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1 **Molecular field analysis of trophic relationships in soil-dwelling invertebrates to**  
2 **identify mercury, lead and cadmium transmission through forest ecosystems**

3

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11

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13 analyses, prey choice, slugs

14

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18

19 Running title:

20 Molecular diet analyses and metal transfer

21

22 **Abstract**

23 Contamination pathways in complex food chains in soil ecosystems can be difficult to  
24 elucidate. Molecular analysis of predator gut content can, however, rapidly reveal previously  
25 unidentified trophic interactions between invertebrates and thereby uncover pathways of  
26 pollutant spread. Here we measured concentrations of the toxic metals lead, cadmium and  
27 mercury in carabid beetle predators and their prey. Invertebrates were sampled at one control  
28 and four heavy metal polluted sites in order to reveal the impact of diet composition and  
29 seasonal variation in prey availability on metal burden in carabids and metal transfer  
30 pathways through forest ecosystems. This is the first report, to our knowledge, of carabid  
31 diet composition based on PCR analysis of gut contents at the forest community level, rather  
32 than in cultivated fields. Extensive screening using group- and species-specific primers  
33 revealed that carabids ate primarily earthworms and slugs, as well as smaller numbers of  
34 woodlice and springtails. Metal concentrations in carabids correlated with seasonal changes  
35 in diet. Mercury accumulated in beetle predators more than in their slug prey. Since  
36 earthworms, slugs and carabid beetles are the major prey of many birds and mammals, prey-  
37 predator transfer and associated toxicity are major risks at mercury-contaminated sites.  
38 Carabids may be useful bioindicators for assessing the impact of pollutants on soil  
39 ecosystems, as long as species and seasonal factors are taken into account.

40

41 **Introduction**

42 Lead, cadmium and mercury are highly toxic metals without any biological function. While  
43 they do occur naturally in the soil at low levels, more than 200 years of industrialisation have  
44 led to elevated levels in the soil, degrading its quality and threatening soil biota (European  
45 Environment Agency & Joint Research Centre 2010). Eco-toxicity of metals correlates with  
46 their bioavailability and accumulation rate in soil biota. Bioavailability of metals depends, in  
47 turn, on the physical and chemical properties of the soil, and bioaccumulation depends on  
48 factors such as the metal tolerance of organisms, species-specific physiology and exposure  
49 history (e.g. van Straalen & van Wensem 1986; Hopkin 1989).

50

51 The main biological factors controlling metal accumulation in terrestrial invertebrates are diet  
52 and the structure and physiology of the digestive system (Hopkin 1989). After ingesting toxic  
53 metals, some organisms may avoid harmful effects by sequestering them in certain tissues  
54 and immobilising them in a form that cannot interfere with vital biological processes (Hopkin  
55 1989). When organisms assimilate large amounts of heavy metals and do not excrete them,  
56 biomagnification up the food chain can occur. By the process of biomagnification, even low  
57 concentrations of some toxins (e.g. DDT, PCBs, organic mercury) can end up in living  
58 organisms at higher concentrations and can be disseminated to distant places through  
59 migration. Biomagnification of some toxic compounds occurs in both aquatic and terrestrial  
60 ecosystems affecting many organisms, such as raptors and otters (e.g. Castro *et al.* 2011;  
61 Mayack 2012), and can be harmful to human health. After mercury discharge in Japan in  
62 1956, mercury contamination of fish and shellfish severely affected humans and animal  
63 populations for decades (e.g. Harada 1995). Although biomagnification of metals is less  
64 common in terrestrial food chains (reviewed in Janssen *et al.* 1993), it does occur in some

65 invertebrate species such as predatory lycosid spiders in the case of cadmium (Hendrickx *et*  
66 *al.* 2003), as well as along some species-specific trophic chains (van Straalen & Ernst 1991).

67  
68 The large diversity of soil invertebrates makes it difficult to identify general patterns in metal  
69 distribution in the soil and in consequence bioaccumulation. Some organisms, such as  
70 earthworms, slugs and isopods accumulate metals in their tissue (e.g. Hopkin 1989; Dallinger  
71 1993), while some hexapods efficiently excrete them (Hopkin 1989; Janssen *et al.* 1991;  
72 Lindquist *et al.* 1995). Taxonomic grouping by itself is not a sufficient basis for predicting  
73 accumulation rates in the environment. Furthermore, different species at the same trophic  
74 level have been found to accumulate different lead and cadmium concentrations (Georgii  
75 1986; van Straalen & van Wensem 1986).

76  
77 Seasonal variations are another complicating factor. The slug *Arion ater*, after feeding in the  
78 laboratory, takes up more metal in July than in September (Ireland 1981). The carabid  
79 *Calathus melanocephalus* takes up more cadmium in autumn than during other times of year  
80 (Janssen *et al.* 1991). Some carabid species active in autumn accumulate more cadmium than  
81 do species active in late spring (Šerić Jelaska *et al.* 2007). In addition to these apparently  
82 species-specific differences, seasonal changes in abiotic factors like temperature, day length  
83 and moisture, as well as seasonal changes in species composition, may cause variations in  
84 metal concentration in soil ecosystems during the year (Ireland 1981; Hopkin 1989; Šerić  
85 Jelaska *et al.* 2007).

86  
87 In addition, to reveal heavy metal distribution and bioaccumulation in soil ecosystems, the  
88 pathways by which metals move from the soil into the biota need to be identified. Soil  
89 ecosystems feature complex food webs and highly biodiverse communities comprising  
90 different developmental stages from eggs and juveniles to adults, as well as numerous

91 microscopic and even cryptic species. These factors make it difficult to identify the many  
92 different trophic interactions involved and hence metal transfer pathways.

93

94 Carabids beetles have been used extensively for studying trophic interactions (Symondson *et al.*  
95 *al.* 1996; Holland 2002; Symondson *et al.* 2000, 2006; Eskelson *et al.* 2011; Hatteland *et al.*  
96 2011; Boreau de Roince *et al.* 2012; Traugott *et al.* 2012; Davey *et al.* 2013) and the  
97 dynamics of heavy metal bioaccumulation and decontamination (Kramarz 1999; Stone *et al.*  
98 2002; Butovsky 2011). They thus provide ecologically highly-connected indicator taxa for  
99 assessing metal contamination in many soil environments. Assessments based on single  
100 predator species may not capture effects on other species in the community, since even  
101 species within the same taxonomic group can show different behavioural, physiological and  
102 morphological responses to environmental stresses.

103

104 As hard-bodied chitinous insects, carabid adults are exposed to metal contamination mostly  
105 through food ingestion. Previous studies based on molecular and microscopic gut content  
106 analysis, as well as field observations, show that predatory carabids feed on a wide range of  
107 other soil invertebrates including earthworms, molluscs, woodlice, springtails and many  
108 insect taxa (e.g. Sunderland 1975; Hengeveld 1980; Sunderland & Sutton 1980; Symondson  
109 *et al.* 1996, 2000; Harper *et al.* 2005; King *et al.* 2010). Thus, their exposure to heavy metals  
110 may depend more on the concentration of the metal in prey tissue than its bioavailable  
111 concentration in the soil. Carabids, earthworms and slugs are in turn important food sources  
112 for birds and mammals.

113

114 Quantifying predation by carabids, through direct observation or microscopic gut screening,  
115 is difficult because they are mainly nocturnal and many of their prey are soft-bodied

116 organisms. As a result, knowledge of their food preferences is limited. Precise information on  
117 trophic interactions between beetles and other parts of the ecosystem is critical for  
118 understanding how different pollutants move from the soil into the biota. Recent advances in  
119 molecular analysis of gut contents allows more precise, *in situ* predation analyses that can  
120 reveal trophic interactions in greater detail (Harper *et al.* 2005; King *et al.* 2010).

121

122 Group- and species-specific PCR primers were used to detect prey choice by carabids among  
123 four dominant prey groups (earthworms, slugs, woodlice and springtails). 25 carabid species  
124 screened were collected in the field in late spring-early summer and in autumn. We quantified  
125 carabid predation in both seasons and we analysed lead, cadmium and mercury  
126 concentrations in carabids and in their main prey (earthworms and slugs) sampled from the  
127 same sites. We tested the hypothesis that prey choice, and lead, cadmium and mercury  
128 concentrations in prey, would explain the heavy metal concentrations in the carabids,  
129 providing a trophic pathway for transport of contaminants from soil into wildlife. We also  
130 assessed the extent to which each carabid species, and their community assemblage as a  
131 whole, might prove useful as indicators for metal pollution assessment.

