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BIAS IN COMMUNITY-WEIGHTED MEAN ANALYSIS RELATING SPECIES ATTRIBUTES TO SAMPLE ATTRIBUTES: JUSTIFICATION AND REMEDY

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Abstract

A common way to analyse relationship between matrix of species attributes (like functional traits of indicator values) and sample attributes (e.g. environmental variables) via the matrix of species composition is by calculating community-weighted mean of species attributes (CWM) and relating it to sample attributes by correlation, regression, ANOVA or other method. This *weighted-mean approach* is used in number of ecological fields (e.g. functional and vegetation ecology, biogeography, hydrobiology or paleolimnology), and represents an alternative to other methods relating species and sample attributes via species composition matrix (like the fourth-corner problem and RLQ analysis).

Here, I point out two important problems of weighted-mean approach: 1) in certain cases, which I discuss in detail, the method yields highly biased results in terms of both effect size and significance of the relationship between CWM and sample attributes, and 2) this bias is contingent upon beta diversity of species composition matrix. CWM values calculated from samples of communities sharing some species are not independent from each other and this lack of independence influences the number of effective degrees of freedom. This is usually lower than actual number of samples entering the analysis, and the difference further increases with decreasing compositional heterogeneity of the dataset. Discrepancy between number of effective degrees of freedom and number of samples in analysis turns into biased effect sizes and inflated Type I error rate in case that significance of the relationship is tested by standard tests, a problem which is analogous to analysis of two spatially autocorrelated variables.

Consequences of the bias is that reported results of studies using rather homogeneous (although not necessarily small) compositional datasets may be overly optimistic, and results of

39 studies based on datasets differing by their compositional heterogeneity are not directly
40 comparable. I describe the reason for this bias and suggest guidelines how to decide in which
41 situations the bias is actually a problem for interpretation of results. I also introduce analytical
42 solution accounting for the bias, test its validity on simulated data and compare it with an
43 alternative approach based on the *fourth-corner* approach.

44

45 **Introduction**

46 *Weighted-mean approach* is a method to analyse link between species attributes and sample
47 attributes by calculating community-weighted means of species attributes (CWM), which can be
48 directly related to sample attributes by correlation, regression, ANOVA or other methods (Fig. 1).
49 *Species attributes* are species properties (traits), behaviour (species ecological optima) or
50 phylogenetic age, while *sample attributes* are characteristics of community samples measured in
51 the field (environmental variables) or derived from matrix of species composition (species
52 richness or positions of samples in ordination diagrams).

53 Weighted-mean approach is used in wide range of ecological fields. In functional ecology,
54 testing the effect of environmental variables on changes in CWM is one of the approaches
55 demonstrating the effect of environmental filtering on trait-mediated community assembly (Diaz
56 *et al.* 1998; Shipley 2010; Laliberté *et al.* 2012). Similarly, CWM are used to predict changes in
57 ecosystem properties, such as biomass production or nutrient cycling (Garnier *et al.* 2004; Vile *et*
58 *al*. 2006), or ecosystem services like fodder production or maintenance of soil fertility (Diaz et al.
59 2007). In biogeography, grid-based means of species properties (like animal body size, range
60 size or evolutionary age) are linked to macroclimate or diversity (Blackburn & Hawkins 2004,

Hawkins & Diniz-Filho 2006, Hawkins et al. 2014). Vegetation ecologists use species indicator values (e.g. those of Ellenberg et al. 1992 or Landolt 1977) to estimate habitat conditions from calculated mean species indicator values of vegetation samples and relate them to soil, light or climatic variables (Schaffers & Sýkora 2000, Wamelink et al. 2002, 2005). In hydrobiology, reliability of saprobic index of Sládeček (1973) based on weighted mean of diatom indicator values, or similar indices (e.g. trophic diatom index, Kelly & Whitton 1995) is evaluated by relating them to measured water quality parameters. Similarly, in paleoecology the method used to reconstruct acidification of lakes from fossil diatom assemblages preserved in lake sediments is based on weighted means of diatom optima along pH gradient (ter Braak & Barendregt 1986) and as one of the transfer functions, (e.g. Birks *et al.* 1990) is considered to be a tool which have "revolutionised paleolimnology" (Juggins 2013). Other, more specific examples include relating community specialization index (mean of species specialization values weighted by their dominance in community) to environmental variables (Clavero & Brotons 2010, Fajmonová et al. 2013), or attempts to verify whether plant biomass can be estimated from tabulated plant heights and species composition as mean of species heights weighted by their cover in a plot (Axmanová *et al.* 2012).

Important thing to note is that although weighted-mean approach is technically relating two sets of variables (CWM and sample attributes), three matrices are in fact involved in the computation background (notation here follows RLQ analysis of Dolédec *et al.* 1996): matrix of *sample attributes* **R** with *m* sample attributes of *n* samples ($n \times m$), matrix of *species composition* **L** with abundances (or presences-absences) of *p* species in *n* samples ($n \times p$) and matrix of *species attributes* **Q** with *s* species attributes for *p* species ($s \times p$). Weighted-mean approach is

just one of possible options how to tackle the problem of relating species attributes (**Q**) with sample attributes (**R**) via matrix of species composition (**L**): it combines **Q** with **L** into matrix of weighted-means **M** and relates it to **R** (Fig. 1). Alternative solution is to combine matrix of sample attributes **R** with species composition **L** by calculating weighted-mean of sample attributes (optima of individual species along given sample attribute or species centroids) and relate these values to species attributes **Q** (e.g. ter Braak & Looman 1986). Third option is to use methods suitable for simultaneously handling all three matrices (**R**, **L** and **Q**), such as the *fourth-corner approach* (Legendre *et al.* 1997), related ordination method called RLQ analysis (Dolédec *et al.* 1996) and other alternatives (Jamil et al. 2013, Brown et al. 2014).

In weighted-mean approach, relationship between CWM and sample attributes is in most cases tested by standard parametric or permutation test. However, not all types of ecological questions, which are usually solved by weighted-mean approach, should actually be tested by standard tests. In certain situations and types of null hypotheses, weighted-mean approach combined with standard tests generates biased results, which are more optimistic than would be actually warranted by analysed data. This bias includes unreliable estimates of effect size (e.g. correlation coefficients in case of correlation or r^2 in case of linear regression) and inflated Type I error rate, leading to more frequent rejection of the null hypothesis than would be expected. The key point before applying the weighted-mean approach is to explicitly decide, based on critical inspection of the context of the study question and tested null hypothesis, what is actually the relationship between species attributes or sample attributes and species composition, and which of these relationships is actually fixed and which is random (more on the terms “fixed” and “random” below). Inspiration for this issue can be seen in application of the fourth-corner

105 approach (Legendre et al. 1997), for which Dray & Legendre (2008) demonstrated the problem
106 of deciding the right permutation test (from five permutation models) to test the actual question
107 in hand, with a risk of inflated Type I error rate in situation of wrong choice. For *weighted-mean*
108 *approach*, this issue was shown by Zelený & Schaffers (2012) in a specific context of relating
109 mean Ellenberg indicator values (species attributes) to sample attributes derived from species
110 composition matrix (like ordination scores or species richness), and also by Peres-Neto et al.
111 (2012) in the context of metacommunity phylogenetics. Both studies proposed numerical
112 solutions: Zelený & Schaffers (2012) introduced modified permutation test, based on permuting
113 species instead of sample attributes, and Peres-Neto et al. (2012) suggested to use *sequential test*
114 (ter Braak et al. 2012) using the *fourth-corner* statistic (Legendre et al. 1997). Additionally,
115 Šmilauer & Lepš (2014) touched on this issue in the context of CWM-RDA method (Kleyer et al.
116 2012).

117 In this study, first I review categories of questions and null hypotheses which are
118 commonly analysed by weighted-mean approach. Using simulated data, I show for which
119 category there is a risk of biased results if tested by standard tests, and describe in detail what
120 exactly causes this bias. Namely, I argue that the bias is caused by mismatch between number of
121 samples in weighted-mean analysis and actual number of effective degrees of freedom, since
122 community samples sharing some of the species with other samples do not count for the full
123 degree of freedom in this analysis. I will also demonstrate that the amount of bias depends on the
124 compositional heterogeneity (beta diversity) of the species composition matrix in a way that with
125 increasing heterogeneity the bias decreases, which makes comparison of results between datasets
126 of different compositional heterogeneity difficult. Note that for numerical simplicity, I ignore

intraspecific variation in species attributes. Finally, I will review methods available for solving the problem of inflated Type I error rate in weighted mean approach (namely *modified permutation test* of Zelený & Schaffers 2012 and solution based on combining *fourth-corner statistic* and *sequential permutation test* in weighted regression as introduced by Peres-Neto et al. 2012) and introduce novel solution, here called *two-step permutation test*. Although the examples, ecological interpretations and reasoning used here are focused on relationship of species functional traits or species indicator values with sample attributes analysed by weighted-mean approach, the general context is valid also for other types of species and sample attributes linked by weighted-mean approach.

