Multidimensional trophic niche approach: gut content, digestive enzymes, fatty acids and stable isotopes in Collembola

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Abstract

1. The trophic niche of an organism is tightly related to its role in the ecosystem and can explain its coexistence with other species. A number of instrumental methods were developed to describe trophic niches of organisms in natural complex and cryptic ecosystems where direct observation is hampered, such as soil. However, these methods are often applied independently from each other, simplifying the multidimensional nature of the trophic niche. Using the example of Collembola, dominant soil animals consuming a wide range of food resources, here we for the first time quantitatively compared information provided by four different but complementary methods: visual gut content analysis, digestive enzyme analysis, fatty acid analysis and stable isotope analysis.

2. From 43 published studies and unpublished data, we compiled the most comprehensive trophic trait dataset to date including 154 collembolan species, each studied with at least one method. We selected fifteen key trophic niche parameters that provide information about links between Collembola and their potential food resources. Data on different methods were uniformly scaled and connected through the species identity. Focusing on interspecific variability we (1) explored correlations of all trophic niche parameters and (2) described variation of these parameters in collembolan species, families and life forms.

3. Correlation between trophic niche parameters of different methods was weak or absent in 45 out of 64 pairwise comparisons, reflecting that only some parameters provide similar information. Gut content and fatty acids provided comparable information on fungivory and plant feeding in Collembola. By contrast, information provided by digestive enzymes differed from information gained by other methods, suggesting that digestive enzymes

had high additional value when combined with other methods. Stable isotope values were related to parameters from other methods only when related to plant versus microbial feeding. Many parameters were affected by family identity but not life form, suggesting phylogenetic (taxonomic) signal in Collembola trophic niches. We highlighted the additional value of method combination by providing a complex description of trophic niches across families and showing emerging evidence that bacterial feeding in Collembola may be more common than it is often assumed.

4. Different methods estimate different feeding-related processes and thus different dimensions of the trophic niche, being complementary rather than redundant. A comprehensive picture of the trophic niche in complex food webs can be drawn by a combination of different approaches. However, the number of studies that applied even two methods simultaneously is very low in soil ecology. Controlled laboratory experiments and description of field populations with several joint methodological approaches is the way towards understanding of food-web functioning and coexistence of species in cryptic environments.

Keywords

diet tracing, food webs, biomarkers, trophic interactions, springtails, method comparison

Introduction

Trophic interactions between organisms define structure and stability of ecological communities, channeling of energy through food webs, and functioning of ecosystems (Estes et al., 2011; Rooney & McCann, 2012; Barnes et al., 2018). The trophic interactions of an organism with other coexisting species can be perceived as its 'trophic niche', stemming from the Hutchinsonian 'ecological niche' concept (Chase & Leibold, 2003; Holt, 2009). Trophic niche can be defined in multidimensional space, where each axis represents an aspect, or dimension, of trophic interactions (Hutchinson, 1978; Newsome, Martinez del Rio, Bearhop, & Phillips, 2007; Machovsky-Capuska, Senior, Simpson, & Raubenheimer, 2016). Such dimensions could represent direct evidence of food objects, describe feeding behavior of individuals, and imply basal resources and trophic level of species in food web with potential interactions with predators. For small organisms in cryptic environments, such as invertebrates living in soil, typically only few trophic niche dimensions are known.

Soil food webs rely on dead plant and animal material, soil organic matter, phototrophic microorganisms, roots and root exudates, inseparably mixed with bacteria and fungi, and altogether generalized as 'detritus' (Moore et al., 2004). Hundreds of species of basal soil animal consumers, known as 'detritivores' and 'microbivores', feed on this mixture and locally co-exist with apparently low specialisation, which inspired J.M. Anderson to formalize the 'enigma of soil animal diversity' almost fifty years ago (Anderson, 1975). Trophic niche differentiation is one of the mechanisms, by which different species can coexist. Such differentiation was found e.g. for Collembola, one of the most abundant soil invertebrate groups (Hopkin, 1997). Traditionally considered as generalistic fungivores, different Collembola species in fact occupy

78 different trophic niches, spanning from algivores to high-level consumers (Rusek, 1998;
79 Chahartaghi, Langel, Scheu, & Ruess, 2005).

80 Understanding of trophic niche differentiation in Collembola and other soil invertebrates
81 was for a long time constrained by the small size and/or cryptic lifestyle of these animals.
82 Traditionally, trophic niches of Collembola in natural environments were studied with visual gut
83 content analysis (Anderson & Healey, 1972; Hagvar & Kjondal, 1981; Table 1). This method is
84 based on microscopic observations of gut content and counting the particles of different types.
85 Despite being laborious, this method provides reliable data on the food objects that were
86 ingested. However, visual methods provide only a snapshot of the diet, are biased towards
87 overestimation of poorly digestible resources, and provide limited information in case of feeding
88 on fluids or soil organic matter.

89 To assess which ingested material may be potentially digested in the gut of soil animals,
90 digestive enzyme analysis was applied (C. O. Nielsen, 1962; Siepel & de Ruiter-Dukman, 1993;
91 Parimuchová et al., 2018). Digestive enzymes, such as cellulase, chitinase and trehalase,
92 represent the ability of an animal to decompose corresponding types of complex organic
93 compounds. Since proportions of different organic compounds differ between resources (Table
94 1), trophic interactions with some resources could be also inferred using this method. In addition,
95 digestive enzymes are linked to foraging strategies of animals; 'grazers' have higher activity of
96 enzymes to degrade structural polysaccharides (cellulose, chitin), while 'browsers' have higher
97 activity of enzymes to degrade storage polysaccharides (e.g. trehalose) (Siepel & de Ruiter-
98 Dukman, 1993). According to the existing data on Collembola, most species fall into the group
99 of 'herbo-fungivorous grazers', i.e. having all three abovementioned enzymes in digestive

system (Berg, Stoffer, & van den Heuvel, 2004), which highlights the difficulty to disentangle their feeding habits with this method only.

While being potentially informative in revealing foraging strategies, digestive enzyme analysis provides only little information on the identity of consumed food items and on whether they were actually assimilated or not. More recently developed biochemical tools, such as neutral lipid fatty acid (FA) analysis, filled this gap by providing information on the food compounds that was assimilated and retained in the body over recent period of time (Ruess & Chamberlain, 2010). Plants, fungi and different groups of prokaryotes are able to synthesise specific membrane lipids, that are often retained unchanged in the fat body of their consumers, a phenomenon called 'dietary routing' (P.M. Chamberlain, Bull, Black, Ineson, & Evershed, 2005a; Ruess & Chamberlain, 2010). By analysing the composition of biomarker FAs in consumers, basal food resources of the trophic chain can be tracked over a period of several weeks, even those retaining in higher trophic levels (Pollierer, Scheu, & Haubert, 2010).

Despite being very informative, FA analysis is laborious and does not provide trophic level estimation of species in the food web. Quantitative comparison among contribution of different resources using FA analysis is still very limited (Kühn, Schweitzer, & Ruess, 2019). An alternative method overcoming these limitations (which also provides information on assimilated food resources) is stable isotope analysis of carbon and nitrogen (Tiunov, 2007). In soil food webs low ^{13}C concentration in bulk body tissue indicates a trophic link to freshly fixed plant carbon (e.g. via herbivory), while high ^{13}C concentration suggests a trophic link to microbially processed organic matter (e.g. via bacterivory or fungivory), thereby differentiating basal resources among species or populations (A. M. Potapov, Tiunov, & Scheu, 2019). In turn, ^{15}N concentration can infer trophic levels in the soil food web, being low in basal resource

consumers and high in predators and mycorrhizal fungi feeders (A. M. Potapov, Tiunov, & Scheu, 2019). In particular, the method revealed that trophic niches of Collembola species may correlate with their taxonomic position and life form (i.e. microhabitat specialisation) (A. M. Potapov, Semenina, Korotkevich, Kuznetsova, & Tiunov, 2016). However, bulk natural stable isotope composition provides only very general information on the trophic position in food web and rarely allows to reconstruct exact feeding interactions in soil. This limitation could be overcome by sequencing of the gut DNA (i.e. molecular gut content analysis), but only few species of Collembola were studied so far with this method (Anslan, Bahram, & Tedersoo, 2018; Zhu et al., 2018).

