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Stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) of soil nematodes from four feeding groups

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Soil nematode feeding groups are a long-established trophic categorisation largely based on morphology and are used in ecological indices to monitor and analyse the biological state of soils. Stable isotope ratio analysis ($^{13}C/^{12}C$ and $^{15}N/^{14}N$, expressed as $\delta^{13}C$ and $\delta^{15}N$) has provided verification of, and novel insights into, the feeding ecology of soil animals such as earthworms and mites. However, isotopic studies of soil nematodes have been limited to date as conventional stable isotope ratio analysis needs impractically large numbers of nematodes (up to 1000) to achieve required minimum sample weights (typically >100 µg C and N). Here, micro-sample near-conventional elemental analysis – isotopic ratio mass spectrometry (µEA–IRMS) of C and N using microgram samples (typically 20 µg dry weight), was employed to compare the trophic position of selected soil nematode taxa from four feeding groups: predators (*Anatonchus* and *Mononchus*), bacterial feeders (*Plectus* and *Rhabditis*), omnivores (Aporcelaimidae and Qudsianematidae) and the plant feeder (*Rotylenchus*). Free-living nematodes were collected from conventionally and organically managed arable soils. As few as 15 nematodes, for omnivores and predators, were sufficient to reach the 20 µg dry weight target. There was no significant difference in $\delta^{13}C$ ($p=0.706$) between conventional and organic agronomic treatments but, within treatments, there was a significant difference in N and C stable isotope ratios between the plant feeder, *Rotylenchus* ($\delta^{15}N=1.08$ to 3.22 mUr, $\delta^{13}C=-29.58$ to –27.87 mUr) and all other groups. There was an average difference of 9.62 mUr in $\delta^{15}N$ between the plant feeder and the predator group ($\delta^{15}N= 9.89$ to 12.79 mUr, $\delta^{13}C=-27.04$ to –25.51 mUr). Isotopic niche widths were calculated as Bayesian derived standard ellipse areas and were smallest for the plant feeder (1.37 mUr^2) and the predators (1.73 mUr^2), but largest for omnivores (3.83 mUr^2). These data may reflect more preferential feeding by the plant feeder and predators, as assumed by classical morphology-based feeding groups, and indicate that omnivory may be more widespread

across detritivore groups i.e. bacterial feeders (3.81 mUr). Trophic information for soil nematodes derived from stable isotope analysis, scaled as finely as species level in some cases, will complement existing indices for soil biological assessment and monitoring, and can potentially be used to identify new trophic interactions in soils. The isotopic technique used here, to compare nematode feeding group members largely confirm their trophic relations based on morphological studies.

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16 # ABSTRACT
17 Soil nematode feeding groups are a long-established trophic categorisation largely based on
18 morphology and are used in ecological indices to monitor and analyse the biological state of soils.
19 Stable isotope ratio analysis ($^{13}C/^{12}C$ and $^{15}N/^{14}N$, expressed as $\delta^{13}C$ and $\delta^{15}N$) has provided
20 verification of, and novel insights into, the feeding ecology of soil animals such as earthworms
21 and mites. However, isotopic studies of soil nematodes have been limited to date as conventional
22 stable isotope ratio analysis needs impractically large numbers of nematodes (up to 1000) to
23 achieve required minimum sample weights (typically >100 µg C and N). Here, micro-sample
24 near-conventional elemental analysis – isotopic ratio mass spectrometry (µEA–IRMS) of C and
25 N using microgram samples (typically 20 µg dry weight), was employed to compare the trophic
26 position of selected soil nematode taxa from four feeding groups: predators (*Anatonchus* and
27 *Mononchus*), bacterial feeders (*Plectus* and *Rhabditis*), omnivores (Aporcelaimidae and
28 Qudsianematidae) and the plant feeder (*Rotylenchus*). Free-living nematodes were collected from
29 conventionally and organically managed arable soils. As few as 15 nematodes, for omnivores
30 and predators, were sufficient to reach the 20 µg dry weight target. There was no significant
31 difference in $\delta^{13}C$ (p=0.706) between conventional and organic agronomic treatments but, within
32 treatments, there was a significant difference in N and C stable isotope ratios between the plant

33 feeder, *Rotylenchus* ($\delta^{15}N$=1.08 to 3.22 mUr, $\delta^{13}C$=–29.58 to –27.87 mUr) and all other groups.
34 There was an average difference of 9.62 mUr in $\delta^{15}N$ between the plant feeder and the predator
35 group ($\delta^{15}N$= 9.89 to 12.79 mUr, $\delta^{13}C$=–27.04 to –25.51 mUr). Isotopic niche widths were
36 calculated as Bayesian derived standard ellipse areas and were smallest for the plant feeder (1.37
37 mUr^2) and the predators (1.73 mUr^2), but largest for omnivores (3.83 mUr^2). These data may
38 reflect more preferential feeding by the plant feeder and predators, as assumed by classical
39 morphology-based feeding groups, and indicate that omnivory may be more widespread across
40 detritivore groups i.e. bacterial feeders (3.81 mUr). Trophic information for soil nematodes
41 derived from stable isotope analysis, scaled as finely as species level in some cases, will
42 complement existing indices for soil biological assessment and monitoring, and can potentially
43 be used to identify new trophic interactions in soils. The isotopic technique used here, to
44 compare nematode feeding group members largely confirm their trophic relations based on
45 morphological studies.
46

47 **Introduction**

48 Nematodes are an abundant and diverse animal group in most soils, especially where
49 decomposition is active (Bongers & Bongers, 1998). Nematodes play major roles in soil
50 processes, both directly and indirectly through elemental cycling and decomposition of organic
51 matter. For example, they mineralise nitrogen and phosphorus, as well as influence other soil
52 organisms involved in nutrient cycling (Ferris et al., 2012), especially by regulating soil
53 microbial populations (Griffiths, 1990). Some soil nematodes feed directly on plants and many
54 are prey for larger soil fauna (Curry & Schmidt, 2007; Heidemann et al., 2011).
55 Soil nematodes are traditionally assigned to feeding groups according to morphology, feeding
56 experiments and gut content analyses (Overgaard-Nielsen, 1949; Wood, 1973; Yeates et al.,
57 1993). Nematode feeding groups, functional guilds and strategy-based indices have been used
58 extensively to document the response of nematodes to soil disturbance as bio-indicators of
59 general biological conditions in soil ecosystems (Neher, 2001; Ferris et al., 2001; Ferris et al.,
60 2012), and, in ecological studies, to assess the importance of nematodes in soil energy pathways
61 (de Ruiter et al., 1998; Zhao & Neher, 2014). The indices developed for soil nematodes have
62 been shown to be applicable to other soil fauna (Sánchez-Moreno et al., 2009).