132 **Materials and methods**

133 1. Field collection

134 Soil and animals were collected from four heavy metal polluted locations (L1-L4) and one  
135 control location (L5) in Croatia and the UK: Rudnik Zrinski – Medvednica, Croatia (L1);  
136 Tusti vrh – Medvednica, Croatia (L2); Llantrisant, Wales, UK (L3); Rudry 1, Wales, UK  
137 (L4); and Rudry 2, Wales, UK (L5). All sites were located in deciduous woodlands, and L1 -  
138 L4 were located near old coal mines and smelters, and consequently had high concentrations  
139 of mercury, lead and cadmium in the soil (Morgan & Morgan 1998; Nahmani *et al.* 2007;  
140 Šerić Jelaska *et al.* 2007). Site L5 was not located near pollution sources and therefore served  
141 as a control site. All sites had acid soil with average pH values ranging from 3.75 at site L1 to  
142 5.85 at site L3 (Table S3).

143

144 Soil and invertebrate samples were collected from the end of May to the end of July and from  
145 mid-September to the end of October in 2007 in Croatia, and from mid-June to the end of  
146 July and in October in 2010 in Wales, UK.

147

148 For metal analyses, 8-16 top-soil samples (3L) per site were taken twice, in late spring and in  
149 autumn. Samples were taken randomly from the top 10 cm across the area at each site.

150 Earthworms, slugs and carabids were collected from the same layer of soil at the same sites  
151 by digging and hand sorting, during three visits per season. Adult carabids were also  
152 collected by pitfall trapping to get substantial number of individuals for PCR analysis of their  
153 gut contents and heavy metal analyses. Five empty traps (0.5 L plastic cups) were left at each  
154 site for two weeks during each season. Traps were emptied every morning and beetles used  
155 for PCR analysis of gut content were placed individually in plastic tubes, transported to the  
156 laboratory in a cooler, killed immediately at -80 °C and stored at that temperature until



157 extracted for molecular analysis. Animals used in metal analyses were left in separate Petri  
158 dishes containing moist filter paper to empty their gut for 48 h and the filter paper was  
159 changed daily. They were killed at -80 °C and stored in the freezer before metal analysis.  
160 Woodlice found by digging and trapped in pitfalls were not stored but their densities were  
161 recorded.

162

163 All carabid beetles, earthworms and adult slugs were identified by morphology to species  
164 level using Cameron *et al.* (1983), Sims & Gerard (1985), Mršić (1997), Freude *et al.* (2004)  
165 and Luff (2007).

166

## 167 2. Molecular analyses

### 168 *DNA extractions*

169 DNA was extracted from the beetle foregut for diet analyses. DNA from earthworm and slug  
170 species was also extracted to serve as positive controls during PCR.

171

172 All primers, including newly designed ones for *Limax cinereoniger*, were tested for cross-  
173 amplification against DNA extracted from 35 soil invertebrate species representing potential  
174 non-target prey in the field, including predator DNA (Table S1). The non-target organisms  
175 were tested individually and no cross-amplifications were found.

176

177 Beetles were thawed to room temperature, the foreguts were removed as described in  
178 Symondson *et al.* (2000), and DNA was extracted using the DNeasy Blood & Tissue Kit  
179 (Qiagen), following the manufacturer's instructions. Negative controls with no added animal  
180 tissue were included in each batch of samples to check for potential DNA carry-over  
181 contamination during extraction.

182

183 To check for the presence of DNA after extraction and to avoid false negatives, extractions  
184 were tested by PCR using general invertebrate primers for a 710-bp fragment of the  
185 mitochondrial cytochrome oxidase I (COI) gene (Folmer *et al.* 1994). Each PCR (10  $\mu$ L)  
186 contained the following: 1  $\mu$ L of template DNA, 0.625 U *Taq* polymerase (Invitrogen), 10  
187  $\mu$ M of each primer, 0.8  $\mu$ L 2.5 mM dNTPs, 10x PCR buffer, 50 mM MgCl<sub>2</sub> (Invitrogen), 10  
188  $\mu$ g bovine serum albumin (New England Biolabs) and dH<sub>2</sub>O (Sigma Aldrich). PCRs were  
189 carried out in a GeneAmp 9700 thermal cycler (Applied Biosystems) using the following  
190 conditions: 94 °C for 3 min; 45 cycles of 94 °C for 30 sec, 47 °C for 1 min, 72 °C for 1 min;  
191 and finally 72 °C for 10 min.

192

### 193 *Selection of primers to identify prey in predator gut*

194 The primers were used to screen the gut contents of 317 field-caught carabid beetles for  
195 earthworms, slugs, woodlice and springtails (Table 1).

196

197 Predation on earthworms was detected using general earthworm primers (Harper *et al.* 2005).  
198 To screen for slugs, we used general *Arion* primers (Dodd 2004; Harper *et al.* 2005) to  
199 amplify DNA of four *Arion* species (*A. hortensis*, *A. distinctus*, *A. silvaticus*, *A. subfuscus*)  
200 and species-specific primers for *Deroceras reticulatum* (Dodd 2004; Harper *et al.* 2005). All  
201 of these species were abundant at the study sites. We also designed species-specific primers  
202 to amplify DNA of the slug *Limax cinereoniger* (Limacidae) (see below), which were  
203 abundant at the two study sites in Croatia. The slugs *A. flagellus*, *A. ater*, *Malacolimax*  
204 *tenellus*, *Testacella maugei* and *T. scutulum* were found at low abundance at the study sites,  
205 and DNA of *A. flagellus* and *A. ater* was not amplified after testing with the general *Arion*  
206 primers. No other adult slug species were recorded in the field surveys, except for *Tandonia*

207 *budapestensis* present at site L3. Since this species is highly toxic for carabid beetles  
208 (Symondson 1997) we assumed that it was not consumed by the beetles.

209

#### 210 *Primer design for Limax cinereoniger*

211 *Limax cinereoniger* COI sequences (accession number FJ606460) were aligned together with  
212 other sequences of slug species from the GenBank (*Limax maximus*, FJ606471; *L. sarnensis*,  
213 FJ606493; *L. wohlberedti*, FJ606481; *Deroceras reticulatum*, FJ481179; *Arion subfuscus*,  
214 AM259721; *A. hortensis*, AM259726) using Clustal W (Thompson *et al.* 1994) as  
215 implemented in BioEdit (Hall 1999). Species specific primers were then designed for *L.*  
216 *cinereoniger*, COI-Lcin-F1 and COI-Lcin-R1 (Table 1), that amplified a 198 bp DNA  
217 fragment. After evaluating their compatibility with NetPrimer (Premier Biosoft), annealing  
218 temperature was optimised in PCR.

219

220 DNA from field-collected *L. cinereoniger* and negative control samples were amplified using  
221 the species-specific primers in a 10 µL reaction containing ca. 200 ng of total genomic DNA,  
222 0.625 U *Taq* polymerase (Invitrogen), 10 µM of each primer, 0.8 µL 2.5 mM dNTPs, 10x  
223 PCR buffer, 50 mM MgCl<sub>2</sub> (Invitrogen), 10 µg bovine serum albumin (New England  
224 Biolabs) diluted up to volume with sterile water (Sigma Aldrich). Thermal cycling conditions  
225 were as follows: 95 °C for 3 min; 35 cycles of 94 °C for 30 s, 61 °C for 45 s, 72 °C for 45 s;  
226 and finally 72 °C for 7 min. This novel primer pair proved to be species-specific under the  
227 optimised PCR cycling conditions with no evidence of cross-amplification, even when tested  
228 with related slug species such as *Limax maximus*.