Types of species and sample attributes

When thinking about possible alternative types of questions which are commonly being analysed using weighted-mean approach, it proves as useful to distinguish whether species and sample attributes are fixed or random. Terminology behind distinction into fixed and random is diverse (Gelman 2005); here I don't use this terms in the sense of ANOVA (fixed vs random terms), neither in the sense inferring their importance (fixed factors are important while random are nuisance factors). As *fixed attributes* I consider those which are specific for given dataset and related to given species or samples, and this specificity is acknowledged by the question/hypothesis being tested in a way that this link is deemed as given, and not further questioned or tested. *Random attributes* represent a subset of some larger pool of values, and their link to species composition is not acknowledged by the question being tested. In the narrow sense of permutation tests, fixed attributes should not be permuted among each other, while

149 random attributes can. For interpretation, effect of fixed attributes is limited only for given set of
150 attribute values and in the context of community datasets included in the analysis and is not
151 likely to be generalized beyond, while in case of random attributes interpretation is focused on
152 the general effect of given attribute, not only the subset of attribute values used in the study.
153 Species traits measured on individuals from plots of given community datasets can be considered
154 as fixed, while species traits taken from large trait databases and measured often in completely
155 different context should more likely be considered as random. Similarly, species richness or
156 sample ordination scores derived from community matrix are more likely fixed sample attribute,
157 while environmental variables measured in the field or derived from GIS layers may be
158 considered as random. Indeed, this distinction is often dependent on author's view of the
159 problem and on the theoretical context of the study, and the same variables can be seen as fixed
160 or random in different context.

161 In the original description of the fourth-corner problem (Legendre et al. 1997), focused
162 on linking fish behavioral and biological characteristics to environmental variables, both species
163 and sample attributes have been considered as fixed, and random was matrix of species
164 composition. Alternative permutation models were then used to test alternative hypotheses about
165 the mechanisms assembling the community (like environmental control over individual species
166 or species assemblages, lottery or random species attributes). In weighted-mean approach,
167 decision about fixed or random nature of attributes directly influences the decision for
168 meaningful way to test the relationship, and is therefore crucial for selecting correct statistical
169 test. Important is to note that all hypotheses tested by weighted-mean approach make (implicit or
170 explicit) assumption that either species or sample attributes are fixed, and their link to species

171 composition is *a priori* acknowledged and therefore not questioned (and not tested). In some
172 cases, whether the species or sample attributes are better considered as fixed or random depends
173 also on the need to generalize the results of the study. If the results should be used e.g. for local
174 application (e.g. whether CWM of species height in given agricultural system can predict well
175 the harvested biomass), species attributes can be seen as fixed, since the results will be used
176 solely in the context of the studied system – if the same values are measured again for the same
177 community, the results should be similar, but not applicable generally to other communities. If
178 the aim is to generalize the results (e.g. to assess whether the species height itself, as tabulated in
179 the floras, can be used as a tool to predict biomass yield), it is more reasonable to treat the
180 species attributes as random and modify the analysis accordingly, so as even local study can
181 contribute to more general description of such pattern.

182 Another useful distinction of attribute types is whether the sample or species attributes
183 are internal or external. The main difference is that internal attributes are numerically derived
184 from matrix of species composition, while external attributes are typically measured or estimated
185 variables, not directly derived from species composition matrix. Internal species attributes are
186 species optima calculated as weighted-means of sample attributes or as species scores on
187 ordination axes, and similarly internal sample attributes are e.g. sample scores on ordination axes,
188 species richness of individual samples or assignment of samples into groups based on
189 compositional similarity (e.g. by numerical classification). External species attributes, in contrary,
190 are measured traits or tabulated species indicator values, external sample attributes are measured
191 or estimated environmental variables or experimental treatments. While the link of external
192 species or sample attributes to species composition may be fixed or random and depends on the

context, internal attributes should always be considered as fixed, since they refer only to the context of the dataset from which they have been derived.

Types of hypotheses tested by weighted-mean approach

Considering the distinction between fixed and random (sample or species) attributes, questions and hypotheses commonly tested by weighted-mean approach fall into one of the three categories (see Table 1 for summary). *Category 1* assumes that while sample attributes are fixed, species attributes are random; *category 2* is opposite to the previous, with sample attributes considered random and species attributes fixed; and, finally, *category 3* assumes that both species and sample attributes are random. Below, I review in detail individual categories with examples of ecological questions/hypotheses for each of them.

Category 1 – species attributes are random, sample attributes are fixed

Hypotheses in this category explicitly acknowledge the link between sample attributes and species composition, or the link is implicit from the context or numerical background of the study, and they focus on testing the link of species attributes to species composition. The null hypothesis which is tested states that species attributes are not linked to species composition, while alternative hypothesis states that they are. Questions focused on relating CWM to internal sample attributes derived computationally from matrix of species composition fall into this category (e.g. relating mean Ellenberg indicator values to sample scores on unconstrained ordination, often used to interpret ecological meaning of ordination axes; Zelený & Schaffers 2012). Also studies with external sample attributes considered to be fixed, like experimental

214 treatments, fall into this category in case that their effect on species composition is
215 acknowledged, and the question is about how does species attributes response to it. Additional
216 level of complexity is added in studies dealing with grid data with both CWM and internal
217 sample attributes (e.g. species richness derived from community data) spatially autocorrelated
218 due to spatial coherence of species distribution (B. Hawkins, *pers. comm.*). Zelený & Schaffers
219 (2012) showed that standard parametric and permutation tests has inflated Type I error rate for
220 this category, and as an alternative introduced *modified permutation test*, permuting species
221 attributes instead of sample attributes as further discussed in this study.

222 *Category 2 – species attributes are fixed, sample attributes are random*

223 Hypotheses in the second category assume that the species attributes are linked to species
224 composition, and the null hypothesis states that sample attributes are not linked to species
225 composition, while alternative hypothesis states that they are. Example are trait-based studies
226 asking whether species traits can explain effect of environmental filtering on species abundances
227 in community; these studies operate with an assumption that species traits (as species attributes)
228 are functional, i.e. they influences the abundances of species in community, and the question
229 being evaluated is whether the sample attributes (environmental factor) acts as an environmental
230 filter on species abundances. Also descriptive studies without ambitions to be more generalized
231 fall into this category – e.g. relationship between CWM of species indicator values (e.g. mean
232 Ellenberg indicator values) and measured environmental variables, if the interpretation is
233 restricted only for the community dataset included in the study. Finally, studies using internal
234 species attributes (derived from species composition, e.g. as weighted-mean of sample attributes
235 or as scores on ordination axes) also belong to this category.

Category 3 – both species and sample attributes are random

This category of hypotheses includes mostly observational studies without prior knowledge or expectations about link between any of matrices. The null hypothesis states that there is no link between species and sample attributes via the matrix of species composition because either species attributes or sample attributes (or both) are not linked to species composition. To reject this null hypothesis means to prove that both species and sample attributes are actually linked to species composition. Empirical studies describing general relationship between sample attributes and species attributes without explicitly or implicitly acknowledging some underlying assumptions or mechanisms belong to this category. Examples are studies relating CWM of functional traits to environmental variables without clear assumption that traits are functional, allowing to question whether particular trait are actually linked to species composition or not. In case of studies with species indicator values, these include relating mean indicator values to environmental variables with aim to generalize the result also out of the studied dataset (e.g. answering the question whether Ellenberg indicator values for soil reaction *per se* are good predictors of measured soil pH, i.e. not only in the context of given community dataset).

Illustration of the bias using simulated community data

In the next section, I will use simulated community data to illustrate performance of standard parametric test, if this is used to test hypotheses from each category defined above. The benefit of simulated data is the possibility to keep certain parameters fixed and to manipulate only those parameters whose effect is studied – in this case fixed are numbers of samples in the dataset, and

manipulated are links between species or sample attributes and species composition, and also compositional heterogeneity of community data.