63 There are, however, discontinuities and uncertainties in the assumed trophic groups of some
64 nematodes. For example, bacterial feeders have been cultured successfully on contrary food
65 sources such as fungi, in laboratory situations, and it is often difficult to assign feeding types at a
66 species level (Yeates et al., 1993; Ferris et al., 2001). Laboratory-based feeding experiments are
67 not always indicative of natural in situ feeding behaviour and, morphology alone may be
68 misleading.
69 Terrestrial and aquatic nematode feeding can be categorised similarly (Moens et al., 2006) with
70 growing support for a collective classification (Moens et al., 2004). Feeding response of
71 nematode trophic groups may not be represented fully, without testing finer resolution taxonomic
72 groups (Neher & Weicht, 2013, Cesarz et al., 2015) and certain groups (i.e. omnivores) may shift
73 trophic level feeding as a result of life stage development (Moens et al., 2006). Omnivorous
74 nematodes are taken as generalist feeders and less so as 'true' omnivores (Moens et al., 2004),
75 however, 'true' omnivory (i.e. feeding across different trophic levels) may be more widespread
76 than once assumed in soil food webs (Scheu, 2002), and nematode communities are no exception
77 to this theory (Moens et al., 2006). Several experts have identified the confirmation of trophic
78 groupings of nematodes as a major gap in free-living nematode research (Scheu, 2002; Neher,
79 2010, Ferris, 2012).
80 In current soil food web studies, the combination of traditional taxonomic and observational
81 techniques with molecular and isotopic advances is yielding novel insights (e.g. Curry &
82 Schmidt, 2007). For trophic studies, stable isotopes provide different, often complementary
83 information to molecular techniques because diet-indicating isotopes are assimilated and hence
84 detectable over longer time spans than ingested nucleic acids of food items (Darby & Neher,
85 2012).
86 To date, isotopic studies have been applied more to aquatic nematode groups than to soil groups
87 and mostly to taxa of larger sizes that yield sufficient sample mass for analysis. For example, in
88 estuarine sediments, C and N isotope measurements showed distinct trophic groupings often
89 coinciding with mouth morphology, but certain assumed deposit feeding taxa without teeth had
90 elevated $^{15}N/^{14}N$ ratios suggesting predatory behaviour (Moens et al., 2005; Vafeiadou et al.,
91 2014). Another example is food selectivity of aquatic, bacteria-feeding nematodes, which were
92 investigated by Estifanos et al. (2013) using isotopically-labelled bacteria, with results
93 suggesting a significant component of algae and diatoms in the diet. Results conflicted so much

94 for Vafeiadou et al. (2014) that they concluded that interpretation of nematode feeding ecology
95 based purely on mouth morphology should be avoided.
96 Soil food webs were traditionally defined with a $\delta^{15}N$ gap of 3.4 mUr (‰) between trophic levels
97 (Ponsard & Arditi, 2000). For soil nematodes, plant-parasitic Longidoridae, were first analysed
98 isotopically at species level by Neilson & Brown (1999), and showed varied $\delta^{15}N$ shifts after 28
99 days on *Petunia sp.* roots when transferred from an isotopically distant host plant, suggesting
100 either different species feeding, metabolism or reproductive mechanisms. Soil food web studies
101 under controlled conditions have analysed entire nematode communities for isotopic
102 comparisons with other fauna groups (Sampedro & Domínguez, 2008; Crotty et al., 2014), but
103 individual soil nematode trophic group studies have been slow to follow. For instance, the energy
104 channel (whether fungal or bacterial) and ^{13}C of soil nematode feeding groups was altered by
105 experimentally raised CO_2 with depleted $\delta^{13}C$ (≈−47 mUr), under different crops, in a study by
106 Sticht et al. (2009). In combination with ^{15}N analysis, fatty acids compositions were used as
107 traceable markers for trophic studies by Ruess et al. (2004), and the same approach was
108 employed later to show trophic links with ^{13}C analysis of individual fatty acids for consumer and
109 predatory soil fauna diets under organic compared with conventional systems (Haubert et al.,
110 2009). While these examples enlighten aspects of nematode feeding and its contribution to the
111 larger soil food web, testing of morphology-based nematode feeding group classification has not
112 been extensively undertaken.
113 Coming closer to this undertaking, Shaw et al. (2016) used ^{13}C labelled roots to highlight the role
114 of higher trophic level nematodes in soil C flow and root decomposition under burnt prairie grass
115 in a greenhouse experiment. And most recently, using conventional isotopic ratio mass
116 spectrometry (IRMS), a study in a boreal forest showed that soil nematodes from four feeding
117 groups had distinct isotopic values ($\delta^{13}C$ and $\delta^{15}N$) at natural abundance level, representing
118 chiefly trophic differences between microbial and predatory feeders (Kudrin et al., 2015).
119 Isotopic analysis of soil nematodes using conventional IRMS has been limited by the amount of
120 tissue required to measure N and C (Darby & Neher, 2012). Recently, Langel & Dyckmans
121 (2014) developed a μEA–IRMS method that analyses microgram samples (as little as 0.6 μg for
122 ^{15}N and 1 μg for ^{13}C). This method has already been used to investigate resource shifts (^{13}C
123 labelled) in soil mesofauna under fertilizer treatments (Lemanski & Scheu, 2014) and the