229

#### 230 *Screening of field-caught predators*

231 Singleplex and multiplex PCRs were used to screen the gut contents of each field-caught  
232 beetle for the presence of earthworm and slug species (Table 1).  
233  
234 Singleplex PCR reactions (10  $\mu$ L) contained 1  $\mu$ L of template DNA, 0.625 U *Taq* polymerase  
235 (Invitrogen), 10  $\mu$ M of each primer, 0.8  $\mu$ L 2.5 mM dNTPs, 10x PCR buffer, 50 mM MgCl<sub>2</sub>  
236 (Invitrogen), 10  $\mu$ g bovine serum albumin (New England Biolabs) and sterile water (Sigma  
237 Aldrich). PCRs were carried out in an AB Veriti 96-well thermocycler (Applied Biosystems).  
238 Cycling conditions for earthworm DNA samples were as follows: 94 °C for 3 min; 35 cycles  
239 of 94 °C for 30 s, 65 °C for 45 s, 72 °C for 45 s and finally 72 °C for 10 min. Cycling  
240 conditions for *L. cinereoniger* DNA samples were as follows: 95 °C for 3 min; 35 cycles of  
241 94 °C for 30 s, 61 °C for 45 s, 72 °C for 45 s; and finally 72 °C for 7 min. Cycling conditions  
242 for amplifying woodlice and springtail DNA were as described in Jarman *et al.* (2006) and  
243 Kuusk & Agustí (2008).  
244  
245 Multiplex PCR reaction (10  $\mu$ L), for amplifying *D. reticulatum* and *Arion sp.* DNA,  
246 contained 1.2  $\mu$ L of extracted DNA, 5  $\mu$ L of Multiplex PCR Master Mix (Qiagen), 0.2  $\mu$ M  
247 each primer, 10  $\mu$ g bovine serum albumin (New England Biolabs), and sterile distilled water  
248 (Qiagen). After initial denaturing step at 95 °C for 15 min, amplification proceeded for 35  
249 cycles at 94 °C for 30s, 53 °C for 1 min 30 s, 72 °C for 1 min 30 s and a final extension at 72  
250 °C for 10 min.  
251  
252 All PCRs included a positive control (target prey) and a negative control (sterile water  
253 instead of DNA). PCR products were separated on a 2% agarose gel for 40 min at 120 V and  
254 visualised with ethidium bromide (0.075  $\mu$ g/mL).  
255

256 All samples were screened for target prey twice using the same PCR conditions and those  
257 that came up positive only once were also accepted as positive and included in further  
258 analyses.

259

### 260 3. Metal analyses

261 Metal analyses were carried out on 206 beetles, 135 earthworms, 96 slugs and 111 soil  
262 samples using the ICP-MS technique. The procedure is described in Supporting Information  
263 (Metal analyses). Accumulation factors (AFs) for each metal were calculated by dividing  
264 mean metal concentrations in predator tissue per season per plot by the mean metal  
265 concentrations in prey.

266

### 267 4. Statistical analyses

268 Statistical analyses and figures were prepared using Statistica 9.1 (Statsoft, Inc. 2010), R  
269 (version 2.11.1, R Development Core Team, 2011) and Gephi 0.8.2 beta [Common  
270 Development and Distribution License (CDDL) & GNU General Public License 2008-2012].  
271 Details of statistical analyses used in the paper are provided in Supporting Information  
272 (Statistical analyses).

273

## 274 **Results**

275 317 carabids were submitted for diet analyses and 206 for metal analyses. A total of 135  
276 earthworms and 96 slugs were analysed for metal concentrations in tissue to investigate  
277 whether they can serve as metal transfer vectors through the food web.

278

279 Among the samples collected there were 25 carabid species, with *Nebria brevicollis*, *Abax*  
280 *parallelepipedus* and *Abax parallelus* accounting for 70% of captured animals (Table S2). *N.*  
281 *brevicollis* and *A. parallelepipedus* account for 77.3% of the carabid population sampled in  
282 the UK, while *A. parallelepipedus* and *A. parallelus* account for 61.4% of the total population  
283 in Croatian samples.

284

#### 285 *Prey consumption by carabids in the field*

286 Carabids were shown to be consuming all four prey groups (earthworms, slugs, woodlice and  
287 springtails). Of 317 tested carabids, 213 (67.2%) contained at least one prey from these  
288 groups in their foregut. The foreguts of most beetles (138) contained only one prey group, 66  
289 contained two, while eight contained three and one was positive for all four. Earthworms  
290 were detected most frequently, with 148 (46.7%) testing positive, while 98 beetles (30.9%)  
291 were positive for slugs, of which 52 beetles were positive for both earthworms and slugs.  
292 Woodlice and springtails were consumed by 35 (11.0%) and 17 (5.36%) beetles respectively.  
293 Prey consumption rates in the UK and Croatia were very similar with only woodlice showing  
294 a significant difference (chi-squared = 7.76, df = 1,  $P = 0.005$ ), being consumed at higher  
295 rates in the UK.

296

#### 297 *Seasonal differences in prey consumption*

298 Percentage of carabids testing positive for slugs or woodlice at the same study site differed  
299 between the two seasons (Fig. 1). Within sites L1 and L2, more slugs were preyed on in  
300 autumn and within sites L4 and L5 more woodlice were preyed on in spring.

301

302 There was little difference in predation on *Arion* slugs between seasons at all study sites. 28  
303 individual beetles in the overall sample were positive for *Arion* sp. in spring and 26 in

304 autumn. However, carabid foregut analysis showed higher predation of *L. cinereoniger* and  
305 *D. reticulatum* in autumn than in spring. 80% and 60% of carabid beetles at L1 (Cro) and L2  
306 (Cro) sites tested positive for *L. cinereoniger* in autumn compared to no positives and 6% of  
307 positives in spring, respectively. 7% and 10% of carabids tested positive for *D. reticulatum* in  
308 autumn at sites L4 (UK) and L5 (UK) respectively, with no positives in the spring-summer  
309 season.

310

311 Of the 25 carabid species recorded on the five study sites, eight were observed during both  
312 seasons. Foregut analysis of these eight species revealed significant seasonal differences in  
313 how much they preyed on slugs (T-test,  $t=3.3$ ,  $df=7$ ,  $P=0.013$ ). The overall proportions of  
314 carabid species tested for the DNA of the four prey groups are listed in Table S2.

315

316 Proportions of earthworms, slugs and woodlice in the environment and in predators testing  
317 positive for these prey (Fig. 2) showed that isopods were taken in clearly lower proportions  
318 than would be expected if the carabids were predated randomly. All other prey were taken in  
319 approximately the ratios expected. Croatian sites were not included in these graphs because  
320 of low numbers of recorded animals (slugs, earthworms and woodlice) in the upper 10 mm of  
321 the soil, after extremely dry weather conditions throughout the year.

322

323

324 *Metal concentrations in invertebrates*

325 Metal concentrations in earthworms, slugs and carabids correlated positively with those in  
326 soil samples from the same sites within the seasons ( $r=0.97$ ,  $0.84$  and  $0.83$  for lead  
327 respectively;  $r=0.86$ ,  $0.81$  and  $0.79$  for cadmium respectively and  $r=0.87$ ,  $0.75$  and  $0.58$  for  
328 mercury respectively). Earthworm tissue showed the highest mean concentrations of lead,  
329 cadmium and mercury of all predator and prey samples tested, while beetle tissue showed the  
330 lowest concentrations of lead and cadmium but not of mercury. Slug tissue showed the lowest  
331 mean concentrations of mercury, both in control and highly polluted sites (Fig. 3). Metal  
332 concentrations were compared using the Kruskal–Wallis test. The median concentrations of  
333 lead, cadmium and mercury differed significantly among invertebrate groups (lead,  $N=437$ ,  
334  $df=2$ ,  $H=220.5$ , chi squared= $184.3$ ; cadmium,  $H=208.9$ , chi square= $173.2$ ; mercury,  
335  $H=73.63$ , chi square= $73.6$ ;  $P<0.001$ ). All groups exhibited significantly higher lead,  
336 cadmium and mercury concentrations on highly polluted sites (L1-L4) compared with the  
337 control one (L5), (Mann-Whitney  $U$  Test,  $P<0.05$ ).

338

339 Lead and cadmium concentrations in carabid tissue correlated strongly with those in  
340 earthworms ( $r=0.94$  for Cd,  $r=0.92$  for Pb,  $P<0.05$ ) and slugs ( $r=0.85$  for Cd and Pb,  $P<0.05$ ),  
341 but not mercury concentration ( $r=0.28$ ,  $P=0.43$  with earthworms;  $r=0.04$ ,  $P=0.92$  with slugs).  
342 The highest AF ( $>1$ ), as the measure of metal transmission from prey to predator, was found  
343 for mercury in the beetle-slug trophic pathway [Friedman ANOVA, chi squared ( $N=9$ ,  $df=5$ )  
344 =  $33.125$ ,  $P<0.01$ ], followed by mercury in the beetle-earthworm pathway [Friedman  
345 ANOVA, chi squared ( $N=9$ ,  $df=4$ ) =  $23.933$ ,  $P<0.001$ ], (Fig. S1, Fig. 4 c). The lowest AF  
346 was recorded for lead in the beetle-earthworm pathway (Fig. S1, Fig. 4 a).