Different methods provide information on different trophic niche dimensions, integrate information over different time periods, and each has its own advantages and drawbacks. However, in combination they could help to overcome some of the drawbacks and provide complementary information. A recent review revealed that only few studies have conducted quantitative comparisons among the dietary methods, and none of them applied several methods simultaneously (J. M. Nielsen, Clare, Hayden, Brett, & Kratina, 2017). This motivated us to compile a large trophic trait dataset across the four different field observational methods mentioned above (Table 1), focusing on Collembola – one of the most abundant soil arthropod groups. We aimed to (1) quantitatively assess the complementarity of data provided by each of the methods; (2) describe multidimensional niches of collembolan species, families and life forms.

Table 1. Description of the methods and list of the trophic niche parameters used in this study to assess trophic niches of Collembola.

Parameter	Description	Provided information
Visual gut content analysis		
Represents a snapshot of ingestion of food material. Assessed via microscopic observations of the gut content. Data is usually given as proportion of the particles ingested (Ponge, 2000; Addison, Trofymow, & Marshall, 2003).		
Proportion of fungi	Proportion of particles of fungal origin (i.e. spores and hyphae) among the particles ingested	A proxy for fungivory
Proportion of plant particles	Proportion of particles of plant origin (e.g. coarse plant detritus, roots or shoots) among the particles ingested	A proxy for herbivory and litter grazing
Proportion of amorphous material	Proportion of fine particles of unclear origin (i.e. fine detritus) among the particles ingested	A proxy for soil organic matter and faecal pellets feeding
Digestive enzyme analysis		
Represents the ability to digest ingested food. Assessed via laboratory analyses of degradation of specific organic substrates. Data are given in masses of reaction products liberated from specific substrate by the homogenate of animal bodies per unit of time and per unit of animal mass (Siepel & de Ruiter-Dukman, 1993; Berg et al., 2004). Non-digestive roles of digestive enzymes are ignored.		
Cellulase activity	Production of glucose per unit of time after cellulose addition. Cellulose is a major component of green plant and algae cell walls	A proxy for herbivory, algivory and litter grazing
Trehalase activity	Production of glucose per unit of time after trehalose addition. Primary storage component of fungal, but also lichen and plant cells	A proxy for fungivore (and herbivore) browsing strategy
Chitinase activity	Production of glucosamine or nitrophenole per unit of time after chitin, or its artificial proxy, addition. Chitin is a major component of fungal cell walls	A proxy for fungivory
Fatty acid (FA) analysis		

Represents retention of the assimilated food, integrated over days to weeks. Assessed via chemical neutral lipid extraction and subsequent gas chromatography. Data is given as proportion of a certain biomarker of the total extracted neutral lipids (Ruess & Chamberlain, 2010; T.-W. Chen, Sandmann, Schaefer, & Scheu, 2017).

Sum of gram-positive bacteria biomarkers	Sum of proportion of the FAs synthesized by gram-positive bacteria only: i15:0, a15:0, i16:0, i17:0, a17:0	A proxy for bacterial feeding
Sum of gram-negative bacteria biomarkers	Sum of proportion of the FAs synthesized by gram-negative bacteria and actinomycetes only: 2-OH 10:0, 2-OH 12:0, 3-OH 12:0, 2-OH 14:0, 3-OH 14:0, 2-OH 16:0, and by gram-negative bacteria only: cy17:0, cy19:0	A proxy for bacterial feeding
Sum of non-specific bacterial biomarkers	Sum of proportion of the FAs synthesized by various bacteria: 16:1ω7, 18:1ω7	A proxy for bacterial feeding
Relative fungal biomarker	Proportion of the FA 18:2ω6,9 that is predominantly synthesized by fungi, but also can be synthesized by plants and animals	A proxy for fungivory
Relative and non-specific plant biomarkers	Proportion of the FA 18:1ω9 that is predominantly synthesized by plants, but also can be synthesized by fungi and gram-positive bacteria. Includes sum of biomarkers: 21:0, 22:0, 23:0, 24:0	A proxy for herbivory
Non-specific biomarker for arbuscular-mycorrhizal fungi	Proportion of the FA 16:1ω5 that is synthesized by arbuscular-mycorrhizal fungi, but also can by synthesized by various bacteria	A (potential) proxy for mycorrhiza feeding
Animal-synthesized FAs	Sum proportion of the FAs, that are synthesized by animals, and may accumulate in trophic chains as was shown for soil nematodes: 20:1ω9, 22:6ω3, 22:2ω6, 22:1ω9, 24:1	A (potential) proxy for predation (e.g. on nematodes)

Bulk stable isotope analysis

Represent retention of the assimilated food, integrated over days to weeks. Assessed via isotope mass spectrometry. Data is given in δ-units, reflecting concentration of the heavier isotope in relation to the international standard (Tiunov, 2007; A. M. Potapov, Semenina, et al., 2016; A. M. Potapov, Tiunov, & Scheu, 2019).		
$\Delta^{13}C$	Relative concentration of ^{13}C isotope in the bulk body tissue. Value is calibrated to the local plant litter to account for variability among ecosystems. Parameter varies among basal resources being relatively low in plants and high in saprotrophic microorganisms and animals.	A proxy for herbivory (low values) versus microbial feeding (high values)
$\Delta^{15}N$	Relative concentration of ^{15}N isotope in the bulk body tissue. Value is calibrated to the local plant litter to account for variability among ecosystems. Parameter increases in food chains, being relatively low in primary consumers, especially algivores, and high in predators	A proxy for algivory and detritivory (low values) versus microbial and animal feeding (high values)

Materials and methods

We selected visual gut content, digestive enzyme, FA and stable isotope analyses as the most commonly used methods that provide estimations of the trophic links between Collembola and their potential food resources in the field. Most of the published field studies applied only one of the methods and only two used a combination of two methods (Haubert et al. 2009; Ferlian, Klarner, Langeneckert, & Scheu, 2015). Therefore, we compiled all available data on each method separately and used species names as index to link them. Data were collected from the personal libraries of the authors and complemented with published literature by searching the Web of Science using keywords “Collembola” or “springtail” and various keywords to identify the specific method.

Visual gut content

Primary screening of the available literature yielded 31 studies that reported data on the gut content of Collembola. Out of these, we selected studies that provided quantitative estimates from natural environments, mostly temperate forests and grasslands. Standardisation of data from the selected studies was complicated since each author made its own classification of the particles found in guts. Therefore, we restricted ourselves to the most commonly reported categories and definitions of these categories – (1) particles of fungal origin, including hyphae and spores, (2) particles of plant origin, mostly coarse plant detritus (excluding pollen and algae), (3) amorphous material of unknown nature, i.e. fine detritus such as soil organic matter. The final dataset included 95 records (species-ecosystem average) on 62 species from 18 studies (Table S1). Raw data were expressed as proportion of the particles of a certain type among the total particles ingested.

Digestive enzymes

To our knowledge, digestive enzymes such as cellulase, trehalase and chitinase were reported only in three published studies on Collembola (Urbasek & Rusek, 1994; Berg et al., 2004; Parimuchová et al., 2018). Despite using the same method, these studies used different chemical protocols, including different ways of glucose detection, which resulted in evident differences (orders of magnitude in some cases) in absolute mean values and variation of substrate production per unit of animal body mass. Thus, we excluded the study of Urbasek & Rusek (1994) and normalized data on chitinase activity to the mean value separately for data coming from Berg et al. (2004) and Parimuchová et al. (2018) before merging them. Z-standardization

180 was done by subtracting mean and dividing by standard deviation using log-transformed data due
181 to high data skewedness. This could have introduced biases to our analyses and thus data on
182 chitinase activity should be interpreted with caution. However, since each study covered the
183 entire community and included species from all major Collembola orders, the normalization bias
184 is likely to be small. The final dataset included 45 records on 27 species (Table S2). Raw data
185 were expressed as mg of reaction products per g of animal mass per hour.
186