124 comparative feeding ecology of oribatid mites in varying regional and forest deadwood types
125 (Bluhm et al., 2015).
126 Here, the µEA–IRMS method was employed for natural abundance, dual stable isotope analysis
127 of feeding group members of free-living soil nematodes collected from a field experiment with
128 conventionally and organically managed arable soil. This pilot study had three main aims; (i) to
129 establish how many nematodes are needed (from different taxa/groups) for sufficient sample
130 mass for natural abundance isotopic analysis (dual ^{13}C and ^{15}N analysis), (ii) to compare
131 members of nematode feeding groups from two different agronomic systems and (iii) to compare
132 isotopically derived functional group results with traditional nematode feeding classifications.
133 Isotopic 'niche spaces' were calculated for: predators (*Anatonchus* and *Mononchus*), bacterial
134 feeders (*Plectus* and *Rhabditis*), omnivores (Aporcelaimidae and Qudsianematidae) and the plant
135 feeder (*Rotylenchus*). We hypothesized that 1) the isotopically represented nematode
136 communities would be altered under the organically amended agronomic treatment and that 2)
137 the isotopic niches of tested nematode groups would largely agree with the traditional
138 classification of feeding groups.
139

140 Materials & Methods

141 The original field experiment consisted of four different agronomic treatments, each treatment
142 was replicated three times according to a randomised plot design and the plot size was 3 m by 10
143 m. The study site was No. 3 field at the Bush estate, Penicuik, Midlothian, Scotland (lat. 55° 51'
144 N, long. 3° 12' W). For full site and soil details, refer to Vinten et al., (1992); Vinten & Lewis
145 (2002). The conventional treatment (i.e. with the use of tillage, synthetic fertilisers, pesticides
146 and herbicides) and the organic treatment (i.e. no fertiliser, herbicides or pesticides, but with the
147 addition of 10 t ha^{-1} of farmyard manure and under-sown with clover) were established in 2007
148 (Aruotore, 2009). Plots from these two treatments were sampled in Autumn 2014 for this study,
149 following a crop of spring barley (*Hordeum vulgare* L.).
150 From each plot, 12 soil cores, 2 cm diameter and 10 cm deep, were extracted using an auger in a
151 stratified random sampling pattern to form a composite sample. Soil samples were stored in
152 plastic bags at 4°C and nematodes were extracted from approximately 100 g soil according to
153 Whitehead & Hemming (1965). The nematodes were collected alive in water every day for 16
154 days and kept in water at 4°C before being identified. Each sample was examined using an

155 inverted microscope at up to x400 magnification. This allowed nematodes to be identified to
156 family/genus level according to mouth and body morphology using Bongers (1988). They were
157 then transferred individually, using the microscope and an eyelash attached to the tip of an
158 entomological needle via parafilm, into previously weighed, miniature tin capsules (8 mm x 5
159 mm, Elemental Microanalysis Ltd.). Additional specimens (for each group), 1 from every 5
160 nematodes identified were preserved in DESS (dimethyl sulphoxide, disodium EDTA and
161 saturated NaCl) (Yoder & Ley, 2006) for confirmatory identification. Tin cups with nematodes
162 were placed inside a multi-well plate with cover but left un-sealed and dried at 37°C overnight.
163 A conservative target of 20 µg dry weight for each nematode taxonomic group was adopted to
164 take advantage of the µEA–IRMS technique (Langel & Dyckmans, 2014).
165 The samples were weighed on a microbalance (Mettler Toledo) to verify if the target weight was
166 reached. If not, more nematodes were counted into the previous day's samples, dried again at
167 37°C for 12-24 hours, and the process continued until the target weight was reached. Tin
168 capsules were then wrapped and placed in a new, clean multi-well plate and shipped for
169 measurement. Some samples that did not reach the target weight were also included for analysis.
170 Measurements of isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) were made with an isotope ratio mass
171 spectrometer (Delta V, Thermo Scientific, Bremen, Germany) coupled to a modified elemental
172 analyser (Eurovector, Milano, Italy) as described by Langel & Dyckmans (2014). Results are
173 expressed in mUr notation after Brand & Coplen (2012). SD of the system was <1 mUr at
174 sample size of 0.6 µg N (Langel & Dyckmans, 2014).
175 Blank correction was performed by measuring additional reference samples of acetanilide ($\delta^{13}C$
176 = -29.6 mUr, $\delta^{15}N$ = -1.6 mUr) and wild boar liver ($\delta^{13}C$ = -17.3 mUr, $\delta^{15}N$ = 7.2 mUr). The
177 results were used to determine the blank amount and isotopic compositions for both C and N in a
178 Keeling-plot type graph as described e.g. in Langel & Dyckmans (2014). The C blank was 2 µg
179 with an isotopic value of -25 mUr, whereas no blank correction was performed for N because N
180 blank was very small (0.2 µg) and variable in isotopic composition. This variability is probably
181 caused by the fact that N is derived from two different sources, atmospheric N_2, on the one hand,
182 (leading to slightly negative isotopic values due to fractionation upon diffusion) and the
183 carryover from preceding samples, on the other hand, which can have different isotopic
184 composition in the oxidation reactor.
185 All statistics and graphics were generated in R (R Development Core Team, 2007). The Siber

186 package within SIAR - Stable isotope analysis in R (Jackson et al., 2011) was used to analyse
187 isotope data with Bayesian statistics. The trophic niches of the sampled nematode communities
188 and groups were inferred from the 'isotopic niche space' occupied by each of the groups on a
189 $\delta^{13}C/\delta^{15}N$ biplot and calculated as the Bayesian standard ellipse areas (SEA with units of mUr^2).
190 In communities, the Bayesian standard ellipse areas (SEA) were probability tested to see if they
191 were significantly different as well as comparing area overlap. Due to the small and varied
192 sample numbers for pooled nematodes groups, area overlap of SEAs and convex hulls (TAs)
193 were compared, both of which indicate niche width. Note that convex hull total area (TA)
194 estimates are less reliable due to small sample sizes (Jackson et al., 2011), while SEA, and
195 expressly sample size corrected standard ellipse areas (SEAc), are less biased when there are low
196 sample numbers (Syväranta et al., 2013). Bayesian estimates of 10^5 were used to generate
197 Standard Ellipse areas in all cases.
198 Animals used in this research (phylum Nematoda) are not endangered, nor subject to animal
199 research ethics regulations in the countries where the work was conducted. Field studies did not
200 require approval by an Institutional Review Board.
201