Each artificial community dataset includes the set of three matrices (sample attributes **R**, species composition **L,** and species attributes **Q**), with the link between species or sample attributes and species composition (or both) broken by permutation of attributes. This creates four scenarios (Fig. 2, identical with scenarios 1-4 of Dray & Legendre 2008): *scenario 1* - both sample and species attributes are linked to species composition, *scenario 2* - sample attributes are linked to species composition, but species attributes are not, *scenario 3* - species attributes are linked to species composition, but sample attributes are not, and *scenario 4* - none of species or sample attributes are linked to species composition. For hypotheses in category 1 defined above, the scenario 2 represents the null hypothesis, for category 2 scenario 3 is the null hypothesis, and for category 3 the scenarios 2, 3 and 4 represent alternative states of null hypothesis (Table 1). Scenario 1 represents the power test for all three categories (i.e. it measures probability of getting significant results if the alternative hypothesis is true). Additionally, I also examined how observed bias depends on compositional heterogeneity (beta diversity) of community matrix, which influences the number of effective degrees of freedom in analysis (as explained in detail further in the section *Justification of the bias*). Note that all analyses in this paper were conducted using R-project (v. 3.2.3, R Core Team 2015); complete R scripts are available in Appendix S5, and all functions have been wrapped into R-packages *weimea* (abbreviation for *wei*ghted *mea*n; source code for v. 0.58 available as Appendix S6).

Description of simulated data

I created an algorithm generating community data which are structured by two virtual ecological gradients (by extending the original one-gradient algorithm of Fridley et al. 2007 based on concept of Minchin 1987). The first gradient has *constant* length for all generated datasets and serves as a surrogate for measured environmental variable; in the analysis, positions of samples along this gradient are used as *sample attributes*, while the optima of species along this gradient are used as *species attributes*. The length of the second gradient is *variable* and its increasing length increases the compositional heterogeneity of species composition matrix. Community samples based on this simulated community ecospace were created by randomly locating samples along the first gradient, and species composition for each sample was derived by random assignment of fixed number of individuals to species identities weighted by relative abundances of species with non-zero probability of occurrence at given location of the gradient (see Appendix S1 for further details). With short second gradient, the resulting simulated community dataset was compositionally relatively homogeneous, with samples located nearby along the first gradient (with similar value of sample attribute) sharing rather high proportion of species. Increasing the length of the second gradient increased the compositional heterogeneity of the dataset, since two nearby samples may have quite different species composition (Fig. S1 in Appendix S1). Note that although scenarios 1-4 are conceptually analogous to scenarios 1-4 in Dray & Legendre (2008) used in the context of the *fourth-corner approach*, the model generating simulated communities is different, since Dray & Legendre (2008) used one-gradient model, which generates rather homogeneous communities, while I used two-gradients model, generating set of communities of increasing compositional heterogeneity.

Weighted-mean approach with standard parametric tests applied on simulated data

Using the algorithm described above, for each of the four scenarios (1-4) I created ten levels of compositional heterogeneity, and for each combination of *scenario* × *level of heterogeneity* I created 1000 datasets (4 scenarios × 10 levels of heterogeneity × 1000 replications = 40 000 datasets). For each dataset I calculated CWM of species attributes, related it to sample attributes using Pearson's *r* correlation and tested its significance using parametric *t*-test (for additional results for least-square regression and r^2 see Fig. S2 in Appendix S2). For each level of community heterogeneity in each scenario I counted proportion of correlations significant at α = 0.05 (note that this proportion is identical to the proportion of significant regressions).

From the three scenarios with no direct link between species and sample attributes (scenarios 2, 3 and 4), analysis of data generated by scenario 2 (Fig. 3) reveals the bias – the correlation coefficient deviates from zero more than in other cases (Fig. 3), and the test of significance shows inflated Type I error rate (Fig. 4). This bias is decreasing with increasing heterogeneity of the species composition matrix (Fig. 3 & 4, Scenario 2): for the most homogeneous dataset (*number of communities* = 1), the range of Pearson's *r* correlation coefficients (expressed as 2.5% and 97.5% quantiles) is between -0.751 and 0.751 with 60% of correlations significant, while for the most heterogeneous dataset with high beta diversity (*number of communities* = 10) the range of Pearson's *r* values is between -0.381 and 0.354 with 15% of correlations significant (compared to 2.5 and 97.5% quantile range values of *r* observed in scenarios 3 and 4 being in average between -0.278 and 0.281, with expected number of significant results being close to 5%). Similarly inflated are values of coefficient of determination (r^2; Fig. S2, Scenario 2) calculated by least-square linear regression.

Dray & Legendre (2008) showed that the *fourth-corner approach*, if tested by the permutation test based on reshuffling sample attributes (or rows of species composition matrix, respectively, model 2 in their paper) also reveals inflated Type I error rate in case of scenario 2. I applied the fourth-corner analysis also on the simulated community data described above. Results show, in line with Dray & Legendre (2008), biased values of the fourth-corner statistic and inflated Type I error rate for the model 2 permutation test (Figs. S3 & S4, Appendix S2), with the bias (and inflated Type I error rate) decreasing with increasing compositional heterogeneity (Fig S3 & S4 with Scenario 2, Appendix S2).

Justification of the bias

Simulation study above showed that if hypotheses in category 1 or 3, for which scenario 2 is relevant, are tested by standard parametric or permutation tests, results are prone to biased estimates of model parameters and inflated Type I error rate, and the bias is contingent upon compositional heterogeneity of the community dataset. In this section, I explore the reasons for this bias, which will be used as a theoretical base for solution proposed in the next section, and also explain the link of bias to compositional heterogeneity. Note that only hypotheses in category 1 and 3 are influenced by this bias, since for the category 2 with fixed species and random sample attributes, scenario 2 is not relevant and the bias therefore does not occur.

A peculiar feature of CWM of species attributes is their "numerical burden", namely that they are calculated from species attributes assigned to individual species and from species composition of individual samples, and therefore inherit part of information from both sources. The numerical difference between calculated CWM values of two community samples is indeed

343 constrained by a difference in species composition of these two samples - if they have identical
344 species composition (or identical relative species abundances), their calculated weighted-means
345 do not differ (because they cannot), and if their species composition differs only slightly, the
346 difference in their weighted-mean values is likely to be small. This property of weighted-mean
347 values, which are not independent from each other, has notable consequences for analysis with
348 sample attributes, if these are themselves related to species composition. In fact the situation is
349 analogous to the analysis of two spatially autocorrelated variables, just the autocorrelation is not
350 happening in the geographical space, but in the compositional space (more about this analogy in
351 the next section). Two values of CWM calculated from community samples sharing some
352 species do not bring two independent degrees of freedom into analysis, because samples used for
353 their calculation are not independent - difference in their CWM are predictable (to some extent)
354 from difference in their species composition in a way that the more similar is species
355 composition of two community samples, the more similar must be also their calculated CWM.
356 This problem scales up to the dataset level: in case of two compositional datasets with the same
357 number of samples used in weighted-mean approach, the dataset which is compositionally more
358 homogeneous has lower number of effective degrees of freedom compared to the more
359 heterogeneous one.

360 Although there are many ways how to quantify compositional heterogeneity of the dataset,
361 promising is to use beta diversity measure based on Whittaker's index of association (Whittaker
362 1952; in Legendre & Legendre 2012 as D_9). This index can be numerically derived from
363 differences in species composition between two calculated CWM values, and therefore quantifies
364 the dissimilarity in species composition which is directly related to weighted-mean approach

365 analysis (Appendix S3). To obtain single value of beta diversity for given dataset, one may use
366 beta diversity metric of Legendre & De Cáceres (2013) quantifying the variation in species
367 composition, which can be calculated also from symmetric matrix of dissimilarities among all
368 pairs of samples (here using Whittaker's index of association). Advantage of this beta diversity
369 metric is that it is independent on the size of the dataset, and the underlying dissimilarity
370 coefficient is directly related to weighted-mean approach.

371 Below, I will first illustrate what I mean by the differences in *effective number of degrees*
372 *of freedom* in analysis. I use a simple example focused on relationship between community-
373 weighted mean of traits with environmental variables, and compare two contrasting sampling
374 designs differing substantially by number of effective degrees of freedom they bring to the
375 analysis. Then, I elaborate in more detail the analogy to analysis of two spatially autocorrelated
376 variables, since it offers deeper insight to the problem and inspiration for potential solutions.

