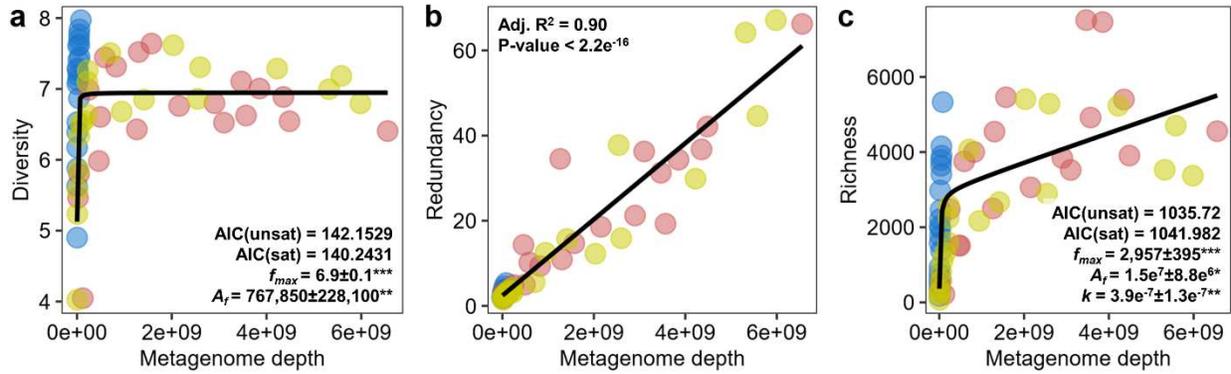
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Figure 2: Functional diversity, redundancy and richness in aerial, aquatic and terrestrial metagenomes of the whole range of microbial density when all functions are considered (All) or when the widespread functions are removed (All w/o wide) (a). Groups followed by the same letter are not significantly different according to the HSD test (P-value > 0.05).



265

266 **Figure 3:** The saturated relationship of functional diversity, linear relationship of functional
 267 redundancy and unsaturated relationship of functional richness to metagenome depth when all
 268 functions are considered. Aerial samples are shown in blue, aquatic in red and terrestrial in
 269 yellow. Asterisks indicate the significance of individual parameters in saturated and unsaturated
 270 models (* = P-value < 0.05, ** = P-value < 0.01 and *** = P-value < 0.001).



271

272 **Figure 4:** The saturated relationship of functional diversity, linear relationship of functional
273 redundancy, and unsaturated relationship of functional richness to metagenome depth when
274 the widespread functions are removed. Aerial samples are shown in blue, aquatic in red and
275 terrestrial in yellow. Asterisks indicate the significance of individual parameters in saturated
276 and unsaturated models (* = P-value < 0.05, ** = P-value < 0.01 and *** = P-value < 0.001).

Figures

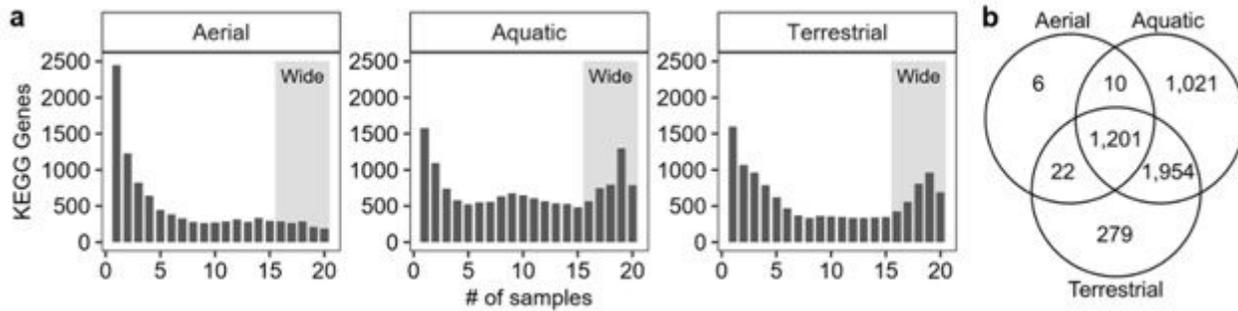


Figure 1

The number of genes with KEGG level 3 functions found in subsets of the 20 aerial, aquatic and terrestrial metagenomes with their wide spread function in at least 16 of 20 samples indicated in grey (a) and the share of widespread functions between the three ecosystems (b).