202 Results

203 Sample sizes and measurement issues

204 The average number of nematodes per sample (Table 1) varied within family/genera groups,
205 some being larger in size/weight and also within samples, since both mature and immature
206 (smaller) individuals were used, once identifiable. In the pooled samples, a priori designation of
207 feeding type by morphology was assigned before analysis and groups included either one or two
208 members (Table 1). Larger-sized omnivore nematodes had ranges as low as 15–25 individuals
209 per sample, while the smaller bacterial feeders had higher ranges of 35–115 individuals to
210 achieve 20 μg target dry weight.

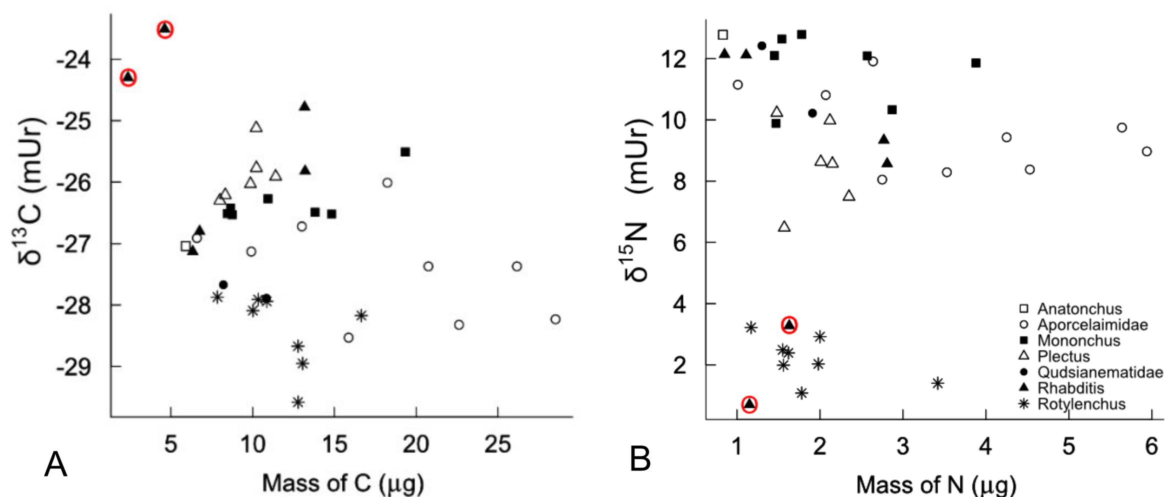
211 **Table 1.** The mean number of nematodes (± SD) used to achieve the target weight per sample for the groups listed,
212 number of measured replicate samples (in brackets), and total number of measured replicate samples in each feeding
213 group (in final column) from conventional and organic arable soils.

Soil nematode taxa			Conventional	Organic	Total
Feeding group ORDER	Family	Genus	Mean no. of nematodes per sample ± SD (n=measured samples)		Number of measured samples
Predators					
MONOCHIDA	Anatonchidae	*Anatonchus*	-	3 (*n=1*)	

MONOCHIDA	Mononchidae	*Mononchus*	50 ± 5 (*n=3*)	25.2 ± 7 (*n=4*)	*n=8*
Omnivores					
DORYLAIMIDA	Aporcelaimidae	-	16 ± 2 (*n=3*)	20 ± 3 (*n=6*)	
DORYLAIMIDA	Qudsianematidae	-	-	33 ± 4 (*n=2*)	*n=11*
Bacterial feeders					
PLECTIDA	Plectidae	*Plectus*	73 ± 46 (*n=2*)	65 ± 37 (*n=4*)	
RHABDITIDA	Rhabditidae	*Rhabditis*	32 ± 33 (*n=3*)	35 ± 14 (*n=3*)	*n=12*
Plant feeder					
TYLENCHIDA	Hoplolaimidae	*Rotylenchus*	97 ± 12 (*n=3*)	84 ± 27 (*n=5*)	*n=8*

214

215 For an initial quality control and check of linearity, all $\delta^{13}C$ and $\delta^{15}N$ (mUr) sample results were
216 plotted against the mass of C and N per sample, respectively (Figures 1A and 1B). Two samples
217 (out of 39 pooled samples measured) were excluded because the C mass was considered too
218 small. There was no significant correlation (Spearman's) between C mass and $\delta^{13}C$ values (r_s= -
219 0.143, p=0.397), or N mass and $\delta^{15}N$ values (r_s = -0.274, p=0.10), once these two samples were
220 removed. Importantly, there was no obvious pattern of systematic sample mass differences
221 explaining isotopic clustering of nematode groups (Figures 1A and 1B).