377 *Two sampling designs: difference in effective number of degrees of freedom*

378 The following example will illustrate the mismatch between number of effective degrees of
379 freedom and number of samples in analysis, using species functional traits. The weighted mean
380 of species functional traits, if combined with environmental variables, is used to investigate
381 whether the environment filters species into community via their trait properties. Assumption
382 behind is that the traits are functional, meaning that they directly influence the probability of
383 species occurrence in community under given environmental conditions, and this hypothesis
384 therefore classifies into the category 2 described above. For example, plant species specific leaf
385 area (SLA, Reich et al. 1992) is known to be related to the plant requirements for light, and
386 shade tolerant species restricted to more shady habitats have larger thinner leafs with higher SLA

387 values (e.g., Lambers et al. 2008). One may therefore expect that light acts as an environmental
388 filter for species entering given community, and this filtering is (at least partly) happening
389 because of species specific SLA. To support this reasoning, let's conduct imaginary experiment:
390 collect a dataset about composition of understory species in the forest and analyse it by the
391 weighted-mean approach. Let's keep things simple in this example and assume that we will
392 compare two forest types, one with open and one with closed canopy, to see whether the closed
393 canopy filters the understory species with high SLA. When preparing the design of data
394 collection, we have two options. The first is to choose two vegetation types (e.g. at the level of
395 association) with contrasting canopy openness, search for them in the study area (this could be
396 just one forest complex with mosaic of both vegetation types, or a larger region where these
397 vegetation types occur), sample their species composition and measure the light in the canopy
398 and SLA of individual species. The second option is to sample open- and closed-canopy forests
399 without any restrictions about their species composition, possibly in wider area. Following the
400 first sampling design we get dataset where samples of open-canopy forest are compositionally
401 similar to each other, as well as samples made in closed-canopy forest (but the composition of
402 open-canopy forest is different from the close-canopy one). In the second scenario we probably
403 get samples which all are having rather different species composition.

404 A simplified example how the species composition of six samples collected by these two
405 sampling designs would look likes in extreme cases is on Fig. 5. In the case of the first sampling
406 design, three and three samples have exactly the same species composition, while in the case of
407 the second design, species composition of each sample is distinctly different from the others (no
408 species are shared among any pair of samples). In the first case, three and three samples have the

409 same weighted-mean values since their species composition is identical. In the second case, three
410 and three samples have also the same weighted-mean values, not because of identical species
411 composition, but because samples from the same canopy cover category (open or closed) are
412 made to have similar distribution of trait values (although the species taxonomic identities are
413 completely different).

414 Indeed, the real data would fall somewhere in between these two cases, but this example
415 illustrates well the concept of effective degrees of freedom in weighted-mean analysis. The
416 relationship of the light availability to weighted-mean of SLA is exactly the same in both cases
417 (if analysed e.g. by one-way ANOVA in this situation, when environmental variable is
418 categorical with two levels, open-canopy forest with more light and close-canopy forest with less
419 light). The difference is in the number of effective samples, which each sampling design offers
420 for answering our question whether light serves as an environmental filter of species occurrence
421 in the community via species SLA. Three community samples with completely different species
422 composition, yet similar CWM of SLA (low for open- and high for closed-canopy forest) offers
423 considerably better information about interaction between and SLA *per se*, then do the three
424 samples with identical species composition (and hence also identical low or high CWM of SLA).
425 Also, what if our assumption is wrong and the SLA is in reality not a functional trait, and hence
426 belongs to category 3 instead of category 2? Let's replace the real trait values by random one (by
427 randomizing species attributes among species in table), calculate CWM and test their difference
428 between open- and closed-canopy stands (and repeat this process 1000 times). In case of the first
429 sampling design, 87% of tests detect significant difference (874 significant results of one-way
430 ANOVA at $P < 0.05$), while in case of the second sampling design the probability is near the

expected Type I error rate of 5% (54 significant results out of 1000). This agrees with the result of weighted-mean approach applied on simulated data saying that inflation of Type I error rate is high for homogeneous datasets (the case of the first sampling design) and decreases with increasing compositional heterogeneity (being effectively zero in dataset with all samples completely dissimilar as in case of the second sampling design).

Analogy to the analysis of spatially autocorrelated variables

The situation when *weighted-mean approach* is based on species composition data, in which some pairs of samples have the same or similar species composition and sample attributes are related to species composition, resembles an analysis of two spatially autocorrelated variables. In case of spatially autocorrelated variables, samples located more close to each other in geographical space have more similar values than expected if the values are randomly selected (Legendre & Legendre 2012). In case of weighted means, it is not the proximity in geographical space, but the proximity in compositional space, which reflects distances between samples expressed as their compositional dissimilarity.

It has been shown that when analysing two positively *spatially autocorrelated* variables, spatial autocorrelation biases the results of statistical tests, inflating the type I error rate and thus resulting into too optimistic results (Legendre 1993). The problem is not with autocorrelation of individual variables themselves, but with spatially autocorrelated residuals when analysing their relationship (e.g. by linear regression). From the point of view of the degrees of freedom, samples located nearby in geographical space are not statistically independent, behaving to a certain degree as pseudoreplications (Legendre & Legendre 2012). A new observation does not bring completely new information, because its value can be partly derived from the value of a

453 nearby site, and the effective number of samples (i.e., effective number of degrees of freedom) is
454 lower than the real number of samples. Since for standard parametric tests the number of degrees
455 of freedom is important for choosing the correct statistical distribution, appropriate for a given
456 sample size, disparity between the real number and effective number of samples leads to
457 selection of narrower confidence intervals and hence a higher probability of obtaining significant
458 results (Bivand 1980; Legendre 1993).

459 The same reasoning applies also for analysis of two *compositionally autocorrelated*
460 variables. Two weighted-mean values calculated from two samples with similar species
461 composition do not bring two full degrees of freedom to the analysis, as would be case of two
462 weighted-mean values calculated from samples with distinctively different species composition.
463 In a simple example with two sampling designs above, if we want to know whether species SLA
464 really increases with decreasing light in the understory, we would learn more about this from two
465 samples in the shaded understory which have different species composition and yet both have
466 high CWM of SLA, than from two samples which both have similar species composition (and
467 hence also similar CWM of SLA).

468 If sample attributes are not related to species composition, than the problem with
469 effective number of degrees of freedom is not present; although weighted-means are still
470 compositionally autocorrelated, sample attributes are not – in case of spatially autocorrelated
471 variables this is analogous to situation when one variable is spatially autocorrelated, but the other
472 is not, in which case the bias caused by autocorrelation doesn't appear. The situation which
473 requires attention due to potential bias is therefore limited to cases when sample attributes are
474 linked to species composition (i.e. they are fixed). This is the case of all internal sample

attributes derived from matrix of species composition, since they are linked to matrix of species composition (fixed) due to their numerical origin, and also the case of some of external sample attributes, if these are considered to be fixed (for examples see section *Types of species and sample attributes*).

Proposed solutions

Analogy between the bias in weighted-mean approach to the bias in analysis of spatially autocorrelated variables suggests potential toolbox for solving the problem. A simple option would be to stratify the dataset to reduce redundancy in species composition among samples, i.e. from pairs of samples with similar species composition remove one of them. Although methods for stratification based on species composition are available (e.g. Lengyel et al. 2011), it potentially results into throwing out a large number of expensive data. Alternative option would be to apply some correction for effective degrees of freedom in analysis, analogously to Dutiluel's method introduced for estimating effective number of samples in case of autocorrelated variables (Dutilleul 1993). The option I will further investigate here is based on comparison of results obtained by weighted-mean approach with those generated by a null model.

Modified permutation test: comparison with the results of a null model

Comparison with results of a null model is an analogy to testing the relationship between autocorrelated variables using toroidal shift, when one variable is permuted in a way that it preserves the original degree of spatial autocorrelation (Fortin & Dale 2005). Alternatively, one can generate random variables with the same degree of spatial autocorrelation as of the original

496 variable (Deblauwe et al. 2012). In case of compositionally autocorrelated variables used for
497 weighted-mean analysis, such variables can be generated for weighted-mean values, by
498 calculating weighted mean from randomized (or randomly generated) species attributes. Such
499 weighted-mean of randomized species attributes inherits the same level of compositional
500 autocorrelation as have the weighted-mean values of the real species attributes, because they are
501 calculated by the same algorithm from the same species composition matrix. One can generate
502 the null distribution of a test statistic (like *t*-value for correlation or *F*-value for regression) for
503 each weighted-mean of randomized species attributes related to original sample attributes, and
504 compare the observed statistic (relating the weighted-mean of real species attributes to sample
505 attributes) to this null distribution. This is identical with the modified permutation test,
506 introduced to test the relationship between weighted mean of species attributes and sample
507 attributes by Zelený & Schaffers (2012) in case of relating mean Ellenberg indicator values with
508 variables derived from ordination/classification based on the same species composition dataset.