347

348 *Seasonal and species variations in metal concentrations*



349 Seasonal comparisons of metal concentrations in animals within each study site revealed  
350 significant differences. Carabids contained higher lead and mercury concentrations in spring  
351 than in autumn, and higher cadmium concentrations in autumn (Mann-Whitney  $U$  Test for  
352 lead:  $U = 95.000$ ,  $P = 0.032$  on location L3 and  $U = 94.000$ ,  $P = 0.036$  on location L5; for  
353 cadmium:  $U = 136.000$ ,  $P = 0.009$  on location L4), (Table S3). Cadmium concentration in  
354 slugs was also higher in autumn, even higher than in the earthworms (Fig. 3). In general,  
355 lead, cadmium and mercury concentrations in animal tissue at highly polluted sites showed  
356 greater seasonal differences in carabids and slugs than in earthworms (Fig. 3).

357

358 Node diagrams (Fig. 4) show the links between mean metal concentrations in carabid species  
359 and the main prey, presented by the size of nodes and predation events in both seasons.

360 Significant differences were found in metal concentrations between carabid species (Kruskal-  
361 Wallis ANOVA,  $P < 0.05$ ), (Table S4). *N. brevicollis* accumulated more lead and cadmium  
362 (Fig. 4, Table S4) than did the two *Abax* species (lead,  $U = 718.000$ ,  $Z = 5.31836$ ; cadmium,  
363  $U = 996.000$ ,  $Z = 3.83615$ ;  $P < 0.001$ ). *A. parallelepipedus* accumulated more mercury than did  
364 *Nebria* species ( $U = 393.000$ ,  $Z = -7.05115$ ,  $P < 0.001$ ). *Abax* species were dominant at the  
365 highly mercury-polluted sites.

366

## 367 **Discussion**

368 Molecular gut content analyses provided detailed insight into carabid diet under field  
369 conditions, allowing the construction of metal transfer pathways and providing possible  
370 explanations for seasonal variations in metal tissue concentrations. We revealed earthworms  
371 and slugs as the main prey within carabid communities with more slugs being consumed in  
372 autumn than in late spring and early summer. We confirmed our main hypothesis that

373 seasonal shift in prey consumed, and lead, cadmium and mercury concentrations in those  
374 prey, influenced metal concentrations in the carabids.

375

376 We observed a decrease in mercury and lead concentrations in predators in autumn, which  
377 coincided with a doubling in the proportion of beetles testing positive for slugs, allowing us  
378 to correlate the decrease in metal concentrations in carabids with the fact that slugs  
379 accumulated lower levels of these metals than did earthworms. In the case of cadmium, the  
380 tissue burden in carabids was higher in autumn than spring, coinciding with higher  
381 concentrations in slugs.

382

### 383 *Diet composition and heavy metal tissue burden*

384 Although many studies have used molecular methods to study natural regulation of pests  
385 (Symondson *et al.* 1996; Chen *et al.* 2000; Nash *et al.* 2008; Eskelson *et al.* 2011; Hatteland  
386 *et al.* 2011; Boreau de Roince *et al.* 2012) they can also elucidate complex food webs and  
387 thereby track pathways of pollutants between trophic levels in ecosystems. For example, data  
388 on metal transmission through trophic interactions have been obtained from feeding  
389 experiments (Janssen *et al.* 1991, Kramarz 1999; Hendrickx *et al.* 2003) and from analysis of  
390 stable isotope ratios (e.g. Cabana & Rasmussen 1994).

391

392 Metal transfer has been measured *in situ* indirectly by measuring metal concentrations in  
393 different food chain compartments (e.g. Notten *et al.* 2005, Roodbergen *et al.* 2008). We used  
394 a similar approach in our study to measure metal concentrations in different food web  
395 compartments (soil, earthworms that ingest soil substrate, slugs as herbivores and carabids as  
396 carnivorous), in order to explore the relationship between the relative proportion of each prey  
397 group in the beetle diet and metal accumulation in beetle tissue. Our results confirmed the

398 expected positive relationship between metal concentrations in predator and in prey.  
399 Earthworms as the main prey with the highest metal concentrations in the tissue represent the  
400 most important link in trophic pathways of contaminants from the soil to predators. In  
401 addition, we observed that carabid beetles had lower concentrations of lead and cadmium  
402 than slugs and earthworms, but had more mercury than slugs ( $AF > 1$ ), raising the possibility  
403 of mercury biomagnification (Fig. S1). Unlike spiders, which lack mechanisms to excrete  
404 cadmium (Hendrickx *et al.* 2003), carabids showed lower Pb and Cd concentrations, with  
405 seasonal and species differences detected.

406

407 Taking into account that each prey contributes differently to metal accumulation in the  
408 predator, the AF could be calculated by multiplying mean metal concentrations in  
409 earthworms and slugs by the percentage of beetles testing positive and adding up the  
410 concentrations of both prey to yield the total metal concentration from prey. Then, the mean  
411 metal concentration in the predator (carabids) would be divided by this total concentration  
412 from both prey. This approach could be useful for assessing metal transfer from multiple prey  
413 combinations to predators and thus calculating more accurate AF values, but predation rates  
414 need to be adjusted for all predator-prey combinations. As it was not possible within this  
415 study to correct for the multiple combinations of primers and predator species in the analyses,  
416 simple AF calculations have been done.

417

418 Although carabid tissue showed lower Pb and Cd concentrations than did their prey, carabids  
419 from highly polluted sites exhibited much higher metal concentrations than did animals from  
420 control sites (Fig. 3). Some differences between sites may be influenced by metal availability  
421 due to chemical and physical properties of the soil (i.e. pH values, Table S3). Still some clear  
422 trends in metal concentrations in invertebrates were evident.

423

424 *Carabid beetles as bioindicators for metal pollution assessment*

425 Carabid species differ in lifespan, as well as in feeding and breeding behaviours (autumn or  
426 spring breeders). Moreover, the same species may even change its diet during the year:

427 *Carabus violaceus* consume more slugs in early spring and again in autumn (Paill 2000). All  
428 these differences may affect metal concentrations and pollution assessment. Species and  
429 seasonal variations in heavy metal tissue burden have previously been described in carabids  
430 collected from the field in other studies (i.e. Janssen *et al.* 1991; Purchart & Kula 2007; Šerić  
431 Jelaska *et al.* 2007; Butovsky 2011).

432

433 *Seasonal differences in metal burden*

434 Carabids had higher lead and mercury concentrations in spring than in autumn, but higher  
435 cadmium concentrations in autumn. In an attempt to explain these differences, we  
436 superimposed changes in heavy metal concentrations in carabid tissue with numbers of  
437 carabids testing positive for each prey group. Since earthworms showed the greatest lead and  
438 mercury accumulation and were also the main carabid prey, we speculate that trophic  
439 pathways involving earthworms should be the main determinant of metal concentration in  
440 carabids at highly polluted sites. Slugs accumulate lead and mercury to a much smaller extent  
441 than do earthworms, and carabids consume a higher proportion of slugs in the autumn than in  
442 spring and early summer (Table S2). This shift in consumed prey may explain why lead and  
443 mercury concentrations in carabid predators were lower in autumn. This same shift may also  
444 explain seasonal variations in cadmium concentrations. Not only carabid predators but also  
445 the slugs showed higher cadmium tissue concentrations on polluted sites in autumn (Fig. 3).  
446 During this time, slugs showed maximal levels of this metal (Table S3), coinciding with the  
447 time when carabids consumed an increasing proportion of them as prey. Although Ireland

448 (1981) found greater cadmium uptake by *A. ater* slugs after feeding trials in July than in  
449 September, here we measured higher concentrations in slugs in autumn on the two cadmium-  
450 rich locations L3 and L4; concentrations at the other sites were higher in spring.

451

#### 452 *Species differences in metal burden*

453

454 Species differences in metal concentrations in carabid tissue were observed, including among  
455 the most abundant *Abax* and *Nebria* species. For example, the highest cadmium  
456 concentrations were measured in *Nebria brevicollis* tissue with 2.2 and 2.4 times more  
457 cadmium on L3 and L4 locations (with higher cadmium concentrations in the soil) compared  
458 with the control L5 location. On the other hand, *A. parallelepipedus* accumulated 1.2 times  
459 more lead at lead-contaminated site L3 compared with control site. In both species, lead  
460 concentrations were higher in spring. *Nebria* accumulated more cadmium than did *Abax*,  
461 especially in autumn when more *Nebria* were trapped, predated more on slugs than did  
462 species collected in spring-summer (Fig. 4). Links in food webs (Fig. 4) gave a good  
463 indication of interaction strengths. Still, some adjustments based upon modeling the decay  
464 curves for each predator-prey combination would probably be needed to obtain more precise  
465 results, although given the large numbers of carabid-prey combinations involved this may  
466 not be practical. At least 100 individual predators would be needed for each trial to model the  
467 decay curve for each predator-prey combination shown in Fig. 4.