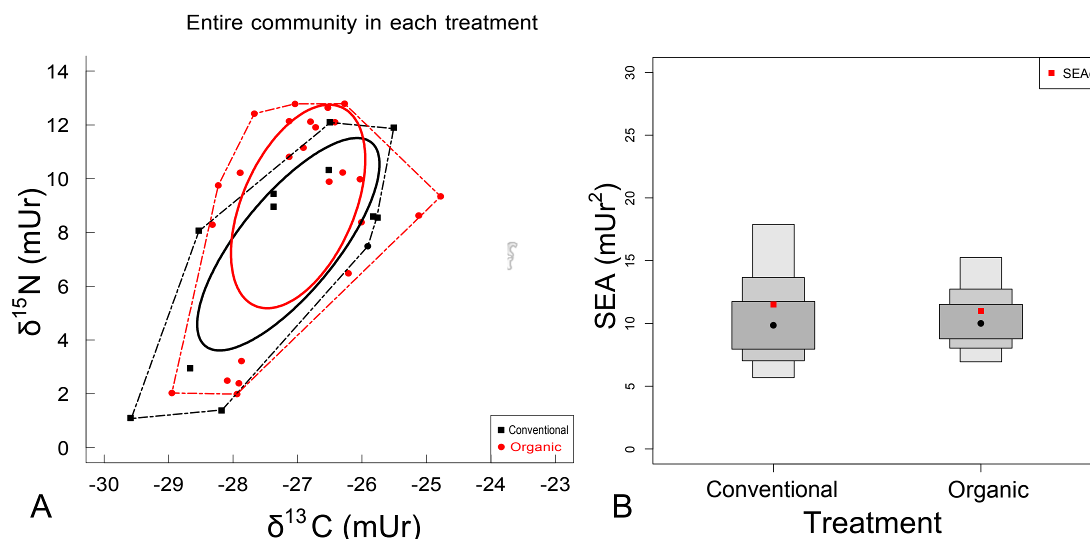


222
223 Figure 1A: Sample mass of C for all samples plotted against the measured $\delta^{13}C$ values. Figure 1B: Sample mass of
224 N for all samples plotted against the measured $\delta^{15}N$ values. Two samples (in red circles) were excluded as outliers.
225

226 **Agronomic system comparison**

227 The $\delta^{15}N$ values for all nematode samples ranged from 1.08 to 12.79, spanning >11.5 units.
228 When examined separately using a multivariate normality test, the conventional (W=0.901,

229 p=0.163) and organic (W=0.940, p=0.1484) treatment groups had normal distributions. Their
230 $\delta^{15}N$ values ranged from 1.08 mUr to 12.09 mUr in the conventional treatment (*n=12*) and from
231 1.99 mUr to 12.79 mUr in the organic treatment (*n=25*).
232 The sample size corrected standard ellipse area (SEAc) of the conventional treatment was 11.51
233 mUr^2, while for the organic treatment it was 10.98 mUr^2. Bayesian generated estimates exhibited
234 a large area overlap (Figures 2A and 2B) between the two treatment groups, suggesting no
235 significant difference between the size of the two SEA treatment areas (p=0.4928). The standard
236 ellipse area overlap from conventional to organic was 69.8% and the convex hull area overlap
237 was 85.3%. In addition, analysis of variance showed no significant difference in $\delta^{15}N$ (p=0.290)
238 or $\delta^{13}C$ (p=0.706) between the two treatments. Since there were no significant differences in any
239 isotopic statistics between the two agronomic treatments, all data were pooled for subsequent
240 feeding group analyses.

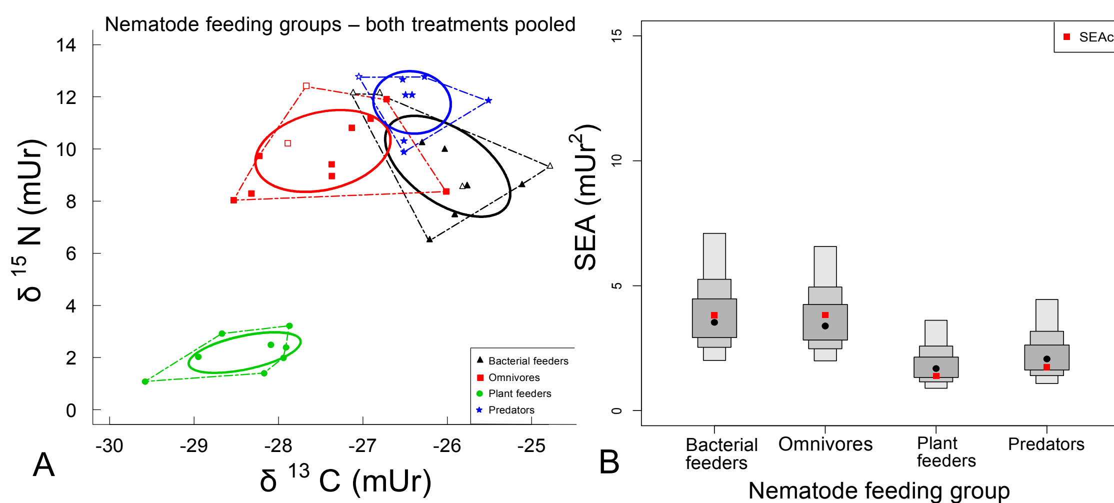


241
242 Figure 2A: All samples in the conventional agronomic treatment (black squares, *n=12 pooled samples*) and all
243 samples in the organic agronomic treatment (red circles, *n=25*). The solid lines represent the Bayesian generated;
244 Standard Ellipse area (SEAc – 40% of the data) and the broken line represent the Convex Hull with 100% of the
245 data. Figure 2B: SIAR density plot, with credible intervals (50% inside dark grey boxes, 75% middle grey boxes,
246 100% outer light grey boxes), for the Bayesian generated ellipses (SEA) (black dots) of the nematode isotope data
247 overlaid with sample size corrected uncertainty around the estimates (SEAc) (red dots).
248

249 Nematode feeding groups

250 When all samples were assigned into four groups by feeding type (Table 1), analysis of variance
251 showed highly significant differences in $\delta^{15}N$ (p < 0.0001) between the plant feeder and other
252 feeders and in $\delta^{13}C$ ($F_{3;33}$=24.18 p < 0.0001) between all groups. The four groups (bacterial
253 feeders (*n=10*), omnivores (*n=11*), plant feeder (*n=8*) and predators (*n=8*)) were assembled