509 To illustrate behaviour of the modified permutation test, I used the set of artificial
510 community data as above, calculated the correlation between weighted-mean of species attributes
511 and sample attributes for all four scenarios in communities of increasing heterogeneity, and
512 tested the significance of this correlation using modified permutation test. Results show that in
513 contrast to standard permutation test, inflated Type I error rate in case of the scenario 2
514 disappears (Fig 6b). At the same time, in case of scenario 3 (species composition related to
515 species attributes, but not to sample attributes) the test is overly conservative for homogeneous
516 datasets. Additional power analysis (Appendix S4) reveals that the modified permutation test
517 loses the power with decreasing sample size and mainly with decreasing number of species

which are being permuted (Fig S5 in Appendix S4). Modified permutation test seems therefore suitable for testing hypotheses in the category 1, which assume that species attributes are random, while sample attributes are fixed (linked to species composition) and for which scenario 2 is relevant for testing the null hypothesis. It is, however, not optimal for hypotheses in the category 3, which assume that both species and sample attributes are not fixed (not linked to species composition), since in scenario 3, which is also relevant as an alternative null hypothesis for this category, the results are overly conservative (although only for the most homogeneous dataset, Fig. 6).

Use of the fourth-corner statistic and the sequential test

Dray & Legendre (2008) noted that the fourth-corner statistic *r*, introduced by Legendre et al. (1997), is "equal to the slope of the linear model, weighted by total species abundances, with the niche centroids as the response variable and the species trait as the explanatory variable". This analogy was further elaborated by Peres-Neto et al. (2012, Appendix A), who presented algorithm how to use the *fourth-corner* statistic in weighted-mean approach. In short, both **R** and **Q** matrices are first centred by weighted mean of row sums of **L** (in case of **R**) and column sums of **L** (in case of **Q**), and rescaled; then, the fourth-corner *r* statistic is the slope of regression between weighted mean of standardized **Q** and standardized **R**, weighted by row sums of **L**. Advantage of the fourth-corner statistic is an option to use *sequential permutation test* introduced by ter Braak et al. (2012), which gives unbiased test of significance for all scenarios (for application on the simulated community data used above, see Fig. S3 & S4 in Appendix S2). This sequential permutation test combines results based on permuting sample attributes (model 2) and species attributes (model 4); if the first one is significant, than the second test is done, and

overall significance of the result is equal to the higher of these two test's *P*-values. Disadvantage, on the other side, is the fact that the combination of fourth-corner statistic and sequential test applies only to the regression between standardized (centred and rescaled) species and sample attributes, weighted by row sums of species composition matrix (**L**), and (to my knowledge) it cannot be used to test correlation, non-weighted regression or ANOVA between non-centred and standardized CWM and sample attributes.

Two-step permutation test

As an analogy to the sequential test used together with the fourth-corner statistic, here I introduce two-step permutation test, which gives unbiased results for relationship between CWM and sample attributes for range of statistical metrics (*t*-value for correlation and *F*-value for linear regression tested here). The test is based on combination of standard and modified permutation test; while both tests give unbiased results for scenario 4, standard test gives unbiased results also for scenario 3 (in which sample attributes are not related to species composition), while modified permutation test gives unbiased results for scenario 2 (where sample attributes are related to species composition). The idea behind the two-step permutation test is to first test whether sample attributes (**R**) are related to matrix of species composition (**L**), without considering (or even knowing) the values of species attributes (**Q**). This could be achieved e.g. by constrained ordination, when sample attributes are used as explanatory variables explaining variation in species composition. Here I introduce more general solution (called *LR permutation test* within this paper as a notice that relationship between **R** and **L** matrices is tested), which can be directly connected to particular test statistic (e.g. *t*-value for correlation). The LR permutation test consists of the following steps: (i) generate artificial set of species attributes as species centroids

562 calculated from real sample attributes, (ii) use these species attributes to calculate CWM, (iii)
563 calculate observed test statistic for relationship between artificial CWM and real sample
564 attributes, and (iv) test this relationship. The test is based on comparing observed values of the
565 test statistic (calculated in step iii) with the null distribution of the test statistic, generated in the
566 following way: 1) randomize sample attributes, 2) use them to calculate species attributes as
567 species centroids from weighted-means of sample attributes, 3) use these species attributes to
568 calculate CWM, and 4) relate these calculated CWM with randomized sample attributes from
569 step 1) to obtain the expected test statistic; repeat steps 1) to 4) *n*-times (*n* = number of
570 permutations). If this test is significant, it means that the sample attributes are related to matrix of
571 species composition, and relationship of CWM with sample attributes is consequently tested by
572 modified permutation test. If the test is not significant, standard permutation test is used. When
573 applied on the set of artificial communities used above, this sequential test gives unbiased values
574 of Type I error rate for all three scenarios (2, 3 and 4) and for all levels of compositional
575 heterogeneity (Fig. 6).

576

577 **Discussion**

578 Main motivation of this study was to show that results of weighted-mean approach critically
579 depend on the correct decision about the test used for statistical inference. To help in this
580 decision process, I suggested that each hypothesis can be classified into one of the three
581 categories, given the explicit (or implicit) assumptions about the role of species and sample
582 attributes. For each category, I suggested optimal strategy for testing the significance of
583 relationship between CWM and sample attributes. The decision about appropriate category is not

584 always straightforward, although the decision whether species attributes should be considered as
585 fixed or random changes classification of the hypothesis from category 2 (with recommended
586 standard parametric or permutation test) into category 3 (with two-step permutation test). For
587 example, trait studies, which are testing whether environment is filtering the species into
588 community via their functional traits, routinely assume that such traits are functional, and in
589 weighted-mean approach are therefore considered as fixed (category 2). However, this
590 assumption may not always be justified; traits included in these analyses are often those readily
591 available in databases and/or relatively easy to measure, but these do not necessarily need to be
592 really the functional ones (Fox 2012, Mlambo 2014). In case of compositionally relatively
593 homogeneous datasets, even the traits with no ecological meaning may show high and significant
594 relationship to environmental variables if tested by standard tests. I believe that this calls for
595 revision of such commonly applied practice.

596 Differences in effective degrees of freedom among datasets complicate comparison of
597 results between studies based on datasets of different compositional heterogeneities. Studies
598 conducted on datasets of relatively low beta diversity may obtain stronger and more likely
599 significant relationship between weighted-mean of species attributes and sample attributes than
600 studies on datasets of relatively higher beta diversity, even in case that the real link of species
601 attributes to species composition is missing (Figs. 3 & 4, Scenario 2). This situation is analogous
602 to biased estimates of model parameters and inflated Type I error rate in analysis of spatially
603 autocorrelated variables. An option how to deal with this problem is to routinely report, in each
604 case-study using weighted-mean approach, some standardized value of compositional
605 heterogeneity. Although this would not remove bias in results of these studies, it would at least

606 allow for comparison of the potential for bias among different studies. Good metric for this
607 purpose should be independent on the sample size, and should pertain dissimilarity in species
608 composition which is relevant for differences in community-weighted means; here, I suggested
609 beta diversity measure based on Whittaker's index of association (Appendix S3) following the
610 approach summarized by Legendre & De Cáceres (2013).

611 Specific question is how to deal with missing values of species attributes for some of the
612 species. Should species with missing species attributes remain in the matrix of species attributes
613 and species composition? And in a case of the modified and two-step permutation tests, should
614 the missing values be kept and permuted among species? The analogy to spatial autocorrelation
615 issue offers clear answers for these questions. Species with missing attribute values are not used
616 for weighted-mean calculation, so they do not contribute to the compositional autocorrelation of
617 weighted-mean values. The point of the modified (and subsequently also two-step) permutation
618 test is to generate random variables with the same compositionally autocorrelated structure as the
619 weighted mean calculated from the original species attributes. For this, matrix of species
620 composition, which inherits the compositional autocorrelation into weighted-mean values,
621 should remain the same also for calculation of weighted-means from randomly generated species
622 attribute values. This would not be the case if the species with missing attribute values remains
623 in both matrices, because permuting missing values would cause the weighted mean of permuted
624 species attributes being calculated every time with different species composition matrix (the
625 species which in given permutation run would be assigned missing values will not be included in
626 this weighted-mean calculation). The solution is hence to remove species with missing species
627 attributes from both species attributes and species composition matrix, and in the case of

628 modified permutation test to permute only existing species attribute values. In case that more
629 species attributes are analyzed (e.g. three different functional traits, or six different species
630 indicator values) and species has missing species attribute value for some attributes and not for
631 the others, the species should be removed from species composition matrix only for the purpose
632 of calculating and testing weighted mean of that species attributes for which the species value is
633 missing, and not for the others.