468

469 Similar seasonal trends were observed in previous studies (reviewed in Butovsky 2011).  
470 These findings suggest that season and carabid community structure should be taken into  
471 account when using these beetles to assess metal pollution in the field.

472

473 *Carabid beetles diet*

474 Earthworms and slugs were the primary components of the carabid diet, with more slugs  
475 consumed in autumn than in late spring or early summer. Some carabid species, such as  
476 *Pterostichus melanarius*, may rely on their earthworm diet to improve their fitness  
477 parameters (Symondson *et al.* 2006). Also, some species of carabids have been shown to  
478 prefer slug eggs and juveniles (Paill 2000; Hatteland *et al.* 2010) because adult slugs have  
479 effective defence mechanisms (McKemey *et al.* 2003). A study by Foltan (2004) showed that  
480 arionid slugs deterred ground beetle attacks more effectively than did limacid slugs. Our data  
481 strongly suggest that the observed number of isopod positive beetles was less than expected  
482 in both seasons (Fig. 2). Again, we have conducted no statistical analyses on these results for  
483 the reasons explained. As earthworms were clearly the most abundant prey available in the  
484 autumn, they represent the most important trophic pathway available at that time.

485

486 Although *Nebria brevicollis* has been described as preying primarily on springtails (Thiele  
487 1977), we detected all four prey groups in its foregut, with earthworms present in more than  
488 40% of individuals. The proportion of carabids in our study positive for woodlice (18.8% in  
489 UK locations) was similar to the 17% reported by Sunderland & Sutton (1980) in grasslands  
490 in UK. The number of carabids testing positive for each prey group could be changed to some  
491 degree by different detection periods following ingestion. Decay rates might be different for  
492 each predator and primer combination, and therefore have to be calculated to get more  
493 precise results and interaction strengths.

494

495 This study has provided insights not only at the level of individual species, but also at the  
496 community level. Molecular gut content analyses allowed us to screen the entire community

497 rapidly for a broad range of prey, elucidate the position of carabids within the ecosystem and  
498 identify nutrient flows while taking into account species composition and abundance.  
499 These findings suggest the possibility of using carabids as bioindicators of heavy metal  
500 contamination in soil-based ecosystems, as long as species and seasonal variations are taken  
501 into account, since these factors affect metal concentrations in carabids. The high  
502 accumulation factor for mercury (>1) on mercury polluted sites highlights the need to analyse  
503 the potential for mercury transmission in other predators.

504

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515

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721 related to body-size and trophic level. *Environmental Pollution*, **42**, 209-221.

722

### 723 **Data Accessibility**

724 Lead, cadmium and mercury concentrations (dry weight) in soil and invertebrates: DRYAD  
725 entry doi:10.5061/dryad.5n02f/1. Raw data of molecular gut content analyses (MGCA) in  
726 carabid beetles: DRYAD entry: doi:10.5061/dryad.5n02f/2 .

727

728

### 729 **Author Contributions Box**

730 LSJ designed the study, collected the samples, performed molecular diagnostics of sampled  
731 material, data analyses and wrote the manuscript; DSB conducted part of molecular gut  
732 content analyses; JJ performed metal analyses; LSJ and IPV performed statistical analysis;



733 WOCS supervised this work from the very beginning, adding valuable suggestions and  
734 revising the manuscript.

735

736

737

738 Figure legends:

739

740 Figure 1. Beetles testing positive for each prey group in spring-early summer (in 2007 and  
741 2010 together) and autumn (in both years together). Box represents mean  $\pm$ SE and whisker  
742  $\pm$ 0.95 confidence interval.

743

744 Figure 2. Number of PCR positive beetles for three prey groups (shown in open bars) and  
745 abundance of each prey group in the environment (shown in black bars). Upper graph showed  
746 data across the three locations in the UK (L3-5) for the spring-summer period, and lower  
747 graph showed data across three locations in the UK for autumn.

748

749 Figure 3. Lead, cadmium and mercury concentrations (mean,  $m \pm SE$ ,  $m \pm 1.96*SE$ ) in  
750 earthworms, slugs and carabids collected in spring-summer 2007, 2010 (open boxes) and in  
751 autumn 2007, 2010 (filled boxes) in control (L5) and polluted locations (L1-4). Mean metal  
752 concentration values from the control site are marked as squares, and those from the polluted  
753 sites are marked as circles.

754

755 Figure 4. Node diagrams of observed trophic interactions between prey groups and predator  
756 species with measured metal concentrations in the tissue. Size of the nodes reflects mean  
757 concentrations of lead (a), cadmium (b), and mercury (c) in earthworms, slugs and carabids.  
758 Yellow circles represent beetles caught in spring-summer, and purple circles represent those  
759 caught in autumn. Arrow thickness represents the percentage of carabid species testing  
760 positive for the given trophic interaction. Black circle represents metal concentration of 0.1  
761 mg/kg, with an arrow thickness representing 10% of carabid species being positive for a  
762 given interaction. Force Atlas model was chosen as a layout. Node sizes range from 20-150

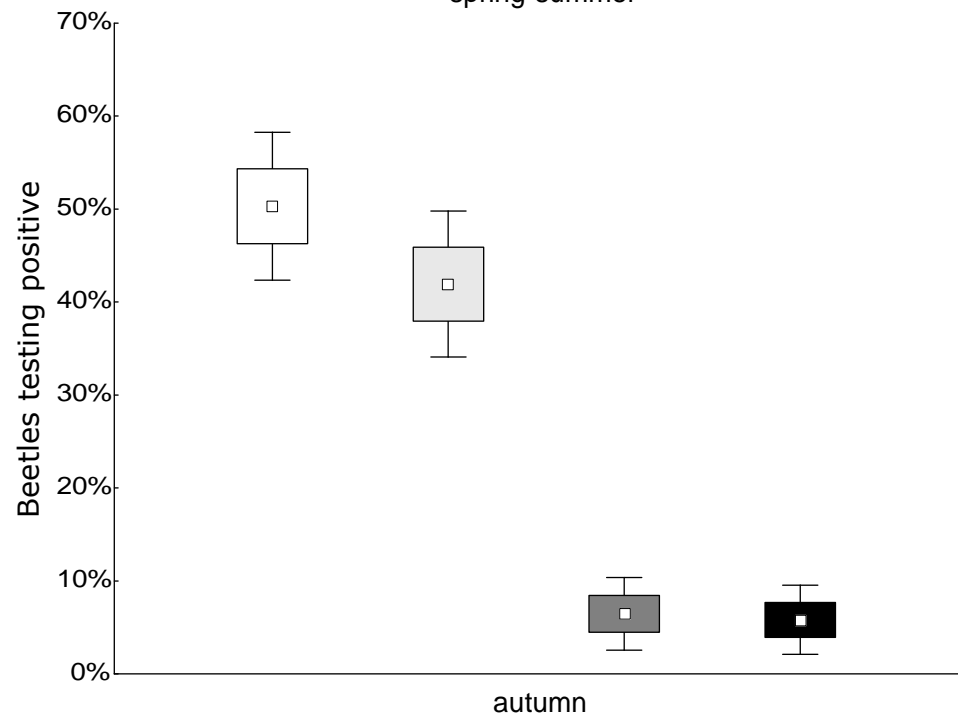
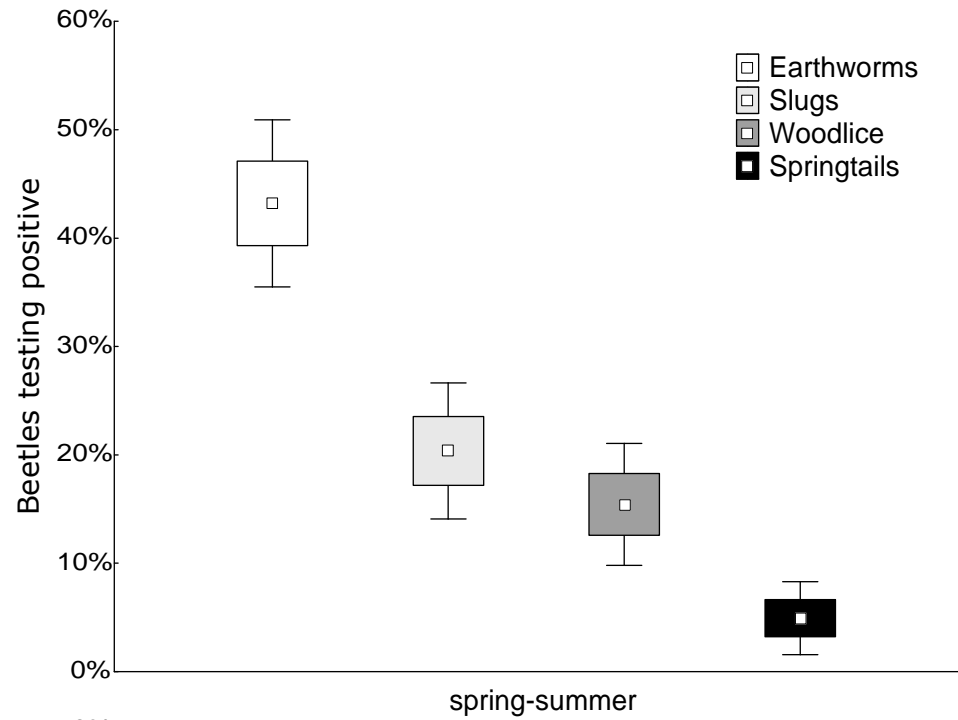
763 for Pb (0.08-623.1 mg/kg), Cd (0.1-56.5 mg/kg) and Hg (0.035-0.666 mg/kg) concentrations  
764 measured in carabids. The same range is chosen for arrow thickness, representing the  
765 percentage (from 0.1 to 1) of PCR positive carabid species tested for each prey within the  
766 season.

