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# Stable isotope analysis ( $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ) 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 ( $^{13}\text{C}/^{12}\text{C}$  and  $^{15}\text{N}/^{14}\text{N}$ , expressed as  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) 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  $>100\ \mu\text{g}$  C and N). Here, micro-sample near-conventional elemental analysis - isotopic ratio mass spectrometry ( $\mu\text{EA-IRMS}$ ) of C and N using microgram samples (typically  $20\ \mu\text{g}$  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  $20\ \mu\text{g}$  dry weight target. There was no significant difference in  $\delta^{13}\text{C}$  ( $p=0.706$ ) 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* ( $\delta^{15}\text{N}=1.08$  to  $3.22$  mUr,  $\delta^{13}\text{C}=-29.58$  to  $-27.87$  mUr) and all other groups. There was an average difference of  $9.62$  mUr in  $\delta^{15}\text{N}$  between the plant feeder and the predator group ( $\delta^{15}\text{N}= 9.89$  to  $12.79$  mUr,  $\delta^{13}\text{C}=-27.04$  to  $-25.51$  mUr). Isotopic niche widths were calculated as Bayesian derived standard ellipse areas and were smallest for the plant feeder ( $1.37\ \text{mUr}^2$ ) and the predators ( $1.73\ \text{mUr}^2$ ), but largest for omnivores ( $3.83\ \text{mUr}^2$ ). 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.

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

3

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15

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 ( $^{13}\text{C}/^{12}\text{C}$  and  $^{15}\text{N}/^{14}\text{N}$ , expressed as  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) has provided  
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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  $>100\ \mu\text{g}$  C and N). Here, micro-sample  
24 near-conventional elemental analysis – isotopic ratio mass spectrometry ( $\mu\text{EA-IRMS}$ ) of C and  
25 N using microgram samples (typically  $20\ \mu\text{g}$  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  $20\ \mu\text{g}$  dry weight target. There was no significant  
31 difference in  $\delta^{13}\text{C}$  ( $p=0.706$ ) 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* ( $\delta^{15}\text{N}=1.08$  to  $3.22$  mUr,  $\delta^{13}\text{C}=-29.58$  to  $-27.87$  mUr) and all other groups.  
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36 calculated as Bayesian derived standard ellipse areas and were smallest for the plant feeder ( $1.37$   
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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  $^{15}\text{N}/^{14}\text{N}$  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  $\delta^{15}\text{N}$  gap of 3.4 mUr (‰) between trophic levels  
97 (Ponsard & Ardit, 2000). For soil nematodes, plant-parasitic Longidoridae, were first analysed  
98 isotopically at species level by Neilson & Brown (1999), and showed varied  $\delta^{15}\text{N}$  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  $^{13}\text{C}$  of soil nematode feeding groups was altered by  
105 experimentally raised  $\text{CO}_2$  with depleted  $\delta^{13}\text{C}$  ( $\approx -47$  mUr), under different crops, in a study by  
106 Sticht et al. (2009). In combination with  $^{15}\text{N}$  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  $^{13}\text{C}$  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  $^{13}\text{C}$  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 ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) 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  $\mu\text{EA-IRMS}$  method that analyses microgram samples (as little as 0.6  $\mu\text{g}$  for  
122  $^{15}\text{N}$  and 1  $\mu\text{g}$  for  $^{13}\text{C}$ ). This method has already been used to investigate resource shifts ( $^{13}\text{C}$   
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  $\mu$ EA–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  $^{13}\text{C}$  and  $^{15}\text{N}$  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.  $55^{\circ} 51'$   
144 N, long.  $3^{\circ} 12' \text{ W}$ ). 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  $10 \text{ t ha}^{-1}$  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  $4^{\circ}\text{C}$  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  $4^{\circ}\text{C}$  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 ( $^{13}\text{C}/^{12}\text{C}$  and  $^{15}\text{N}/^{14}\text{N}$ ) 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 ( $\delta^{13}\text{C}$   
176 = -29.6 mUr,  $\delta^{15}\text{N}$  = -1.6 mUr) and wild boar liver ( $\delta^{13}\text{C}$  = -17.3 mUr,  $\delta^{15}\text{N}$  = 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  $\text{N}_2$ , 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  $\delta^{13}\text{C}/\delta^{15}\text{N}$  biplot and calculated as the Bayesian standard ellipse areas (SEA with units of  $\text{mUr}^2$ ).  
 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  $\mu\text{g}$  target dry weight.