187 *Fatty acids*

188 Screening of the available literature yielded 10 studies that reported data on the neutral lipid FA
189 composition of Collembola. Those were complemented with unpublished data collected by
190 Melanie M. Pollierer and students under her supervision. Studies varied in completeness of FA
191 profiles, but most of them reported data on 16:1ω7 and 18:1ω7 (general bacteria biomarkers),
192 18:2ω6,9 (relative fungal biomarker), 18:1ω9 (relative plant biomarker) and several gram-
193 positive and gram-negative bacterial biomarker FAs. If reported, we also used 16:1ω5 as the
194 biomarker related to feeding on arbuscular-mycorrhizal fungi (Ngosong, Gabriel, & Ruess, 2012)
195 and sum of 20:1ω9, 22:6ω3, 22:2ω6, 22:1ω9 and 24:1 FAs that were shown to exist in high
196 concentrations in metazoan animals (nematodes) (Tanaka et al., 1996; J. Chen, Ferris, Scow, &
197 Graham, 2001; Paul M Chamberlain & Black, 2005; P.M. Chamberlain, Bull, Black, Ineson, &
198 Evershed, 2005b). Raw data was compiled as proportions of the total neutral lipid FAs of the
199 organism. Individual biomarker FAs were summed up to generate seven conventional trophic
200 niche parameters, which are listed in the Table 1. The final dataset included 130 records on 47
201 species from 13 studies and unpublished ones (Table S3).
202

Stable isotopes

The dataset was based on the previous compilation reported by Potapov et al. (2016) and complemented with data on grassland communities and few recent publications. For each study, isotopic baseline (i.e. plant litter) was recorded and used to calculate baseline-calibrated $\Delta^{13}C$ and $\Delta^{15}N$ values (A. M. Potapov, Tiunov, & Scheu, 2019). The final dataset included 378 records on 96 species from 10 studies (Table S4). Raw data were expressed in conventional δ units.

Data analysis

Fifteen trophic niche parameters were collected across methods (Table 1). In total, we collected data on 154 species, each analysed with at least one of the methods. All calculations were done in R 3.5.3 (R Core Team, 2019) with R studio interface 1.0.143 (RStudio, Inc.). We conducted species-based analyses. Data averaging for each parameter of each species followed two steps: First, individual measurements, if present, were averaged to calculate mean values for each species in each ecosystem (representing population from a locality with a certain ecosystem type); second, the records were averaged to calculate overall species average across ecosystems and studies. Species averages of different parameters were interlinked using species names. Proportional data (gut content analysis and FA analysis) were logit-transformed using *logit* in the *car* package prior to calculation of average values and further analyses; 0 and 1 proportions were adjusted to 0.025 and 0.975, respectively. Data on digestive enzyme activity were $\log_{10}$-transformed due to high skewness prior to calculation of average. To align taxonomic names, the table on each method was compared against the gbif taxonomic backbone using the species matching tool (https://www.gbif.org/tools/species-lookup). Therefore, in the study we focused on

225 interspecific variability only, ignoring the variation within species. Raw data used in the study
226 will be available on Dryad digital repository.

227 To have the same data representation across all fifteen parameters, we scaled data on each
228 parameter separately between 0 (lowest observed value of the parameter) and 1 (highest
229 observed value of the parameter). All the analyses and results were based on the scaled data.

230 Three analyses were performed. In the first analysis we tested pairwise correlations among all
231 trophic niche parameters. Spearman rank correlation was applied using *cor* function. The number
232 of points for each correlation varied among parameters according to the number of shared
233 species for which the data was present in both parameters (Table S5). Correlation tests were
234 conducted for those with more than five data points (i.e. at least six shared species in pairwise
235 parameter comparison).

236 In the second analysis we selected species with available data for most of the trophic niche
237 parameters studied. Unfortunately, only two species had data across all fifteen parameters. Thus,
238 we removed rare parameters, which yielded six species with data across seven parameters
239 present with focus on gut content and fatty acid data. Data on these species were used to run
240 principal component analysis (PCA) using *prcomp* function, to explore the association of species
241 with certain niche parameters and differentiation in their multidimensional trophic niches.

242 In the third analysis, we tested the effect of family identity (as the proxy of phylogenetic group)
243 and life form identity (as the proxy of microhabitat preference) on each of fifteen trophic niche
244 parameters separately using *lm* function (*parameter~family* or *parameter~life form*,
245 respectively). Groups with fewer than 3 species were removed from the analysis. Surface-living
246 families of Symphypleona (Dicyrtomidae, Katiannidae, Sminthuridae and Sminthurididae) were

247 analysed together due to low number of species replicates and trophic similarity (A. M. Potapov,
248 Semenina, et al., 2016). Life forms followed Gisin (1943) as defined by A. M. Potapov,
249 Semenina, et al., (2016), so that we included atmobiotic (aboveground and surface dwellers),
250 epedaphic (surface and upper litter dwellers), hemiedaphic (litter dwellers) and euedaphic (lower
251 litter and soil dwellers). For each parameter we used R^2 and p values from the model output to
252 compare predictability of different trophic niche parameters among high-rank phylogenetic
253 (taxonomic) groups and among ecological classifications (i.e. life-forms). We further divided the
254 best replicated family Isotomidae in epedaphic versus hemiedaphic and euedaphic species to
255 assess the effect of microhabitats on this family.

256

257 **Results**

258 *Correlation between trophic niche parameters*

259 In 45 out of total 64 performed pairwise tests (excluding within-method tests), correlation
260 between trophic niche parameters was weak or absent ($R^2 < 0.15$; Fig. 1, more details provided
261 in the Supplementary Fig. S1). Among strong positive correlations, species with high proportion
262 of fungal and plant particles in their guts had high proportions of fungal-synthesised and plant-
263 synthesised FAs in their bodies ($R^2 = 0.56$ and0.50), respectively. A high proportion of fungal
264 particles in the gut, however, negatively correlated with retained FA synthesised by gram-
265 negative bacteria, but the number of replicates was low.

266 Trehalase activity in guts positively correlated with the proportions of retained FAs synthesized
267 by fungi ($R^2 = 0.10$), gram-negative bacteria ($R^2 = 0.16$), plants ($R^2 = 0.22$) and animals ($R^2 =$
268 0.72, low replicates) in the bodies, but negatively with the proportions of FAs synthesised by

gram-positive bacteria (R^2 = 0.24) and non-specific bacteria biomarkers (R^2 = 0.28). Cellulase activity positively correlated with the proportion of arbuscular-mycorrhizal fungi (R^2 = 0.37) and non-specific bacteria FAs (R^2 = 0.21). Similar to trehalase activity, chitinase activity negatively correlated with the proportion of FAs synthesised by gram-positive bacteria (R^2 = 0.49) and positively with the proportion of FAs synthesised by gram-negative bacteria (R^2 = 0.22).

The $\Delta^{13}C$ values were negatively correlated with cellulase activity (R^2 = 0.64), chitinase activity (R^2 = 0.17) and proportion of plant-synthesised FAs (R^2 = 0.16). The $\Delta^{15}N$ values were negatively correlated with proportion of plant particles in gut (R^2 = 0.20) and plant-synthesised FAs (R^2 = 0.12), but positively with trehalase activity (R^2 = 0.18).