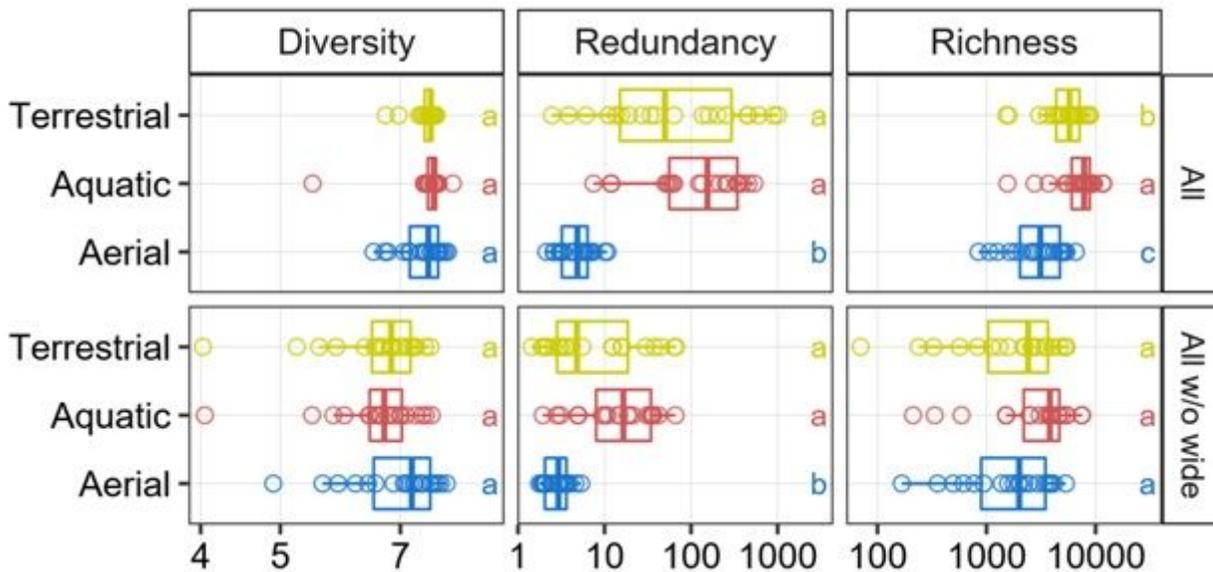


Figure 2

Functional diversity, redundancy and richness in aerial, aquatic and terrestrial metagenomes of the whole range of microbial density when all functions are considered (All) or when the widespread functions are removed (All w/o wide) (a). Groups followed by the same letter are not significantly different according to the HSD test (P-value >0.05).

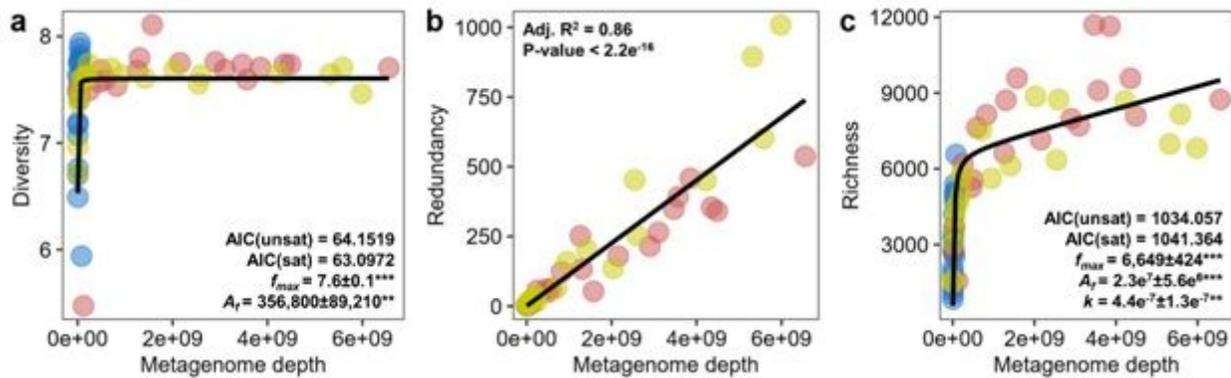


Figure 3

The saturated relationship of functional diversity, linear relationship of functional redundancy and unsaturated relationship of functional richness to metagenome depth when all functions are considered. Aerial samples are shown in blue, aquatic in red and terrestrial in yellow. Asterisks indicate the significance of individual parameters in saturated and unsaturated models (* = P-value < 0.05, ** = P-value < 0.01 and *** = P-value < 0.001).

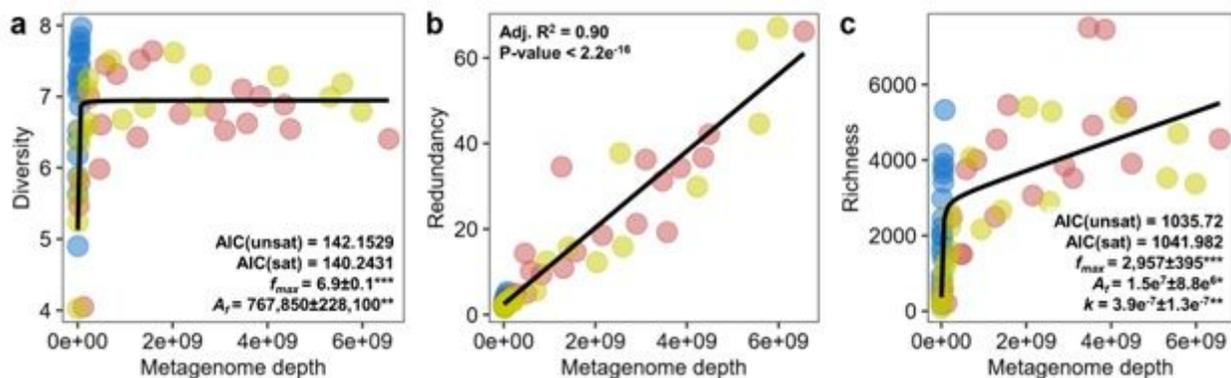


Figure 4

The saturated relationship of functional diversity, linear relationship of functional redundancy, and unsaturated relationship of functional richness to metagenome depth when the widespread functions are removed. Aerial samples are shown in blue, aquatic in red and terrestrial in yellow. Asterisks indicate the significance of individual parameters in saturated and unsaturated models (* = P-value < 0.05, ** = P-value < 0.01 and *** = P-value < 0.001).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.xlsx](#)