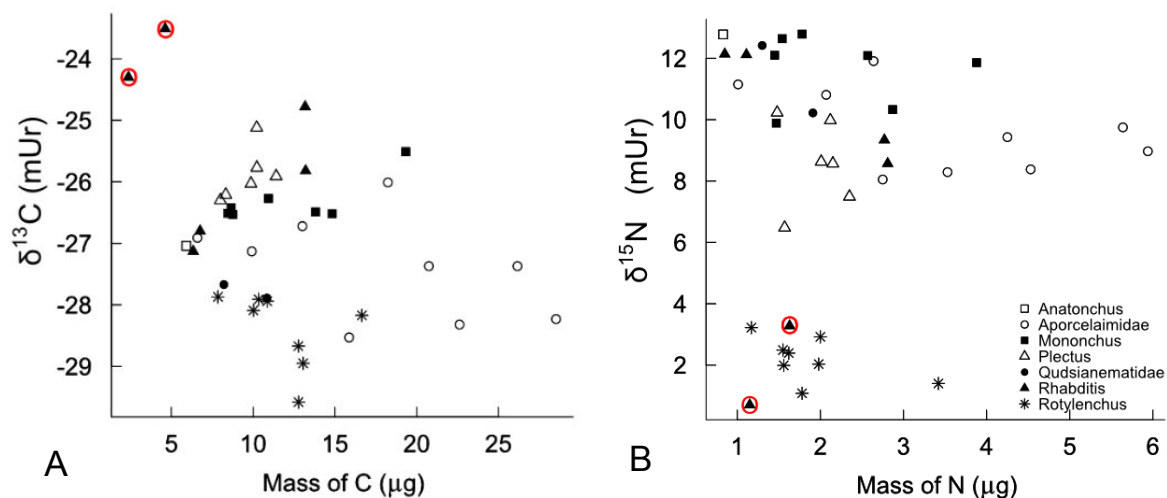
211 **Table 1.** The mean number of nematodes ( $\pm$  SD) used to achieve the target weight per sample for the groups listed,  
 212 number of measured replicate samples (in brackets), and total number of measured replicate samples in each feeding  
 213 group (in final column) from conventional and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

Soil nematode taxa			Conventional	Organic	Total
Feeding group			Mean no. of nematodes per sample $\pm$ SD (n=measured samples)		Number of measured samples
ORDER	Family	Genus			
<b>Predators</b>					
MONOCHIDA	Anatonchidae	<i>Anatonchus</i>	-		3 (n=1)

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

214

215 For an initial quality control and check of linearity, all  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  (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  $\delta^{13}\text{C}$  values ( $r_s = -$   
 219 0.143,  $p=0.397$ ), or N mass and  $\delta^{15}\text{N}$  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  $\delta^{13}\text{C}$  values. Figure 1B: Sample mass of  
 224 N for all samples plotted against the measured  $\delta^{15}\text{N}$  values. Two samples (in red circles) were excluded as outliers.  
 225