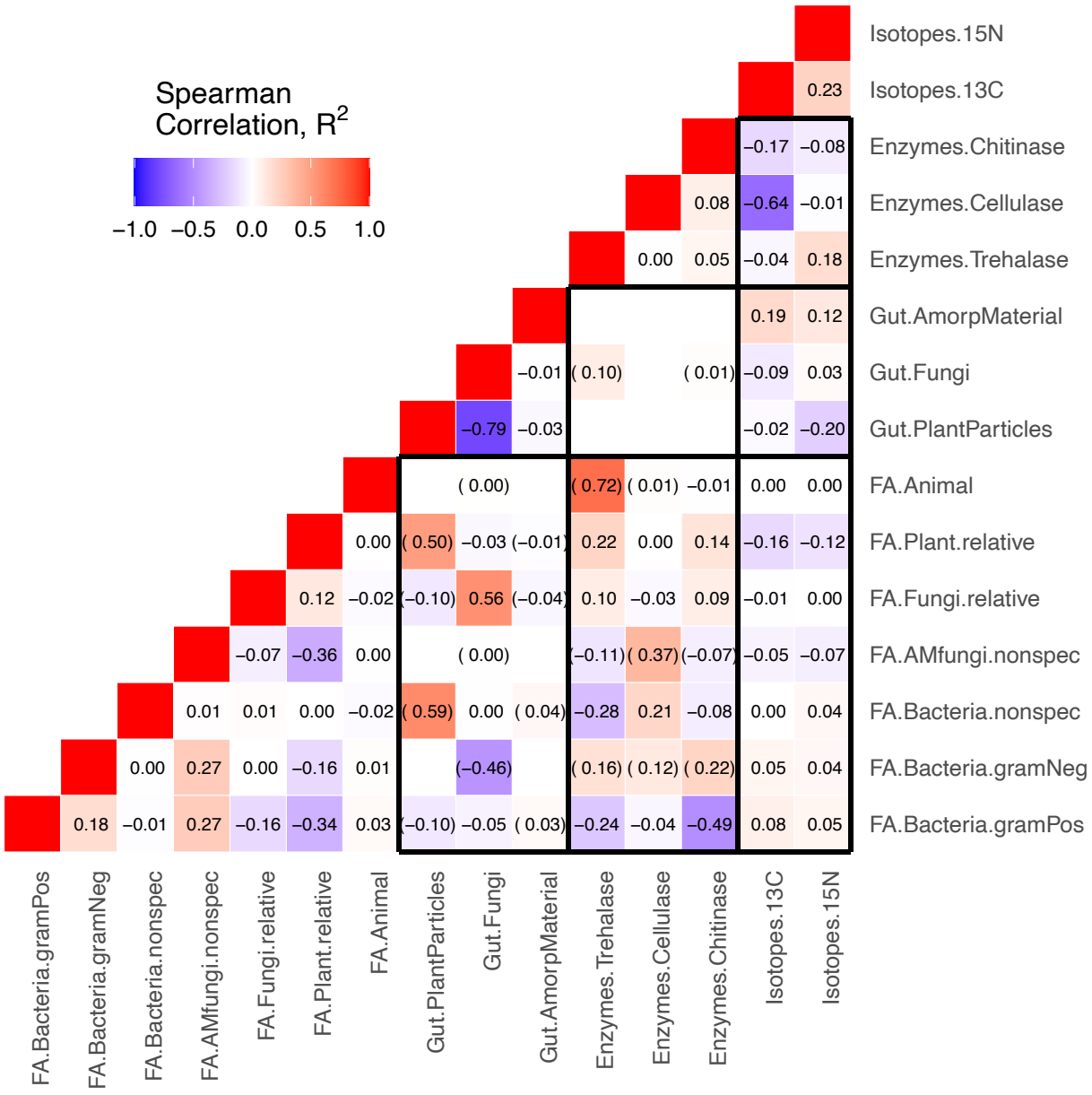

Figure 1. Correlation among fifteen trophic niche parameters in Collembola. Red colour represents positive correlations, blue colour represents negative correlations; to display negative correlations R^2 was multiplied by -1. Correlations based on < 6 species were removed, correlations based on 6–9 species are given in brackets; in other cases n = 10–88 (Table S5). Correlations among parameters derived from different methods are framed within black

284 rectangles. FA data and gut content data are proportional and thus intercorrelations among
285 parameters within the same method should be interpreted with caution.

286

287 *Multidimensional trophic niches of Collembola species*

288 The strongest distinction in multidimensional trophic niche space (joint fatty acid and gut content
289 analysis) on both PC1 and PC2 was observed between *Orchesella flavescens* (surface-dwelling
290 species of the family Entomobryidae) and *Protaphorura* (soil-dwelling genus belonging to
291 Onychiuridae; Fig. 2). The former species was associated with high proportions of plant-
292 synthesised FAs and plant particles in the gut, the latter with high proportions of fungi-
293 synthesised FAs and fungi in the gut. *Tomocerus minor* (Tomoceridae) was associated with plant
294 and non-specific bacteria parameters. *Lepidocyrtus lignorum* (litter-dwelling species of
295 Entomobryidae) was related to fungi in the gut on PC1. *Parisotoma notabilis* (litter-dwelling
296 species of Isotomidae) and *Onychiurus* (soil-dwelling genus of Onychiuridae) were associated
297 with gram-positive bacteria FAs and amorphous material in the gut.

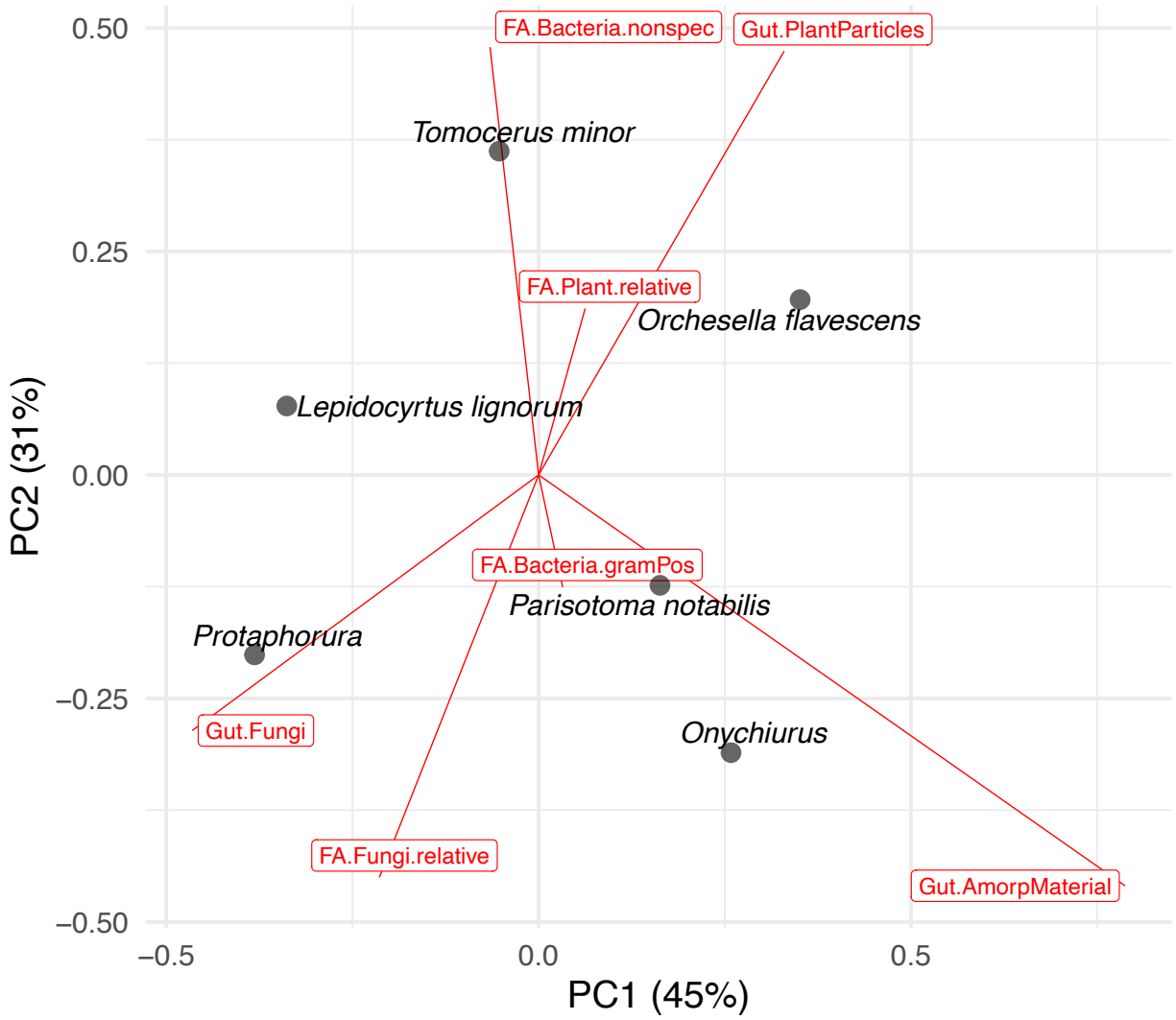

Figure 2. Principal component analysis based on seven selected trophic niche parameters that were collected from six common Collembola species. Parameters are shown with red vectors, species are shown in grey.