634 Power test using simulated dataset showed that the power of both two-step as well as
635 modified permutation test decreases with decreasing number of species in the dataset (and less
636 strongly also with decreasing number of samples). This makes these tests less suitable for
637 smaller and relatively homogeneous datasets with few species (e.g. less than 40), since the
638 probability of Type II error (i.e. not rejecting the null hypothesis which is false) strongly
639 increases. Similarly, both two-step and modified permutation tests are overly conservative for
640 scenario 3. For modified permutation test this is not a problem, since for the hypotheses for
641 which the scenario 3 is null hypothesis (category 2) the modified permutation test is not
642 recommended method (see Table 1). The two-step permutation test is also overly conservative,
643 but only only in case of the most homogeneous community dataset, and with increasing
644 compositional heterogeneity this issue diminishes (Fig. 6 and Table S2).

645 In this study, I explicitly ignored intraspecific variation in species attributes, focusing
646 only on use of dataset-wide mean species attribute values. Indeed, intraspecific variation may be
647 important; e.g. in the context of functional traits, the intra-specific variation gains an increasing
648 attention (Albert et al. 2012), and relevant question is whether the inclusion of intra-specific
649 variation (e.g. by including trait values which are sample-specific, not dataset-wide) influences

650 the potential bias reported in this study or not. This question requires further examination, which
651 goes beyond this study, but in my opinion including another source of variation (species-level
652 variation in species attributes) does not remove the problem of the bias itself, but makes the
653 estimation of the bias and its correction more complex.

654 Finally, relevant consideration is whether the weighted-mean approach is actually the best
655 analytical solution for question which is being explored. In some cases, the question is explicitly
656 focused on relating community-level values of species attributes, like mean Ellenberg indicator
657 values (serving as an estimates of ecological conditions for individual sites) or CWM of traits (as
658 one of the functional-diversity metrics and as a community-level trait value), and use of
659 weighted-mean approach is fully justified. Yet, in other cases, when the question is focused on
660 relating individual species-attributes to sample attributes, weighted-mean approach may not be
661 the best analytical choice. Use of alternative options, like fourth-corner or RLQ analysis, for
662 which the problem of inflated Type I error rate and choice of suitable permutation test have been
663 already solved, can be a better solution.

664

665 **Conclusions**

666 In this study, I attempted to draw attention to the problem in weighted-mean approach which I
667 believe is largely overlooked and generally not acknowledged, although it represents a source of
668 potentially serious misinterpretations. Since in certain fields the weighted-mean approach gains
669 increasing momentum (e.g. in functional ecology with CWM of species functional traits as one
670 of the functional-diversity indices), I suggest that time is ripe to critically asses in which
671 situations and for which types of hypotheses the commonly used standard parametric or

672 permutation tests are not appropriate, since they yield results which may be overly optimistic. I
673 offer simple guidelines how to decide whether in given context of a study the standard
674 methodology gives correct or biased results, and suggest solutions available in case that it does
675 not.

676

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681

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795

796 **Supplementary materials**

797 **Appendix S1.** Description of an algorithm generating artificial community data along two
798 environmental gradients.

799 **Appendix S2.** Weighted-mean approach applied on simulated data: additional results.

800 **Appendix S3.** Dissimilarity index between two CWM values and beta diversity assessment.

801 **Appendix S4.** Evaluation of permutation tests using simulated data from Dray & Legendre
802 (2008).

803 **Appendix S5.** R-code for all analyses.

804 **Appendix S6.** Source code for the R library *weimea*, version v. 0.58 (actual version can be found
805 on https://github.com/zdealveindy/weimea/).

806

807 *Table 1*

808 Overview of the characteristics for the three categories of hypotheses tested by *weighted-mean*
809 approach. For each situation, corresponding assumption about link between sample attributes (**R**)
810 or species attributes (**Q**) to species composition (**L**) is given, as well as null vs alternative
811 hypothesis, scenario relevant in the context of given category (see Fig. 2), and recommended test.

Category	Assumption	Null hypothesis	Alternative hypothesis	Relevant scenario	Recommended test
1	sample attributes fixed (**R** <--> **L**)	**Q** <-//-> **L**	**Q** <--> **L**	Scenario 2	modified permutation test
2	species attributes fixed (**Q** <--> **L**)	**R** <-//-> **L**	**R** <--> **L**	Scenario 3	standard parametric or permutation test
3	no assumptions	**R** <-//-> **Q**, i.e. **R** <-//-> **L** and/or **Q** <-//-> **L**	**R** <--> **Q**, i.e. **R** <--> **L** and **Q** <--> **L**	Scenarios 2, 3 and 4	two-step permutation test

812

813

Figure captions

Figure 1. Computational schema of the weighted-mean approach to analyse relationship between species attributes and sample attributes via matrix of species composition. **R** - matrix of sample attributes (e.g. environmental variables), **L** - matrix of species composition ($\mathbf{L_s}$ – **L** standardized by sample totals to simplify the equation), **Q** - matrix of species attributes (e.g. traits, species indicator values), **M** - matrix of weighted means of species attributes (e.g. CWM). The colour gradient within the matrix M (weighted mean of species attributes) from dark to light grey illustrates that this matrix includes information from both matrix of species composition (dark grey) and matrix of species attributes (light grey).

Figure 2. Schema showing conceptual differences between scenarios 1-4 in weighted-mean approach. In scenario 1, both sample attributes (**R**) and species attributes (**Q**) are fixed, linked to matrix of species composition (**L**), while in the other three scenarios one (or both) of attributes are considered random, without the link to species composition. In simulated data example, the link of attributes to species composition is cancelled by permuting the values of species attributes (scenario 2), sample attributes (scenario 3) or both (scenario 4). In the schema, matrix of species attributes is transposed (**Q'**) to match the dimension of matrix of species composition (**L**).

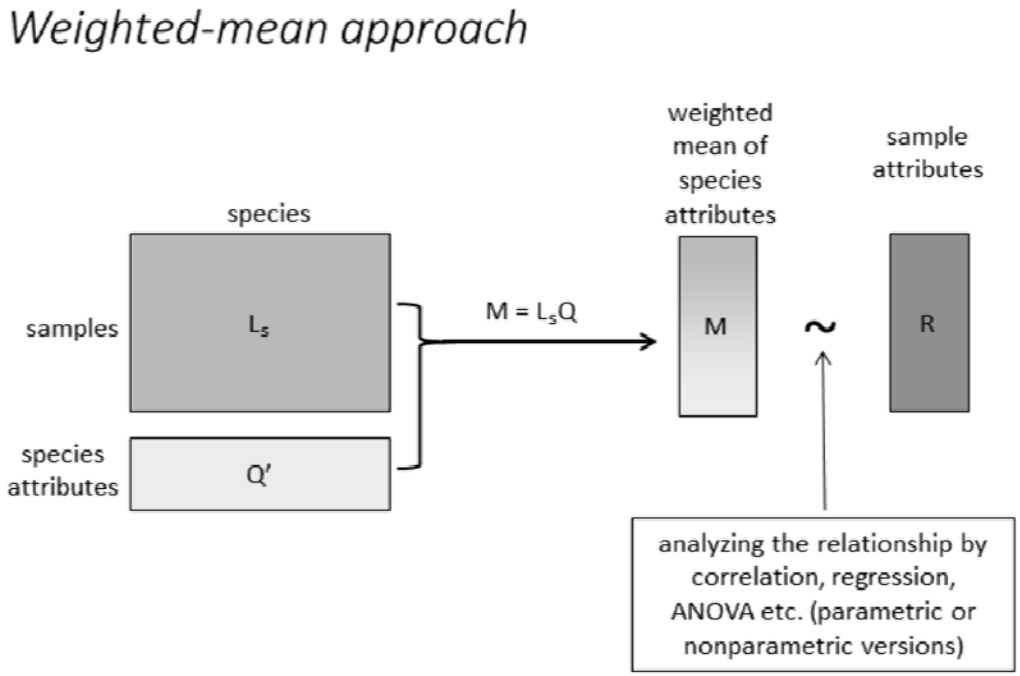
Figure 3. Pearson's *r* correlation coefficients among CWM and sample attributes for each of the four scenarios and ten levels of compositional heterogeneity of species matrix (1000 correlations for each combination have been conducted). Grey horizontal bars are outliers.