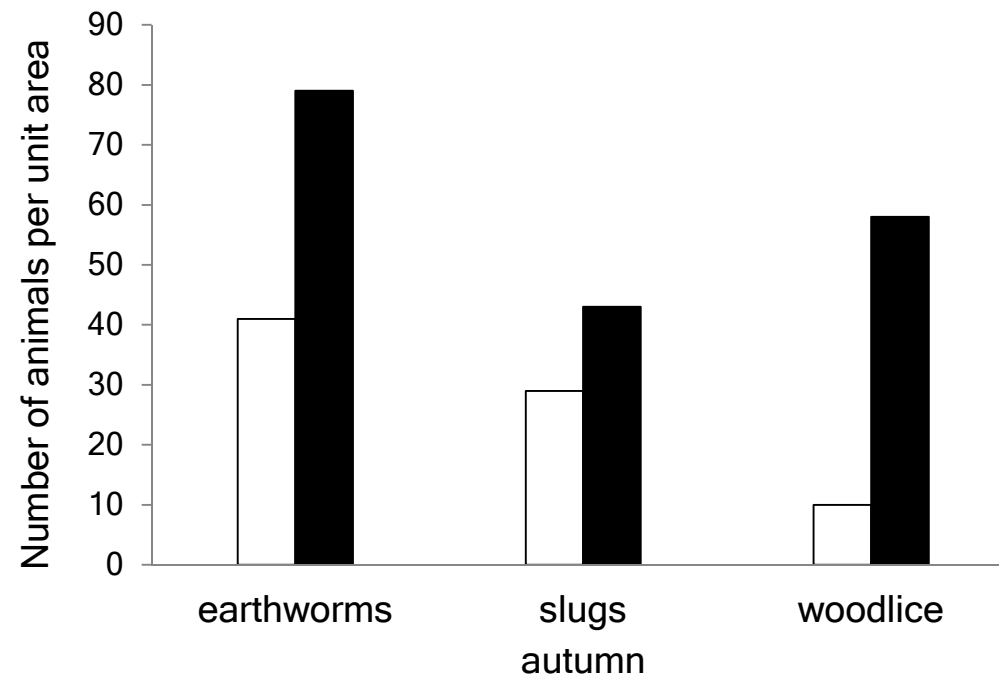
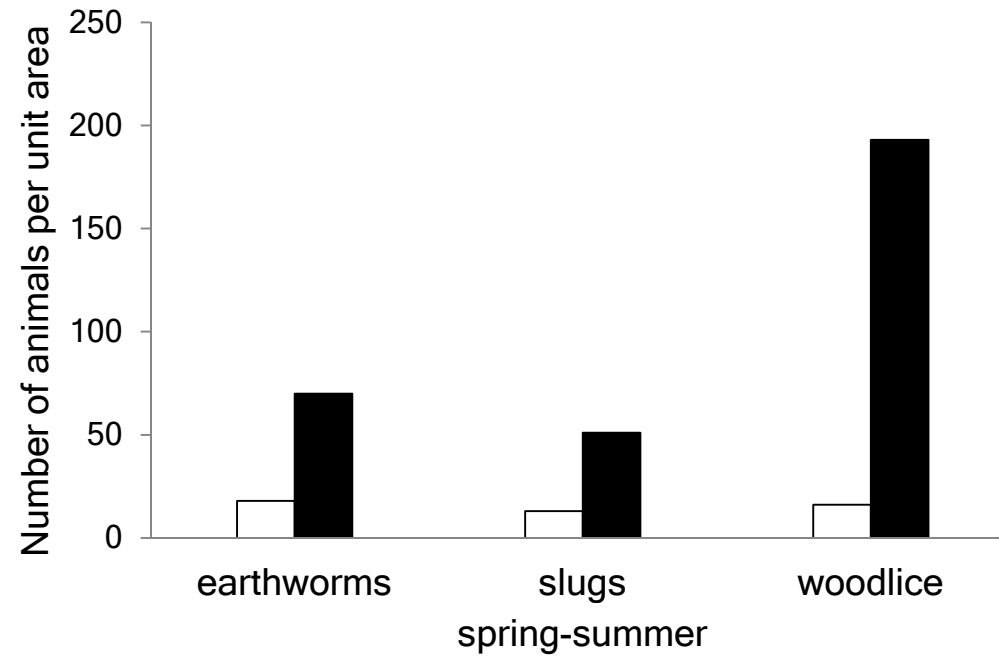
767

768 Tables

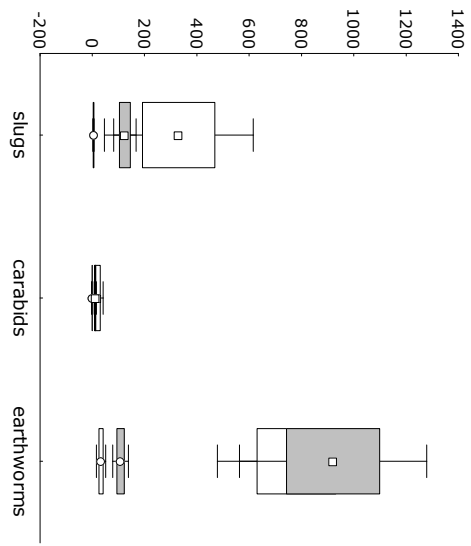
769 Table 1. Sequences of primers (5'-3') used for multiplex (M) and singleplex (S) PCR analyses.

Species	PCR	Primer name	Primer sequence	Amplicon size (bp)	Reference
Earthworms	S	185F	TGTGTACTGCCGTCGTAAGCA	225-236	Harper <i>et al.</i> 2005
		14233R	AAGAGCGACGGGCGATGTGT		
<i>Deroceras reticulatum</i>	M	Dr11F	CTATACACAATTTTTAAATAAG	109	Dodd 2004, Harper <i>et al.</i> 2005
		DRF29RC	GCTTCTGGTTTATCTATTATTTGGT		
<i>Arion sp.</i>	M	Ai1F	CACATAAATGATAGTCACC	208-221	Dodd 2004, Harper <i>et al.</i> 2005
		AR2R	ATACTTACAAGTCCATCTTT		
<i>Limax cinereoniger</i>	S	COI-Lcin-F1	TGAACTGTATACCCGCCTTTG	198	This study
		COI-Lcin-R1	CCTGCCAATACAGGAAGCG		
Springtails	S	Col3F	GGACGATYTTRTTRGTTTCGT	177&272	Kuusk & Agustí 2008
		Col4F	GCTACAGCCTGAACAWTWG		
		Col5R	TCTTGGCAAATGCTTTCGCAGTA		
Woodlice	S	IsopodNSSf1	TCATGATTYATGGGATGT	201-278	Jarman <i>et al.</i> 2006
		IsopodNSSr1	AAGACCTCAGCGCTCGGC		