Multidimensional trophic niches in Collembola families and life forms

Family identity best explained stable isotope composition ($\Delta^{13}C$ and $\Delta^{15}N$ values), and also explained part of the variation in gram-positive and non-specific bacterial FA parameters,

306 proportion of plant and fungi in the gut, and trehalase activity ($R^2 \geq 0.24$, $p < 0.05$ except for
307 trehalase Fig. 3). Cellulase activity and proportion of amorphous material in gut were very
308 poorly related to the family identity ($R^2 = 0.03$), but only four or five families were analysed for
309 these parameters due to a low number of replicates in the other families.

310 Symphypleona species had high average proportion of FAs synthesised by gram-negative
311 bacteria, plants and fungi, high chitinase and cellulase, but very low trehalase activity in
312 comparison to other families (Fig. 3). Tomoceridae had high average proportions of plant
313 particles in the gut, plant-synthesised FAs and non-specific bacteria FAs and the lowest $\Delta^{13}C$
314 values. Entomobryidae had the highest trehalase activity among the other families but low values
315 of bacteria-related parameters. In comparison to previous families, Isotomidae had lower values
316 of fungi-related parameters and enzyme activities. A further separation of Isotomidae into
317 epedaphic and (hemi)edaphic lifeforms indicated that epedaphic Isotomidae had lower $\Delta^{13}C$
318 values than edaphic ones (0.35 versus 0.49), lower proportion of plants (0.55 versus 0.72) but
319 higher proportion of fungi in the gut (0.42 versus 0.20). Hypogastruridae had remarkably high
320 proportion of fungi in gut and high chitinase activity, but low proportion of plant particles in gut.
321 Neanuridae had the highest $\Delta^{13}C$ and $\Delta^{15}N$ values and proportions of FAs synthesized by gram-
322 positive bacteria and arbuscular-mycorrhizal fungi. Onychiuroidea had high $\Delta^{15}N$ values, FAs
323 synthesized by non-specific bacteria and fungi, and high cellulase activity.

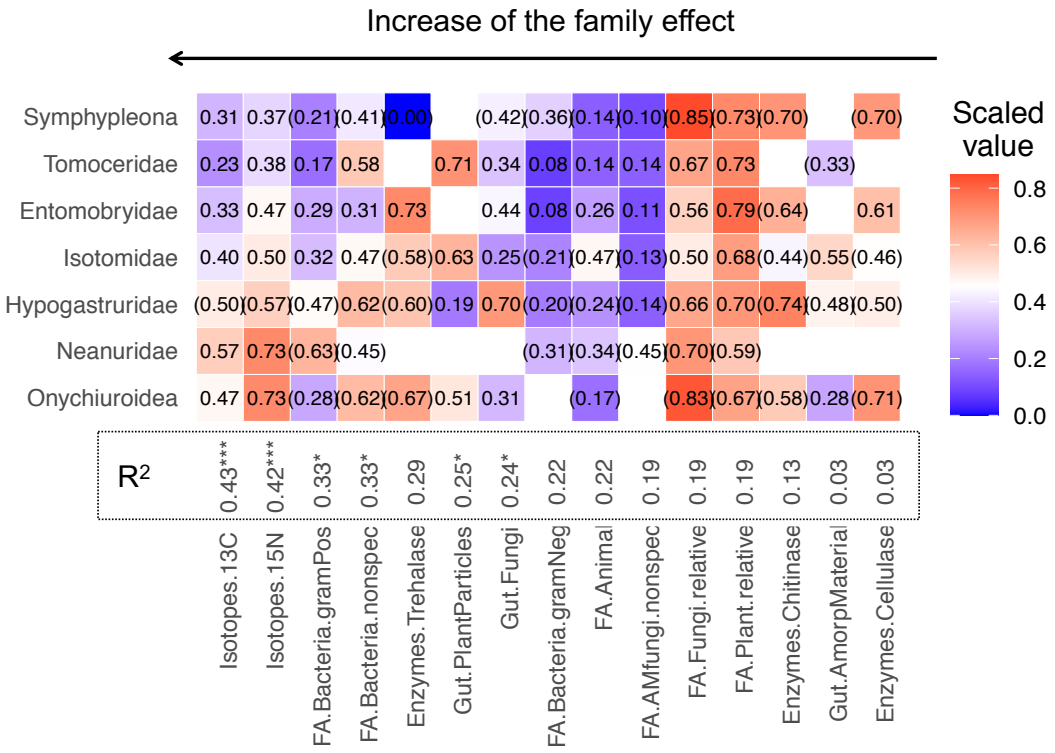

Figure 3. Mean values of different trophic niche parameters in Collembola families. All parameters and scaled between 0 (minimum, intense blue) and 1 (maximum, intense red). Means based on < 3 species were removed, means based on 3–5 species are given in brackets; in other cases n = 6–20. Numbers next to parameters show R^2 explained by family identity. Families are sorted according to the orders; parameters are sorted according to the R^2 values. Surface-living families of Symphypleona (Dicyrtomidae, Katiannidae, Sminthuridae and Sminthurididae) were analysed together due to a low number of species replicates and trophic similarity between families. Onychiuroidea includes Onychiuridae and few Tullbergiidae species.

334 Life form best explained chitinase activity, but the effect was not significant due to a low number
335 of replicates. Life form also well explained $\Delta^{15}N$ values and proportion of FA synthesised by
336 gram-negative bacteria ($R^2 \geq 0.25$; Fig. 4). Most of the other parameters were moderately or not
337 at all related to life form.

338 Atmobiotic species had high average activity of all digestive enzymes and high proportion of
339 FAs synthesized by gram-negative bacteria and plants (Fig. 4). Epedaphic species had in general
340 intermediate values for all parameters, but high proportions of fungi in the gut and high
341 proportions of animal-synthesized FAs. Hemiedaphic species had high $\Delta^{13}C$ values and
342 proportion of FAs synthesized by gram-positive bacteria, but low activity of chitinase and
343 cellulase. Euedaphic species had the highest $\Delta^{15}N$ values, high trehalase activity, high
344 proportions of FAs synthesised by fungi and non-specific bacteria biomarker FA.

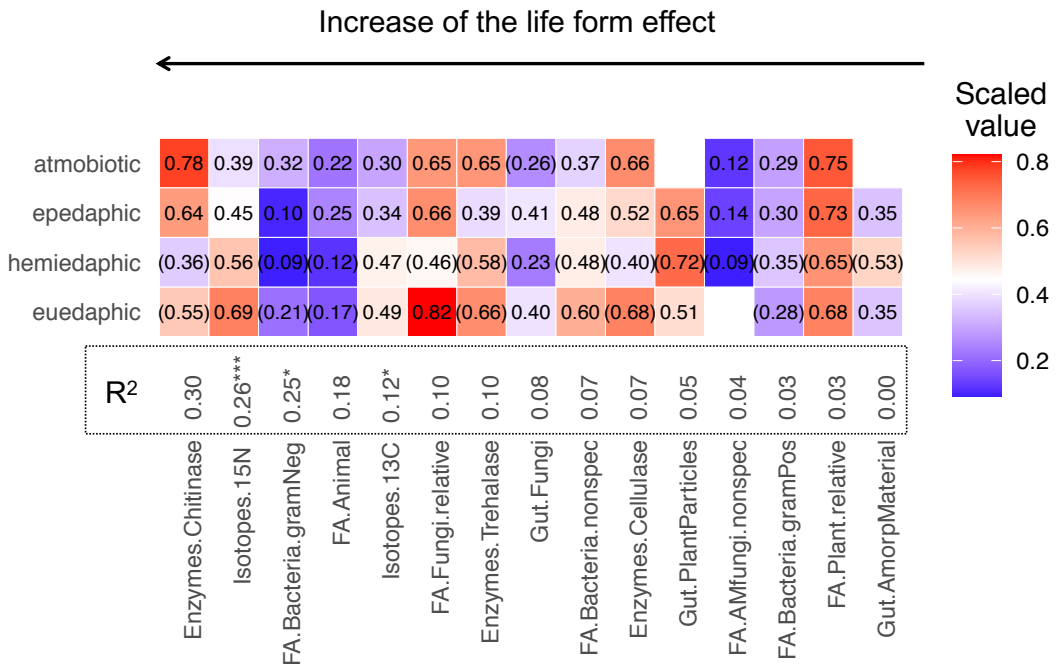

Figure 4. Median values of different trophic niche parameters in Collembola life forms. All parameters were scaled between 0 (minimum) and 1 (maximum). Means based on < 3 species were removed, means based on 3–5 species are given in brackets; in other cases n = 6–36. Numbers next to parameters show R^2 explained by life form identity. Life forms are sorted according to the species vertical stratification along the soil profile; parameters are sorted according to the R^2 values.