Figure 4. Proportion of significant correlations ($P < 0.05$) between CWM and sample attributes, tested by standard parametric *t*-test. For each of the four scenarios and ten levels of compositional heterogeneity of species matrix, 1000 tests have been conducted.

836 **Figure 5.** Simplified example of two community datasets (each with six samples) collected in
837 plots of two different environmental conditions (A and B, e.g. open- vs closed-canopy forest).
838 First sampling design (a) restricts choice into only two vegetation types (one open- and one
839 closed-canopy forest), and results into three and three plots with identical species composition.
840 Second sampling design (b) does not restrict the sampling by choice of community type (any
841 forest with open- or closed-canopy can be sampled), resulting in situation when none of six
842 samples share any species in common. For each dataset, three matrices are presented:
843 sample × species compositional matrix, matrix of sample attributes (in this case with two-level
844 categorical variable) and matrix of species attributes (quantitative variable in range 1 to 5). x -
845 presence of species in the sample.

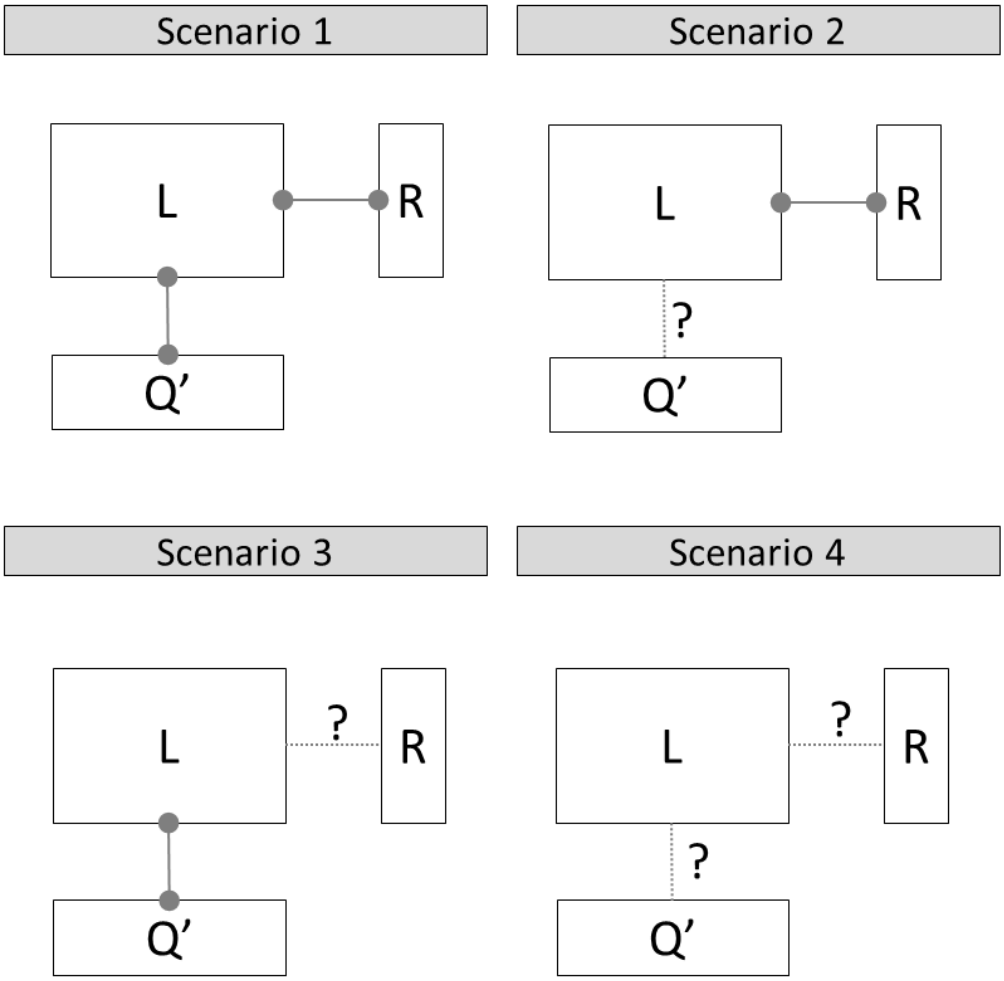
846 **Figure 6.** Proportion of significant correlations ($P < 0.05$) between CWM and sample attributes,
847 tested by three different permutation tests: standard, modified and two-step. For each of the four
848 scenarios and ten levels of compositional heterogeneity of species matrix, 1000 tests have been
849 conducted.
850

851 *Figure 1*



852

853 *Figure 2*



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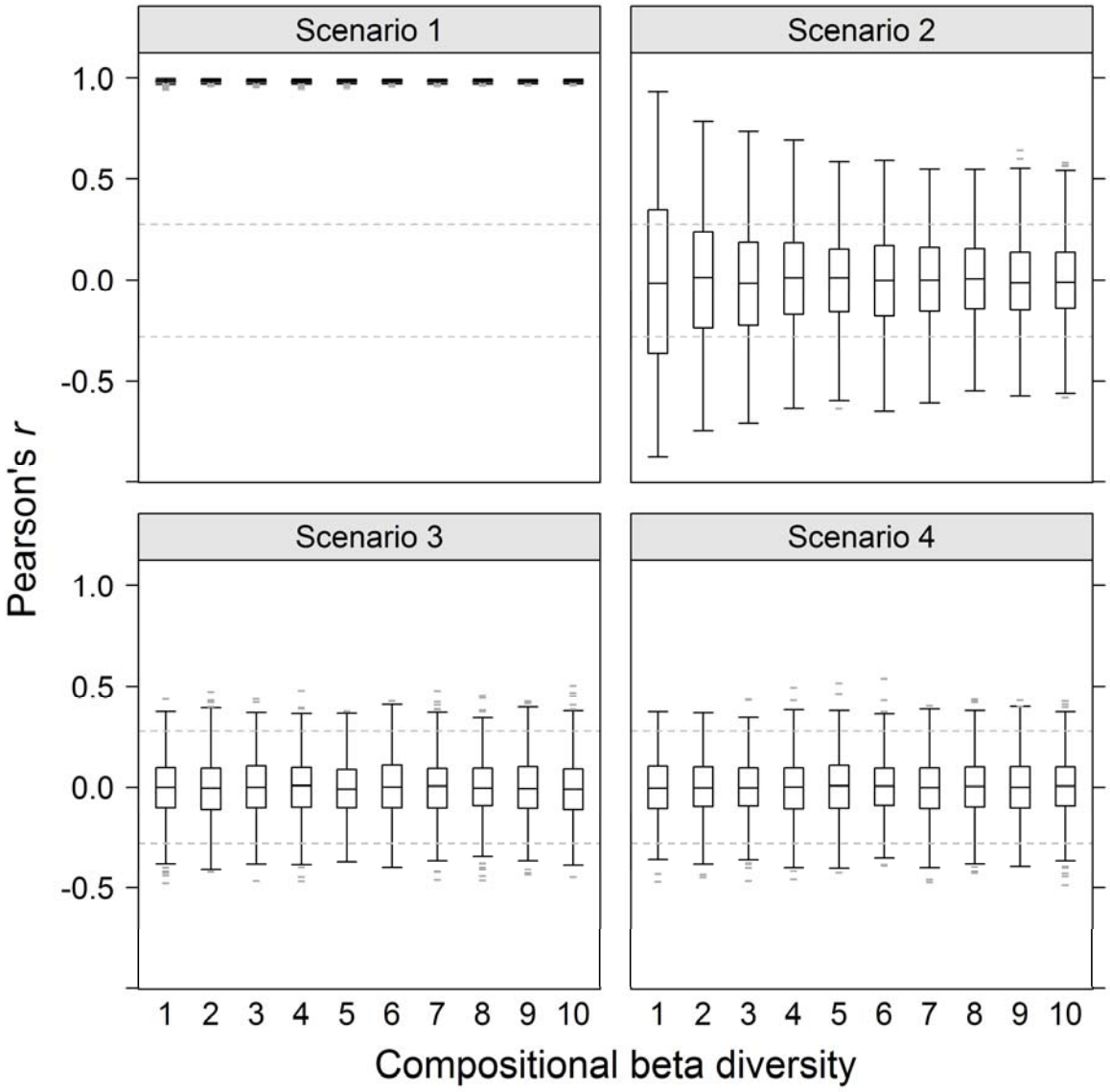
855