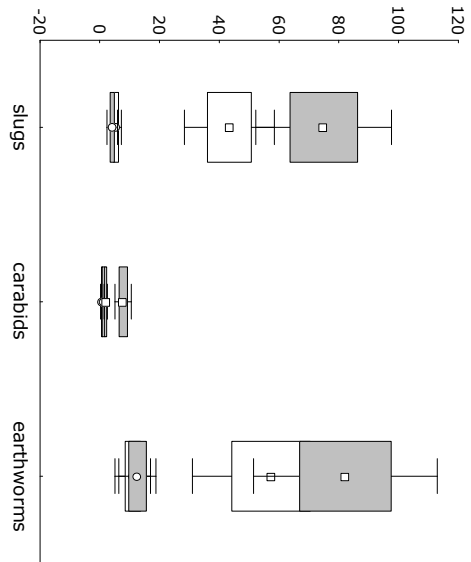




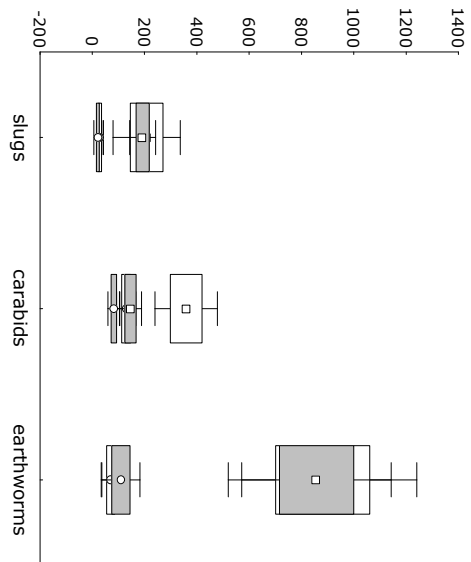
Pb (mg/kg) on control (L5) and polluted locations (L1-4)

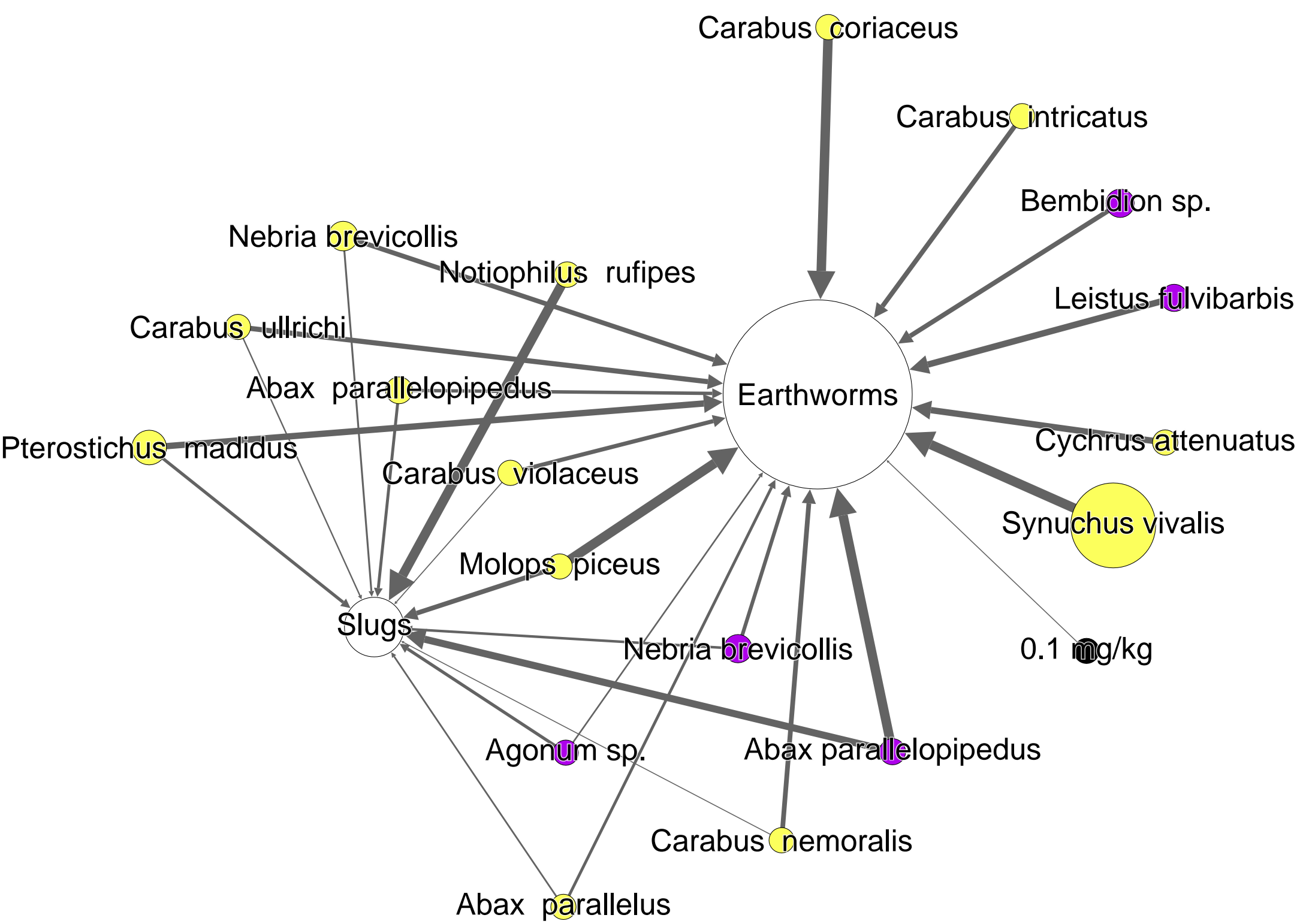


Cd (mg/kg) on control (L5) and polluted locations (L1-4)