254 from pooled individuals from the two treatments and also from one or two different
255 genera/families (Table 1) but with similar assumed feeding. These groups individually showed
256 mutivariate normal distributions.
257 Data are graphed on a biplot (δ^{13}C and δ^{15}N) in 'isotopic niche space' (Figure 3A). A significant
258 difference in N and C stable isotope ratios between the plant feeder (*Rotylenchus*) and all other
259 groups is apparent (Figure 3A and 3B). The plant feeder had δ^{15}N values between 1.08 and 3.22
260 mUr, while the predators were between 9.89 and 12.79 mUr, showing an average gap of 9.62
261 mUr in δ^{15}N. Average C isotope ratios were also more positive (by 1.99 mUr) for the predator
262 group (–27.04 to –25.51 mUr) compared to the plant feeder (–29.58 to –27.87 mUr). The
263 omnivorous group had δ^{13}C (–28.53 to –26.01 mUr) and δ^{15}N value ranges (8.05 to 12.42 mUr)
264 between that of the plant feeder and predators, but were elevated in δ^{15}N (a difference of 7.75
265 mUr) compared to the plant feeder. The bacterial feeding group had a δ^{15}N value range of 6.48 to
266 12.14 mUr and δ^{13}C range of –27.13 to –24.78 mUr.



267
268 Figure 3A: Biplot showing δ^{13}C and δ^{15}N of soil nematodes with Standard Ellipses (solid curved lines) and Convex
269 Hulls (dashed straight lines) for four feeding groups: Bacterial feeders (*Plectus* (solid black triangles) and *Rhabditis*
270 (open black triangles)) (*n=10 pooled samples*), Omnivores (Aporcelaimidae (solid red squares) and
271 Qudsianematidae (open red squares)) (*n=11 pooled samples*), Plant feeder (*Rotylenchus* (solid green circles)) (*n=8*)
272 and Predators (*Mononchus* (solid blue stars) and *Anatonchus* (open blue star)) (*n=8 pooled samples*). Figure 3B:
273 SIAR Density plots of Standard Ellipses areas (black dots) for the four groups with credible intervals (50% inside
274 dark grey boxes, 75% middle grey boxes, 100% outer light grey boxes), overlaid with sample size corrected SEAc
275 (red dots).
276
277 The sample size corrected Standard Ellipse Area (SEAc), representing 'trophic niche width', and
278 Convex Hull total area (TA) were largest for omnivores (respectively 3.83 and 6.9 mUr^2), while
279 the plant feeder had the smallest (1.37, 1.96 mUr^2) (Tables 2 & 3). Predator SEAc and TA were

280 also small (1.73, 2.33 mUr^2). The SEAc or TA of the plant feeder did not overlap with any of the
281 other groups. There was some TA overlap between the bacterial feeders and the omnivores (23-
282 28%) and between the bacterial feeders and predators (15-38%), but minimal overlap between
283 the omnivores and predators (5-15%) (see Table 3). There was no significant overlap in SEAc's
284 between bacterial feeders and omnivores (1%), however they were in the same $\delta^{15}N$ range
285 (representing trophic level) and there was a small SEAc overlap between bacterial feeders and
286 predators (<8-18%).

287 **Table 2.** SEA – Bayesian generated Standard Ellipse Areas (SEAc 40% of the data, in mUr^2), with area and
288 percentage overlaps. BF = Bacterial feeders and PF = Plant feeder. 1 and 2 in parentheses represent, respectively, the
289 first and second feeding group mentioned in the first column of the table.

Feeding group (1) & (2)	Area (1)	Area (2)	Area overlap	% overlap
PF & Predators	1.37	1.73	0	0
Omnivores & PF	3.83	1.37	0	0
BF & PF	3.81	1.37	0	0
Omnivores & Predators	3.83	1.73	0	0
BF & Omnivores	3.81	3.83	0.037	<1%
BF & Predators	3.81	1.73	0.31	8-18%

290

291 **Table 3.** Convex Hull (100% of the data, in mUr^2) with area and percentage overlaps. BF = Bacterial feeders and PF
292 = Plant feeder. 1 and 2 in parentheses represent, respectively, the first and second feeding group mentioned in the
293 first column of the table.

Feeding group (1) & (2)	Area (1)	Area (2)	Area overlap	% overlap
PF & Predators	1.96	2.33	0	0
Omnivores & PF	6.94	1.96	0	0
BF & PF	5.82	1.96	0	0
Omnivores & Predators	6.94	2.33	0.34	5-15%
BF & Omnivores	5.82	6.94	1.61	23-28%
BF & Predators	5.82	2.33	0.90	15-38%

294

295 Discussion

296 Sample sizes and measurement issues

297 The near-conventional µEA–IRMS technique allows the use of microgram samples, reducing the
298 time-consuming effort for enumerating nematode groups experienced by Moens et al. (2005) and
299 others. Nematodes from four feeding groups were included in this study. Fungal feeders were
300 omitted because of their small body size (hence practically unattainable numbers required to
301 reach target weight), low abundances and the difficulty in identifying live specimens at the

302 required taxonomic resolution. The numbers necessary to reach the sample weight for
303 conventional isotopic analysis are difficult to achieve, especially by the approach used here. For
304 example, because of this difficulty, Kudrin et al. (2015) used nematode sample weights as low as
305 11 μg despite using conventional IRMS for isotope analysis. Bayesian community metrics, more
306 conservative methods than convex hull area, were used for inference of trophic behaviour to
307 redress the limitations of small sample numbers.

308

309 **Nematode feeding groups**

310 Prior studies have used isotopic analysis to decode nematode contribution to soil food webs but
311 none has attempted to test members of the traditional soil nematode feeding groups composed by
312 Yeates et al. (1993). To this end, the present study somewhat parallels that of Kudrin et al.
313 (2015) on one forest soil in Russia, with the exception of the use of the μEA–IRMS method, the
314 inclusion of two arable treatments and the successful analysis of a plant-feeding group. Based on
315 dual C and N natural isotope abundance measurements of members of the soil nematode
316 community, results from Kudrin et al. (2015) and the present study conform to (independently of
317 each other) major aspects of the widely used feeding group concept. For the most part, there is
318 agreement between isotopic and traditional feeding groups emerging from both these studies,
319 largely agreeing with morphology-based categorisation to feeding groups. However, isotopic
320 compositions indicate that some members diverge from assumed feeding, which is further
321 discussed below. Many of the uncertainties discussed here may be caused by pooling of species
322 and higher taxa, and these uncertainties will be resolved in future studies that measure better
323 delineated genera or even species of soil nematodes. Life stage of individuals may also be taken
324 into account.