Discussion

Different methods provide information on different dimensions of trophic niche. However, studies quantitatively comparing different methods are very scarce, and usually involve two methods only (Nielsen et al., 2017). Here, we compared data on fifteen trophic niche parameters derived from four methods using Collembola as the model organisms. First, we showed that many trophic niche parameters are weakly correlated reflecting complementarity, rather than redundancy, of different methods. This was not surprising because different methods not only reflect different processes, but moreover differ in their currency, in the time interval which they reflect, and likely in the way they evolved. Second, we outlined trophic niche parameters based on species taxonomic cluster (i.e. family identity) and associated with the microenvironment species are living in (i.e. life form). Finally, we provided the most detailed information on the trophic niches of Collembola, cryptic but very abundant soil arthropods, available to date.

The additional value of method combination

It was shown that outcomes provided by different dietary methods only in part overlap (Nielsen et al., 2017). Pessimistically, this reflects biases embedded in each method – optimistically, it means that different methods may inform us on more trophic niche dimensions (Hambäck, Weingartner, Dalén, Wirta, & Roslin, 2016). Different methods track different processes, embrace different time frames, and assess niches on different resolution scales. We found weak correlation among trophic niche parameters derived from different methods in 70% of the performed tests. Some of the correlations may be improved if the material would come from the same animals, but it is unlikely that correlations that are now absent would become

374 strong. In addition, our results are likely realistic as weak correlations are expected in many
375 cases. Soil decomposers, including Collembola, ingest heterogeneous detritus with the
376 assimilation efficiency being on average only 20% (Jochum et al., 2017). In other words, only
377 about 20% of the ingested material, found during visual gut content observations, is being
378 assimilated and may be retained in the body. This is likely the case for soil-feeding Collembola,
379 whereas species feeding on fungi presumably select their food, suggesting that the ingested
380 material will reflect well what they assimilate. Other methods, such as stable isotope analysis,
381 are less informative in comparison with visual gut content observations as diet becomes more
382 heterogeneous – more food items can be visually observed in the gut, but the same number of
383 isotopic parameters are recorded and used to track many, or few, food items (Nielsen et al.,
384 2017).

385 Various types of interactions among the methods and parameters were observed in our
386 study, including confirmation (redundancy), controversies, complementarity, and clarification.
387 Some methods provided in part redundant information – for instance, proportion of plant and
388 fungal particles in gut correlated very well with retention of plant and fungal FAs, respectively.
389 Thus, both visual gut content analysis and FA analysis reflect well herbivory and fungivory in
390 Collembola; applying one of the methods can be sufficient to trace these feeding strategies. Both
391 $\Delta^{15}N$ and $\Delta^{13}C$ values negatively correlated with plant-related parameters provided by gut
392 content and FA analyses, with the latter negatively correlated also with cellulase activity. This
393 result is also confirmatory – high $\Delta^{15}N$ values reflect high trophic level and thus few plant
394 material in the diet; high $\Delta^{13}C$ values reflect feeding on microbially-decomposed organic matter,
395 and not freshly-fixed plant carbon, thus also reflecting a small proportion of plants in the diet (A.
396 M. Potapov, Tiunov, & Scheu, 2019). However, these correlations were not strong reflecting that

397 bulk stable isotope composition are affected by several different factors (Boecklen, Yarnes,
398 Cook, & James, 2011) and this method may complement gut content and FA analyses.

399 Complementarity to other methods was evident for digestive enzyme analysis, although
400 more data are needed to verify our results. For example, we expected a positive correlation
401 between chitinase activity (allowing to degrade chitin in fungal cell walls) or cellulase activity
402 (allowing to degrade cellulose in plant and algae cell walls) and fungi- or plant-related
403 parameters provided by other methods. Controversially, none of such correlations, especially for
404 cellulase, were strong. However, the proportion of fungi in gut, and proportions of plant- and
405 fungi-synthesized FAs were positively correlated with trehalase activity. Since trehalose is the
406 storage component of fungal, lichen and plant cells, these correlations suggest that Collembola
407 species most actively feeding on plant material and fungi are getting energy and nutrients from
408 storage, rather than structural, polysaccharides. In relation to the foraging behaviour, these
409 species are likely to be browsers, rather than grazers (Siepel & de Ruiter-Dukman, 1993).

410 As another example for complementarity of methods, activity of trehalase and chitinase
411 was negatively correlated with proportions of FAs synthesised by non-specific and gram-positive
412 bacteria. Apparently, some Collembola species have a specific feeding strategy where they rely
413 more on bacterial feeding and thus can invest less in the fungi-related digestive enzymes.
414 Detritivores have been shown to largely rely on amino acids synthesised by free-living and gut
415 symbiotic bacteria if the food quality is low, for example if they feed on soil organic matter
416 (Larsen et al., 2016; A. M. Potapov, Tiunov, Scheu, Larsen, & Pollierer, 2019). Some soil
417 animals repeatedly consume decomposing litter and soil, using free-living microorganisms that
418 produce digestive enzymes to release the nutrients from recalcitrant organic compounds, a
419 strategy termed "external rumen" (Swift, Heal, & Anderson, 1979). Higher $\Delta^{15}N$ values in soil-

420 dwelling Collembola species (A. M. Potapov, Semenina, et al., 2016) may indicate repeated
421 ingestion of soil organic matter and thus trophic level inflation (Steffan et al., 2017). In trend,
422 $\Delta^{15}N$ values were positively correlated with the proportion of FAs synthesised by non-specific
423 and gram-positive bacteria in our study, which may point to the utilization of external and gut
424 symbionts in some Collembola species. Actinobacteria, a gram-positive group of prokaryotes,
425 were shown to dominate in the gut of *Folsomia candida*, that is adapted to soil lifestyle (Zhu et
426 al., 2018). Gram-positive bacteria like Actinobacteria and Firmicutes in soil are associated with
427 small soil fractions, inhabiting small pores (Mummey, Holben, Six, & Stahl, 2006; Hemkemeyer,
428 Dohrmann, Christensen, & Tebbe, 2018), and thus are likely accessible mostly for soil-feeding
429 species. As low activity of digestive enzymes was often observed in species with a high
430 proportion of gram-positive bacteria FAs, this combination could point to feeding on soil organic
431 matter.

432 We avoid further extensive discussion of correlations since many of them were based
433 only on several points. This clearly shows a need for standardized studies, exploring
434 multidimensional trophic niches of species with several methods simultaneously (J. M. Nielsen
435 et al., 2017). In summary, our results showed that FA and visual gut content analysis provide
436 similar estimations of herbivory and fungivory in Collembola. Among these two methods, visual
437 gut content analysis has lower equipment requirements, but does not allow to trace some feeding
438 interactions, especially in the case of fluid-feeding, or feeding on soil organic matter. Besides,
439 data of different studies that recorded gut content of Collembola are very heterogeneous and in
440 many cases are not comparable. Further studies employing this method should ideally follow a
441 single described protocol with clearly defined particle categories. Similar improvement is
442 important for future studies on digestive enzymes since different laboratories are using different

443 chemical protocols which result in estimations differing over an order of magnitude (see
444 methods). Digestive enzyme analysis provides not very concrete information about trophic
445 interactions taken as itself since various food resources can be degraded with the same enzyme.
446 However, this method has a high additional value in combination with other methods, allowing
447 insights in foraging behaviour and animal-microbial interactions. Stable isotope analysis
448 provides integrative parameters, such as estimations of trophic level and plant versus microbial
449 feeding. Considering that this method is the least laborious among the studied ones, it can serve
450 as an appropriate tool for rapid assessment of trophic niches. However, to improve its value,
451 calibration studies with other methods, especially in soil consumers, are needed.