226

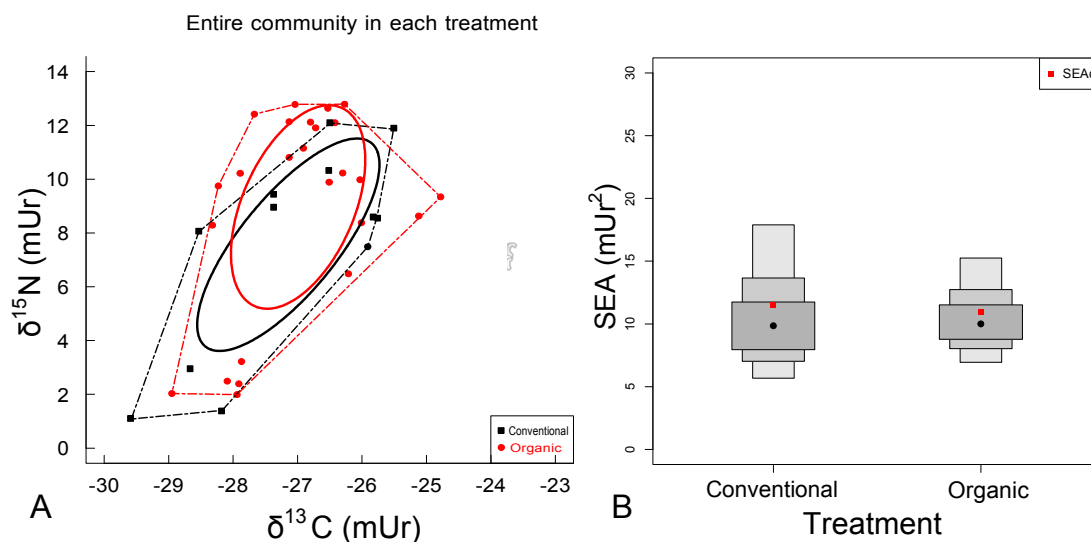
### 226 Agronomic system comparison

227 The  $\delta^{15}\text{N}$  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  $\delta^{15}\text{N}$  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  $\text{mUr}^2$ , while for the organic treatment it was 10.98  $\text{mUr}^2$ . 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  $\delta^{15}\text{N}$  ( $p=0.290$ )  
 238 or  $\delta^{13}\text{C}$  ( $p=0.706$ ) 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.