325 ***Plant feeders***: Soil food webs are characterised by two distinct resources, living plant roots and
326 detritus (De Ruiter et al., 1993), with the majority of soil groups consuming from the detrital
327 food web (Korobushkin et al., 2014). The δ^{15}N of non-plant feeders, namely, saprophagous
328 omnivores, bacterial feeders and fungal feeders, in soil food webs are elevated through the
329 assimilation of microbially-processed organic matter with a marked isotopic distance from plant
330 matter (Hendrix et al., 1999a). In addition, predators are distant from primary plant resources via
331 consumption of δ^{15}N-elevated prey. A resource distinction is clearly evident in the nematode
332 data between the assumed plant feeder and all other groups (Figure 3A).

333 Plant feeders ostensibly have the same or slightly enriched $\delta^{15}N$ values as their resources, and
334 depleted C and N isotope ratios compared with other soil fauna usually reflect feeding on plants
335 or fresh plant residues (Schmidt et al., 2004; Illig et al., 2005, Maraun et al., 2011), as displayed
336 by *Rotylenchus* in this study. Here, what is most apparent is a distinct dual trophic grouping,
337 encompassing predators, omnivores and bacterial feeders presumably feeding on detritivore
338 resources and another grouping with the plant feeder directly consuming plant roots. *Rotylenchus*
339 was depleted in both ^{15}N and ^{13}C compared to all other groups suggesting that categorization of
340 the group as plant feeding is correct.
341 The plant feeder had the smallest SEAc, reflecting a narrow niche width with a singular food
342 source, with their role as direct plant feeding. This may change seasonally due to changing plant
343 nutrient supply (Cesarz et al., 2013) or be affected by the management of the crop in an arable
344 system. As only one genus is represented here, it cannot be inferred that this will be the case for
345 all plant feeders.
346 ***Predators***: At the other extreme, the predatory group (mainly *Mononchus*) had the most elevated
347 $\delta^{15}N$ of the nematode groups, as is common for predators in soil food web studies where they are
348 at the top of the food web and are relatively ^{15}N enriched in relation to their diet (Scheu & Falca,
349 2000; Maraun et al., 2011). The isotopic $\delta^{15}N$ distance between predators and omnivores or
350 bacterivores does not clearly indicate a full step in trophic level between these three groups, but
351 the $\delta^{15}N$ spacing between the plant feeder and predators suggests an apparent difference of 3-4
352 trophic levels within the soil nematodes tested. This distance might indicate that predators have a
353 feeding preference for prey from higher trophic levels than plant feeders. As such, the predators
354 likely feed more on other predators, omnivores and bacterial feeders (and presumably fungal
355 feeders) and less so on plant feeders.
356 Predatory feeders displayed a small SEAc, suggesting that their diet is not general but specific to
357 feeding on small, higher trophic level soil animals, reflected by their elevated $\delta^{15}N$ values (9.89
358 to 12.79 mUr). This feeding presumably involves intraguild predation (Illig et al., 2005), by
359 contrast if the plant feeder ($\delta^{15}N$ 1.08 to 3.22 mUr) was being consumed, the values would have
360 been expected to be lower. On the other hand, predator $\delta^{15}N$ was expected to be markedly more
361 enriched than that of bacterial feeders. Consumption of plant feeders by predators could be one
362 explanation for this. Also, the more negative $\delta^{13}C$ of predators compared to bacterial feeders
363 could be explained by biochemical differences rather than feeding habits, for example predators

364 could have larger lipid reserves that are more negative in $\delta^{13}C$ compared to proteins and
365 carbohydrates (Schmidt et al., 2004). It must also be noted that here mainly one genus,
366 *Mononchus*, is represented. As both mature and immature specimens were used, life stage
367 feeding may be a factor affecting the isotopic composition of the group i.e. immature
368 Monochidae are thought to feed on bacteria (Yeates, 1987).
369 ***Omnivores***: Omnivores had a larger SEAc (isotopic niche width) suggesting a wider trophic
370 niche and thus assimilation of a variety of resources, adhering to their definition in nematology
371 as generalist feeders. This reflects the feeding by omnivores reviewed by McSorley (2012) and
372 assumed by Yeates et al. (1993) who described omnivores as feeding widely on fungal, deposit,
373 bacterial and predatory reserves from non-nematode and nematode sources. Using the biplot and
374 Convex hull (Table 3) overlaps between omnivores and bacterial feeders, there is a suggestion
375 that omnivores and bacterivores occupy the same trophic level (second highest). This is at odds
376 with Kudrin et al. (2015), where the omnivores and predators appear to share the highest trophic
377 level. This could be explained by different members representing the omnivore families from the
378 two studies or by different behaviour in different habitats.
379 The overall sequence of groups (bacterial feeders, omnivores and predators) on the $\delta^{13}C$ and
380 $\delta^{15}N$ bi-plot and therefore in 'trophic niche space', in this arable study corresponds somewhat
381 with that of the Kudrin et al. (2015) study, from a taiga spruce forest soil but is not the same. The
382 SEAc and TA overlaps of these three feeding groups might support the theory that 'true'
383 omnivory is more prevalent in other than just omnivores (Moens et al., 2006).
384 ***Bacterial feeders:*** Not all a priori groupings, based on morphology clearly fit to Yeates's (1993)
385 feeding categorisation. The SEAc of bacterial feeders was comparatively large and they had
386 isotopic values that were somewhat ambiguous with a small degree of 'trophic niche' overlap
387 with predators. The bacterial feeders were more ^{15}N and ^{13}C enriched than expected. Two genera
388 were represented in the group. Diverse feeding between the two genera may have influenced the
389 size of the SEAc as well as the overlap. Bacterivores ^{13}C enriched could reflect grazing on
390 bacteria that are colonizing older elevated ^{13}C food resources in soil (Schmidt et al., 2004) and
391 were ^{15}N enriched which could suggest some predatory behaviour like aquatic deposit feeding
392 nematodes in the study by Moens et al. (2005). Present samples were taken from post harvest
393 soils where there were fewer inputs from a growing crop, so older carbon may be accessed from
394 bacteria colonizing plant residues, applied manure and soil organic carbon with elevated ^{15}N as