856 *Figure 3*



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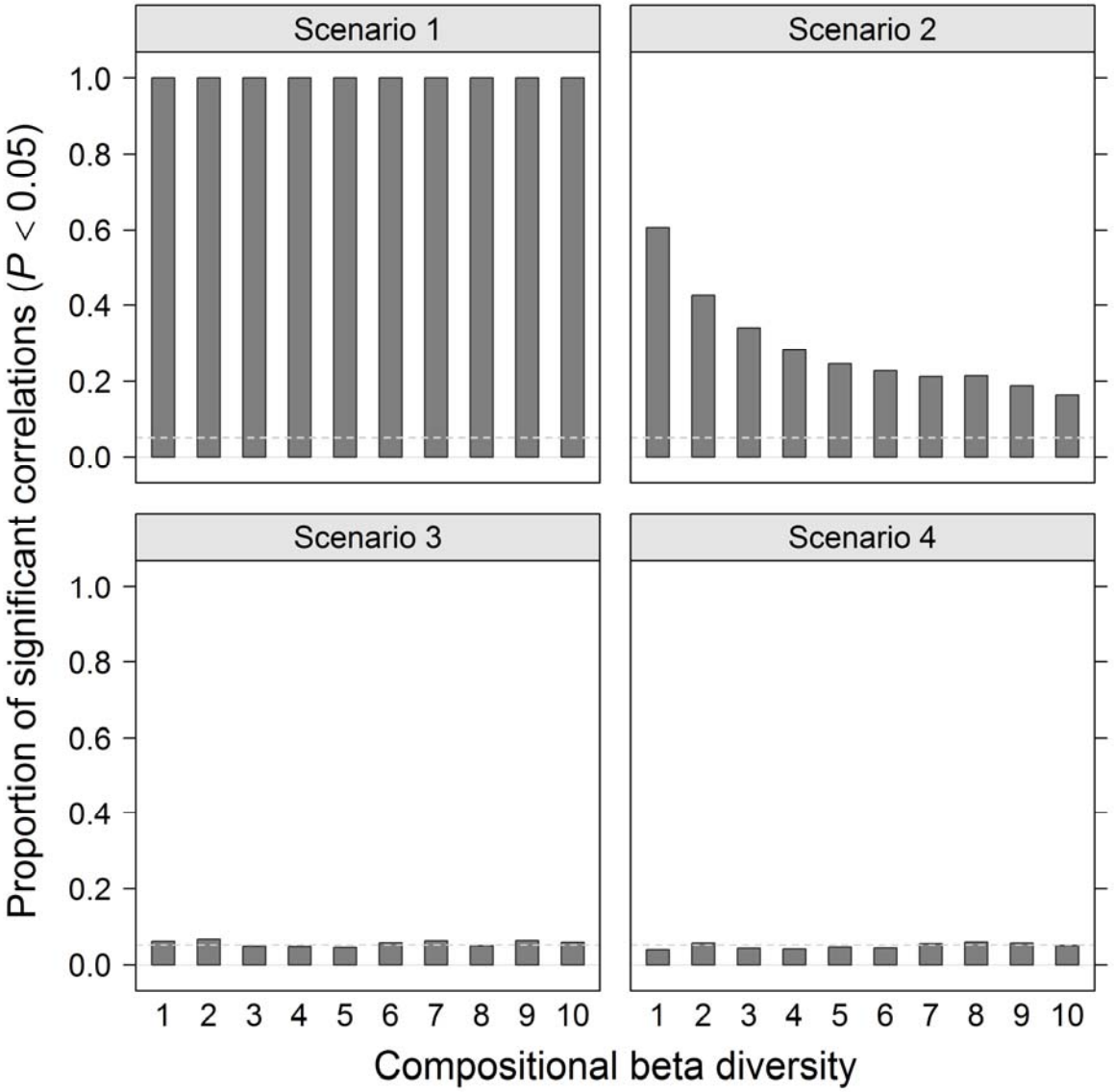
859 *Figure 4*



860

861

862 *Figure 5*

(a)

	species	
	1111:	
	12345678901234	env
sample_1	xxxxxxx	A
sample_2	xxxxxxx	A
sample_3	xxxxxxx	A
sample_4	xxxxxxx	B
sample_5	xxxxxxx	B
sample_6	xxxxxxx	B
spec.attr.	11122233344455	

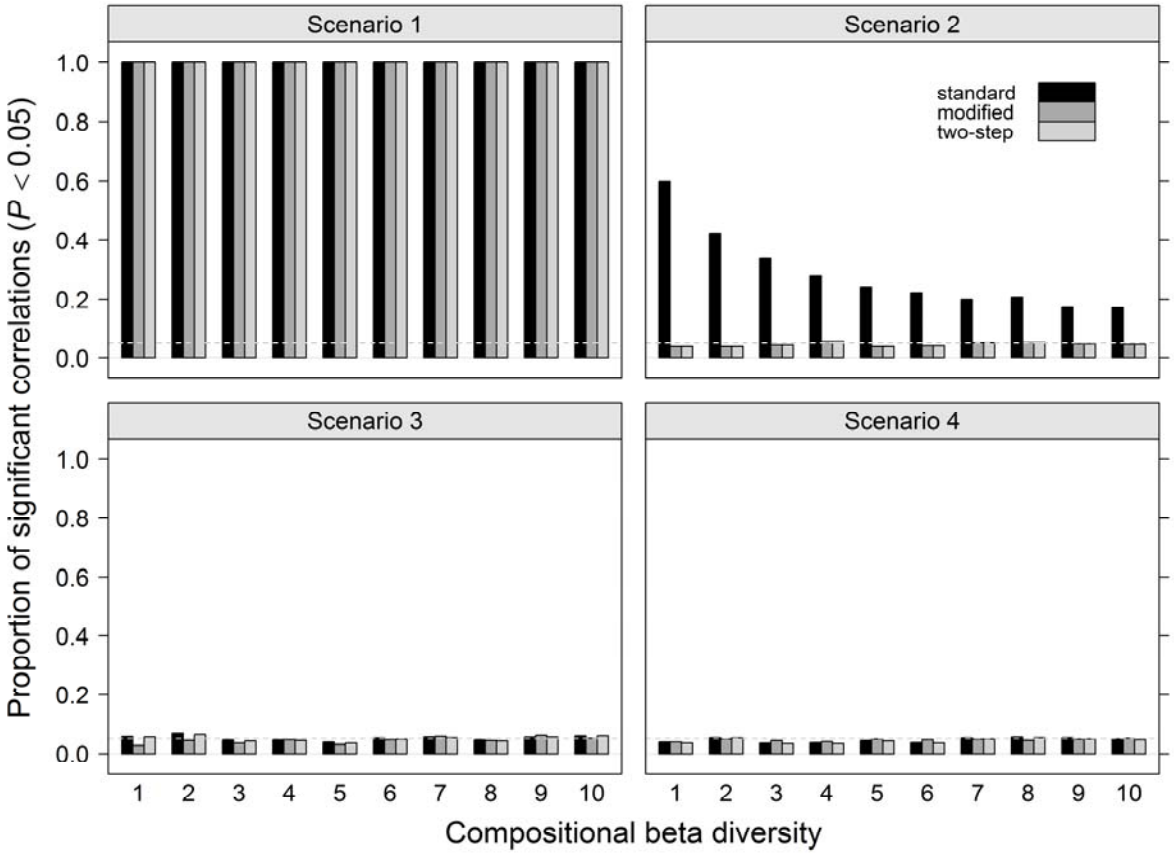
(b)

	species	
	1111:11111222222222233333333444	
	12345678901234567890:234567890123456789012	env
sample_1	xxxxxxx	A
sample_2	xxxxxxx	A
sample_3	xxxxxxx	A
sample_4	xxxxxxx	B
sample_5	xxxxxxx	B
sample_6	xxxxxxx	B
spec.attr.	11122231112223111222333444553344455334445	

863

864

865 *Figure 6*



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