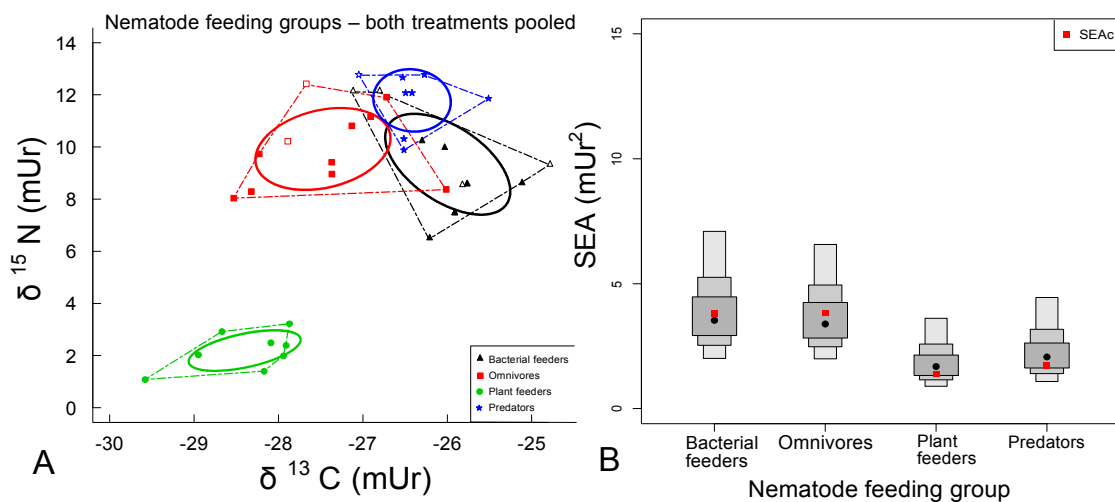


241 Figure 2A: All samples in the conventional agronomic treatment (black squares,  $n=12$  pooled samples) and all  
 242 samples in the organic agronomic treatment (red circles,  $n=25$ ). The solid lines represent the Bayesian generated;  
 243 Standard Ellipse area (SEAc – 40% of the data) and the broken line represent the Convex Hull with 100% of the  
 244 data. Figure 2B: SIAR density plot, with credible intervals (50% inside dark grey boxes, 75% middle grey boxes,  
 245 100% outer light grey boxes), for the Bayesian generated ellipses (SEA) (black dots) of the nematode isotope data  
 246 overlaid with sample size corrected uncertainty around the estimates (SEAc) (red dots).  
 247  
 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  $\delta^{15}\text{N}$  ( $p < 0.0001$ ) between the plant feeder and other  
 252 feeders and in  $\delta^{13}\text{C}$  ( $F_{3,33}=24.18$   $p < 0.0001$ ) 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 multivariate normal distributions.  
 257 Data are graphed on a biplot ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) 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  $\delta^{15}\text{N}$  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  $\delta^{15}\text{N}$ . 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  $\delta^{13}\text{C}$  ( $-28.53$  to  $-26.01$  mUr) and  $\delta^{15}\text{N}$  value ranges (8.05 to 12.42 mUr)  
 264 between that of the plant feeder and predators, but were elevated in  $\delta^{15}\text{N}$  (a difference of 7.75  
 265 mUr) compared to the plant feeder. The bacterial feeding group had a  $\delta^{15}\text{N}$  value range of 6.48 to  
 266 12.14 mUr and  $\delta^{13}\text{C}$  range of  $-27.13$  to  $-24.78$  mUr.



267  
 268 Figure 3A: Biplot showing  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  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<sup>2</sup>), while  
 279 the plant feeder had the smallest (1.37, 1.96 mUr<sup>2</sup>) (Tables 2 & 3). Predator SEAc and TA were

280 also small (1.73, 2.33 mUr<sup>2</sup>). 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  $\delta^{15}\text{N}$  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<sup>2</sup>), 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<sup>2</sup>) 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  $\mu\text{EA-IRMS}$  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  $\mu\text{g}$  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  $\mu\text{EA-IRMS}$  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  $\delta^{15}\text{N}$  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  $\delta^{15}\text{N}$ -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  $\delta^{15}\text{N}$  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  $^{15}\text{N}$  and  $^{13}\text{C}$  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  $\delta^{15}\text{N}$  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  $^{15}\text{N}$  enriched in relation to their diet (Scheu & Falca,  
349 2000; Maraun et al., 2011). The isotopic  $\delta^{15}\text{N}$  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  $\delta^{15}\text{N}$  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  $\delta^{15}\text{N}$  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 ( $\delta^{15}\text{N}$  1.08 to 3.22 mUr) was being consumed, the values would have  
360 been expected to be lower. On the other hand, predator  $\delta^{15}\text{N}$  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  $\delta^{13}\text{C}$  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  $\delta^{13}\text{C}$  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  $\delta^{13}\text{C}$  and  
380  $\delta^{15}\text{N}$  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  $^{15}\text{N}$  and  $^{13}\text{C}$  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  $^{13}\text{C}$  enriched could reflect grazing on  
390 bacteria that are colonizing older elevated  $^{13}\text{C}$  food resources in soil (Schmidt et al., 2004) and  
391 were  $^{15}\text{N}$  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  $^{15}\text{N}$  as

395 shown by Scheunemann et al. (2010). Bacterivores could also acquire elevated  $\delta^{15}\text{N}$  values by  
396 feeding on bacteria fuelled by livestock manures that can be highly  $^{15}\text{N}$  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  $\delta^{15}\text{N}$ ) 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  $^{15}\text{N}$  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  $\geq 4$  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  $\mu\text{EA-IRMS}$  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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