452

453 *Multidimensional trophic niches of Collembola*

454 In our study we compiled available information on the trophic niche parameters of
455 Collembola and explored how they vary across different taxa and life forms. Family identity
456 explained part of the variation in 6 out of 15 trophic niche parameters, suggesting that trophic
457 niches in Collembola are, at least in part, likely phylogenetically structured (A. M. Potapov,
458 Semenina, et al., 2016; T.-W. Chen et al., 2017). Life form, as a proxy for the microenvironment
459 species live in, explained variation only in three parameters with mostly low R^2 values.
460 However, this result should be interpreted with caution since life form is based on morphology
461 and provides only limited information on the real microhabitat (Ellers et al., 2018). Nevertheless,
462 species sharing the same microenvironment may share, or differentiate in their trophic niches.
463 Part of this variation may be explained by phylogenetic signal; thus a combination of phylogeny
464 with morphological traits associated with environmental adaptations and mouthpart morphology
465 (Raymond-Léonard, Gravel, & Handa, 2019) is a promising approach to predict trophic niches in

466 Collembola. This was demonstrated by the difference observed between epigeic and edaphic
467 Isotomidae in our study. Unfortunately, for each life form – family combination only few species
468 were present in the compiled dataset.

469 Both family and life form identities explained only some trophic niche parameters. The
470 effect of family explained most variation in stable isotope composition, and in bacteria-related
471 FA parameters. These parameters are all related to biochemical processes in the body and thus
472 may in part be affected by shared physiology of the phylogenetically-related species, calling for
473 controlled experiments exploring how the physiological effects may bias trophic inferences
474 (Larsen, Ventura, Damgaard, Hobbie, & Krogh, 2009). In turn, cellulase activity was not related
475 to family identity, suggesting that the ability to degrade cellulose is an evolutionary highly
476 variable trait in Collembola (although cellulase activity was generally low and more data is
477 needed to verify this). Life form explained variation in chitinase activity, showing that this
478 enzyme is synthesised mostly by surface-dwelling species, but the effect was not significant. Life
479 form also had a large effect on trophic level ($\Delta^{15}N$ values) and proportion of gram-negative
480 bacteria FAs, which may reflect the vertical distribution of the potential food objects. However,
481 the majority of trophic niche parameters were poorly related to life form, reflecting that a variety
482 of feeding strategies are available for Collembola in different microenvironments.

483 As practical demonstration of the additional value of method combination, in the
484 following sections we described trophic niches derived from multiple methods in conjunction for
485 each Collembola family. The order Symphypleona (representing a conglomerate of several
486 surface-dwelling families) on average had high proportions of fungal FAs, high chitinase activity
487 allowing to degrade fungal cell walls, and a high proportion of FAs synthesised by gram-
488 negative bacteria. Lichens are comprised of fungi, microalgae and cyanobacteria, with latter

being able to synthesise hydroxylated FAs, i.e. biomarkers for gram-negative bacteria (Dembitsky, Shkrob, & Go, 2001; Gugger, 2002) Together with low $\Delta^{15}N$ values typically found in algivores (A. M. Potapov, Korotkevich, & Tiunov, 2018), these values suggest that lichen grazing, or a combination of fungivory and herbivory may be widespread among Symphypleona.

Tomoceridae had a relatively well-defined trophic niche; the combination of parameters points to consumption of plant material with the help of bacteria. They had the highest average proportion of plant particles in the gut among all families and a relatively high proportion of plant-synthesised FAs. In addition, they had a high proportion of non-specific bacteria FAs, suggesting that they may graze on freshly fallen litter and assimilate it with the help of gut symbionts. Clarification of their trophic niche could be advanced with enzyme analysis, but only one record of *Tomocerus minor* was present in our database – this species had the highest cellulase activity among all records in the database, confirming plant litter grazing as the foraging behaviour.

Entomobryidae include many species that are morphologically resembling Tomoceridae, however, these two families differed in a number of trophic niche parameters (Fig. 3). Entomobryidae had more fungi in the gut, remarkably high trehalase activity, but lower proportion of non-specific bacteria FAs. Overall, these differences suggest that they are likely browsers, rather than grazers. *Lepidocyrtus lignorum* can serve as a good illustration – this species preferred fungi (Fig. 2), having high trehalase, but low cellulase and limited chitinase activity (Table S2).

Isotomidae had lower average values of fungi-related parameters and lower enzymatic activity than the previous two families. They also had high $\Delta^{13}C$ values, pointing to consumption of organic material in advanced stages of decomposition (A. M. Potapov, Tiunov, & Scheu,

512 2019), potentially including invertebrate faeces. All these features were the most expressed in
513 hemiedaphic and euedaphic Isotomidae species, inhabiting decomposing litter and soil (e.g.
514 *Parisotoma notabilis* on Fig. 2). It is likely, that edaphic Isotomidae species rely more on
515 bacteria, e.g. via using the "external rumen" feeding strategy (detailed discussion is given in the
516 section above). They usually have a small body size and their gut often contains humus (i.e. soil
517 and faecal material) (Poole, 1959; Ponge, 2000). Interestingly, hemiedaphic and euedaphic
518 Isotomidae such as species of the genera *Isotomiella*, *Folsomia*, *Parisotoma, Folsomides*,
519 dominate in collembolan communities in many ecosystems (M. Potapov, 2001). Thus, bacterial
520 feeding may be more common in Collembola than it is assumed in traditional soil food web
521 models (Hunt et al., 1987; de Vries et al., 2013) as also was observed in isotope labeling
522 experiments with bacteria (Murray et al., 2009; F. V. Crotty, Blackshaw, & Murray, 2011). To
523 verify this hypothesis further studies employing FA analysis calibrated for absolute comparison
524 of resource contributions (Kühn et al., 2019), or compound-specific stable isotope analysis
525 (Pollierer et al., 2019), are needed.

526 Hypogastruridae had the highest proportion of fungi and the lowest proportion of plants
527 in the gut across all families and low activity of cellulase, suggesting that they feed selectively
528 on microorganisms and not the plant material. Species of this family were reported to live and
529 feed on fungi (Sawahata, Soma, & Ohmasa, 2001), and to have relatively high proportions of
530 fungi-synthesised FAs (Ferlian, Klarner, Langeneckert, & Scheu, 2015). Taken together it is
531 likely that many Hypogastruridae are fungivores, or, more broadly, microbivores.

532 Neanuridae have no molar plate and are thus unable to chew the food. This family was
533 long recognised to have a distinct trophic niche from other families (Singh, 1969; Berg et al.,
534 2004; Chahartaghi et al., 2005). However, their exact food objects remain enigmatic. Early

535 studies hypothesised that Neanuridae feed by sucking up the content of fungal hyphae (Poole,
536 1959; Singh, 1969). Stable isotope analysis discovered that Neanuridae have an outstandingly
537 high trophic level, suggesting that they may feed on other animals, such as nematodes
538 (Chahartaghi et al., 2005). More recently, Neanuridae were show to successfully breed on slime
539 moulds (protists with mycelial stage) in laboratory cultures (Hoskins, Janion-Scheepers, Chown,
540 & Duffy, 2015). Since slime moulds are abundant in various ecosystems (Swanson, Vadell, &
541 Cavender, 1999), and tend to live in the rotten wood, as well as Neanuridae, they potentially
542 serve as the food for this collembolan family in natural environments. In our study we showed
543 that Neanuridae have high average values of bacteria- and fungi-related parameters, high $\Delta^{13}C$
544 and $\Delta^{15}N$ values, but very low plant supplementation, supporting all abovementioned
545 hypotheses. Interestingly, they also had a very high proportion of FAs synthesized by arbuscular
546 mycorrhizal fungi, but this biomarker is not specific and can also be synthesized by bacteria
547 (Table 1; Fig. 1). It is likely that Neanuridae are high-level consumers among Collembola,
548 feeding on various groups of microfauna and microorganisms, thus receiving energy from both
549 bacteria and fungi as basal resources.