395 shown by Scheunemann et al. (2010). Bacterivores could also acquire elevated $\delta^{15}N$ values by
396 feeding on bacteria fuelled by livestock manures that can be highly ^{15}N enriched due to gaseous
397 losses of isotopically light N during storage (Schmidt & Ostle, 1999). The bacterial
398 feeder/predator overlap could also be accounted for by direct microbial feeding by predators
399 (Wardle & Yeates, 1993).
400 The overlap with predators may also be due to a lower than expected N fractionation. More
401 information is becoming available on trophic distances between feeding groups in soil food webs,
402 as evinced by a recent stable isotope meta-analysis (Korobushkin et al., 2014)., However, the
403 'trophic distance' in soils is less clear than between trophic levels (i.e. 3.4 mUr for $\delta^{15}N$) in other
404 systems (Hendrix et al., 1999a), with soil food webs having more trophic levels than other food
405 webs (Digel et al., 2014). In addition, the underlying body-diet spacing of consumers are poorly
406 documented and can be affected by the type of trophic level, feeding guilds within feeding
407 groups, or by environmental or physiological factors (Schneider et al., 2004; Maraun et al., 2011).
408 For instance, a meta-analysis suggested that the ^{15}N enrichment can be higher in detritivores and
409 lower in herbivores relative to their food source, and that the type of N excretion of different taxa
410 can have an influence on trophic distance (Vanderklift & Ponsard, 2003). Moens et al. (2014),
411 however, observed spacings as high as ≥ 4 mUr between microalgae and nematode grazers in
412 soft sediments.
413
414 **Agronomic system comparison**
415 The hypothesis that the nematode feeding ecology reflected by isotopic data would show a
416 difference between conventional and organic agronomic treatments was not supported. Organic
417 systems have been shown to cause a shift in trophic responses compared with conventional
418 (Haubert et al., 2009; Sánchez-Moreno et al., 2009), for instance because external carbon inputs
419 such as manure strongly influence the energy pathway in soil food webs (Crotty et al., 2014). In
420 agricultural soils, management and resource availability have a large influence on the resulting
421 energy pathway (Zhao & Neher, 2014). The energy pathway (plant, bacterial or fungal based, see
422 Neher, (2010)) in a detrital consumer soil system can influence the number of trophic levels (Illig
423 et al., 2005). However, Neher (1999) found little difference in nematode maturity and trophic
424 diversity indices from organic to conventional cropped fields. Similarly, in the present study the
425 agronomic treatments did not vary significantly, which could reflect the time lag before

426 management changes have an effect on the soil system or the fact that baseline food resources in
427 the two systems were essentially the same.
428 **Applications for soil ecology**
429 The present work is in line with prior studies and upholds many long held assumptions of trophic
430 behaviour of members of certain nematode feeding groups. By using the µEA–IRMS technique,
431 it is now possible to confirm on a scale as fine as species level (for larger species at least) the
432 feeding behaviour of identifiable soil nematodes. This will further highlight nematode feeding
433 and their role in the complexity of the wider soil food web. Such is the power of isotopic
434 techniques for trophic inference, future studies may find terrestrial genera/species that clearly do
435 not fit the assumed morphological and ecological feeding previously assigned to them, as was
436 the case in aquatic studies (Moens et al., 2005; Estifanos et al., 2013; Vafeiadou et al., 2014).
437 Considering the close relationship between terrestrial and aquatic nematode feeding groups, the
438 present work also has relevance to the feeding ecology of aquatic nematodes.
439 One unique feature of the soil food web is the co-existence of many decomposer groups (Illig et
440 al., 2005). Year round active nematodes encompass many of the wide range of feeding types
441 found within the soil food web and as such are an excellent soil bioindicator group (Ferris et al.,
442 2001; Ferris et al., 2012; Ritz & Trudgill, 1999; Neher, 2010). Trophic information can help to
443 identify 'sentinel' nematode taxa that reflect aspects of soil ecosystem function on landscape
444 monitoring scales (Neher, 2010). Isotope techniques can be used to look at temporal changes in
445 nematode feeding in response to different ecological contexts or management, such as pollution
446 monitoring and habitat restoration (Neher, 2010) or climate change (Sticht et al., 2009).
447 The validity of morphology (mouthparts) linking form to function (Ritz & Trudgill, 1999) is
448 confirmed here by isotopic analysis on certain nematodes. Although many taxa have yet to be
449 tested, feeding group members were isotopically confirmed by Kudrin et al. (2015) as well as the
450 present study, further substantiating the effectiveness of nematode indices based on feeding
451 strategies. The small sample sizes needed for trophic analysis and demonstrated here could
452 complement functional food web detail at a genus/species level that is usually lacking from
453 guild-based indices systems.
454 Species level isotopic investigations of soil nematodes can resolve many of the uncertainties
455 discussed here caused by pooling of species or higher taxa. For quantitative studies, the same
456 analytical approach used here could be combined with isotopic labelling of plants or other food

457 sources (e.g. Crotty et al., 2014, Schmidt et al., 2016, Shaw et al., 2016). Such studies can
458 estimate the flow of C and N from resources (e.g. bacteria, algae, plant roots) to nematode taxa,
459 but at a finer taxonomic resolution. This would offer a better understanding of the feeding
460 ecology of nematodes and their trophic interactions in soil food webs.
461

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465

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