550 Onychiuroidea are soil-adapted Collembola that have no eyes, no pigment and no furca.
551 They are associated with plant roots, presumably by feeding on root tips or mycorrhizae
552 (Endlweber, Ruess, & Scheu, 2009; Fujii et al., 2016; A. M. Potapov, Goncharov, Tsurikov,
553 Tully, & Tiunov, 2016). We found intermediate values for the proportion of plant and fungi
554 particles in their gut in comparison to other families. However, they had high proportions of FAs
555 synthesised by fungi and bacteria (non-specific) and high cellulase activity. Potentially,
556 Onychiuroidea may combine root and soil feeding. Besides, small-sized Tullbergiidae that were

557 analysed together with Onychiuridae to increase replication in our analysis, may not rely much
558 on root-derived resources (Li et al., 2020), calling for more studies on this superfamily.

559 Using comparisons across multiple methods, we provide a general overview of high-rank
560 Collembola taxa trophic niches at the family level. These large-scale patterns will not necessarily
561 be supported on species-level, but rather represent the average trends. In our study we also
562 ignored the variability among ecosystems, which does affect trophic positions of soil animals
563 (Berg et al., 2004; Felicity V. Crotty, Blackshaw, Adl, Inger, & Murray, 2014; Susanti, Pollierer,
564 Widyastuti, Scheu, & Potapov, 2019). This study is the first cross-method compilation of
565 available data on trophic traits in Collembola that clearly shows not only the gaps, but also
566 advantages of the multidimensional trophic niche approach. Different methods are not redundant
567 but rather have a high additional value by compensating individual drawbacks when combined.
568 Simultaneous application of several methods to the same population, and across different groups
569 and ecosystems, is the way to understand the functioning of food webs and the coexistence of
570 species in cryptic environments, such as soil.

571

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Authors' contributions

AMP and TWC developed the idea and study design. SS compiled data on the gut content analysis, VS compiled data on the digestive enzyme analysis, MMP compiled data on the fatty acid analysis, AMP compiled data on the stable isotope analysis. AMP did the analysis and drafted the manuscript. All authors critically revised the analysis and text.

Data accessibility

Raw data supporting the results of the study are provided in the Supplementary materials and we intend to archive it to the Dryad digital repository, should the manuscript be accepted for publication.

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818 **Supplementary materials**

819 - Table S1 – dataset, gut content data (provided separately)

820 - Table S2 – dataset, enzyme data (provided separately)

821 - Table S3 – dataset, FA data (provided separately)

822 - Tables S4 – dataset, stable isotope data (provided separately)

823 - Table S5 – overlap between different trophic-niche parameters

824 - Figure S1 – pairwise correlation between trophic-niche parameters, full version

825

826 **Table S5**. Overlap between different trophic-niche parameters. The numbers of species that has

827 records on each of two parameters for each pairwise comparison are given.

	FA.Bacteria.gramPos	FA.Bacteria.gramNeg	FA.Bacteria.nonspec	FA.AMfungi.nonspec	FA.Fungi.relative	FA.Plant.relative	FA.Animal	Gut.PlantParticles	Gut.Fungi	Gut.AmorpMaterial	Enzymes.Trehalase	Enzymes.Cellulase	Enzymes.Chitinase	Isotopes.13C	Isotopes.15N
FA.Bacteria.gramPos	41														
FA.Bacteria.gramNeg	34	34													
FA.Bacteria.nonspec	41	34	47												
FA.AMfungi.nonspec	33	33	33	33											
FA.Fungi.relative	41	34	47	33	47										
FA.Plant.relative	41	34	47	33	47	47									
FA.Animal	36	29	40	29	40	40	40								
Gut.PlantParticles	6	2	6	2	6	6	5	40							
Gut.Fungi	11	7	11	7	11	11	9	40	54						
Gut.AmorpMaterial	7	3	7	3	7	7	6	32	34	34					
Enzymes.Trehalase	10	9	10	9	10	10	9	3	7	3	24				
Enzymes.Cellulase	10	9	10	9	10	10	9	3	5	3	22	22			
Enzymes.Chitinase	10	9	11	9	11	11	10	3	7	3	22	20	23		
Isotopes.13C	22	17	24	17	24	24	22	14	19	11	14	13	12	90	
Isotopes.15N	22	17	24	17	24	24	22	14	19	11	14	13	12	88	88

828

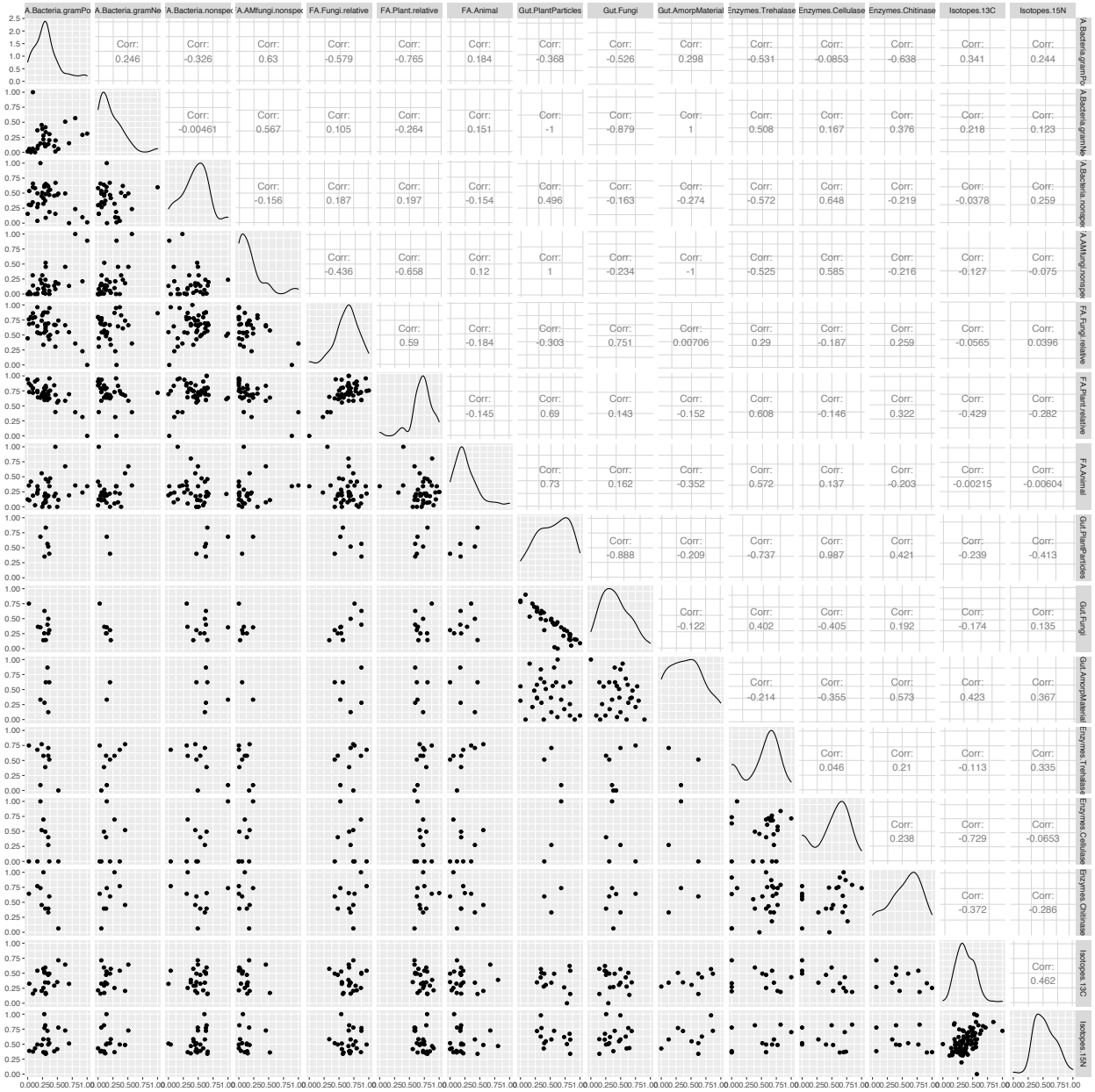

Figure S1. Correlation among fifteen trophic niche parameters in Collembola produced with *ggpairs* in *GGally* package. Number of points varies from 0 ("NA") to 88, depending on the method overlap. Spearman correlation coefficients ("Corr:") are shown in the upper triangle. Diagonal shows density of the data distribution for corresponding parameters. Figure provides more detailed view of the analysis presented on the Fig. 1 in the main text.