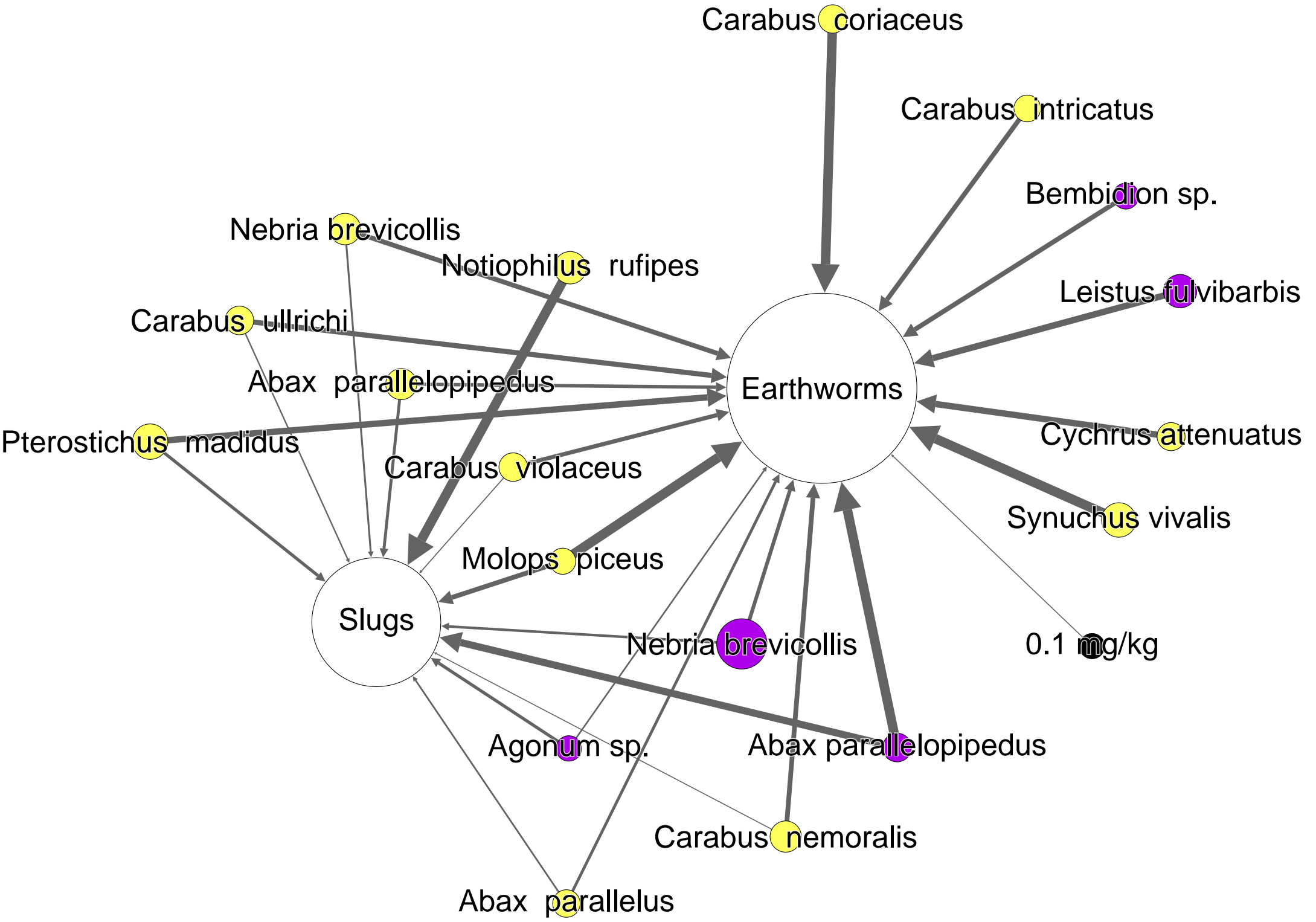


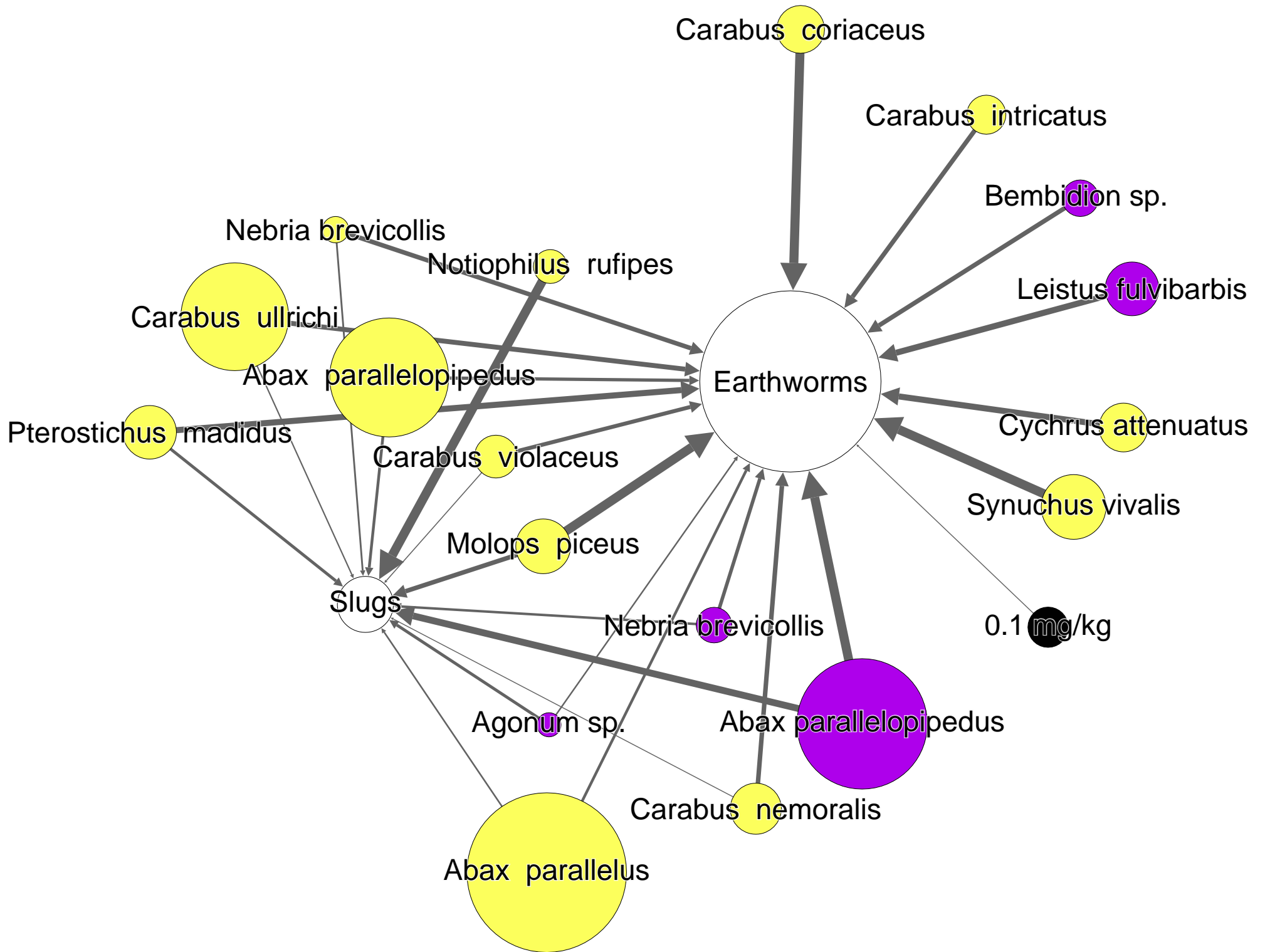
Hg ( $\mu$ g/kg) on control (L5) and polluted locations (L1-4)











## **Supporting information**

Additional supporting information may be found in the online version of this article.

## **Metal analyses**

Lead, cadmium and mercury were analysed by inductively coupled plasma – mass spectrometry (ICP-MS) using the Agilent 7500cx (Agilent Technologies, Waldbronn, Germany).

Whole animals were washed with ultrapure water (18 M $\Omega$  cm; obtained with a GenPure system, TKA, Germany), weighed in a quartz digestion vessels, dried, weighed again and digested in 50% (v/v) nitric acid (3-5 mL, depending on the weight of the samples) in an UltraCLAVE IV microwave digestion system (Milestone, Italy). Soil samples were air-dried and sieved through 2 mm meshes. Dried powdered soil samples (approximately 0.1 g) were weighed into PTFE vessels and digested in 2 mL conc. HNO<sub>3</sub> and 0.4 mL conc. HF, according to UltraCLAVE IV application instructions for the digestion of soil (*UltraCLAVE Application Note N. UC-17. In: UltraCLAVE IV user manual Rev. 02/2008. Leutkirch: MLS-Milestone GmbH Mikrowellen-Laborsysteme*).

Digested samples were adjusted to 30 mL and just before the analysis by ICP-MS those samples were additionally diluted with 1% nitric acid in ultrapure water by a factor of 10-50, depending on the type and starting dry mass of the sample. All standard solutions (for external calibration and internal standards) were prepared from 1000 mg/L PlasmaCAL (SCP Science, Canada) single element standards. Isotopes <sup>114</sup>Cd, <sup>202</sup>Hg and <sup>208</sup>Pb were used for element quantification, while <sup>103</sup>Rh, <sup>153</sup>Tb and <sup>193</sup>Ir were used as internal standards to correct for instrument drift and matrix effects.

Certified standard reference materials were used in duplicate with each sample digestion series for quality assurance. BCR185R Bovine Liver and BCR186 Pig Kidney (Institute for Reference Materials and Measurements, Geel, Belgium) were used to control measurement accuracy of earthworm and slug samples, while San Joaquin Soil 2709 (National Institute of Standards and Technology, USA) was used to control for soil sample measurement accuracy. The results agreed well with the certified values - recoveries ranged from 92% to 110%, with the exception of Hg in San Joaquin Soil NIST 2709 sample (84-92%).

### **Statistical analyses**

Prior to statistical analysis, all data were checked using Shapiro-Wilk's test to see whether they followed a normal distribution; if not, they were transformed [ $\log(x+1)$ ]. The Levene post-hoc test was used to test the assumption of equal variances. If the transformed data followed a normal distribution based on Shapiro-Wilk's test, they were analysed using parametric statistical tests. If not, they were analysed using the following nonparametric tests: Friedman ANOVA and the Mann-Whitney U test, to compare two independent samples; the Kruskal-Wallis test, to compare multiple independent samples followed by multiple comparisons of mean ranks for all groups.

Friedman ANOVA with a significance threshold of  $P < 0.05$  was used to compare lead, cadmium and mercury concentrations factors between carabids and earthworms and between carabids and slugs at the five study sites in the two seasons. The Kruskal-Wallis test was used to test the null hypothesis of no difference in group median metal concentrations among earthworms, slugs and carabids among the sites. The Mann-Whitney U Test was used to compare the metal concentrations in each group of animals (earthworms, slugs and carabids) between the two seasons. Two tailed T-test was used to check for differences in predation

events within the same predator-prey combinations between two seasons. Correlations between prey consumption and metal concentration in carabids at different study sites and during both seasons were calculated using the Pearson correlation test.

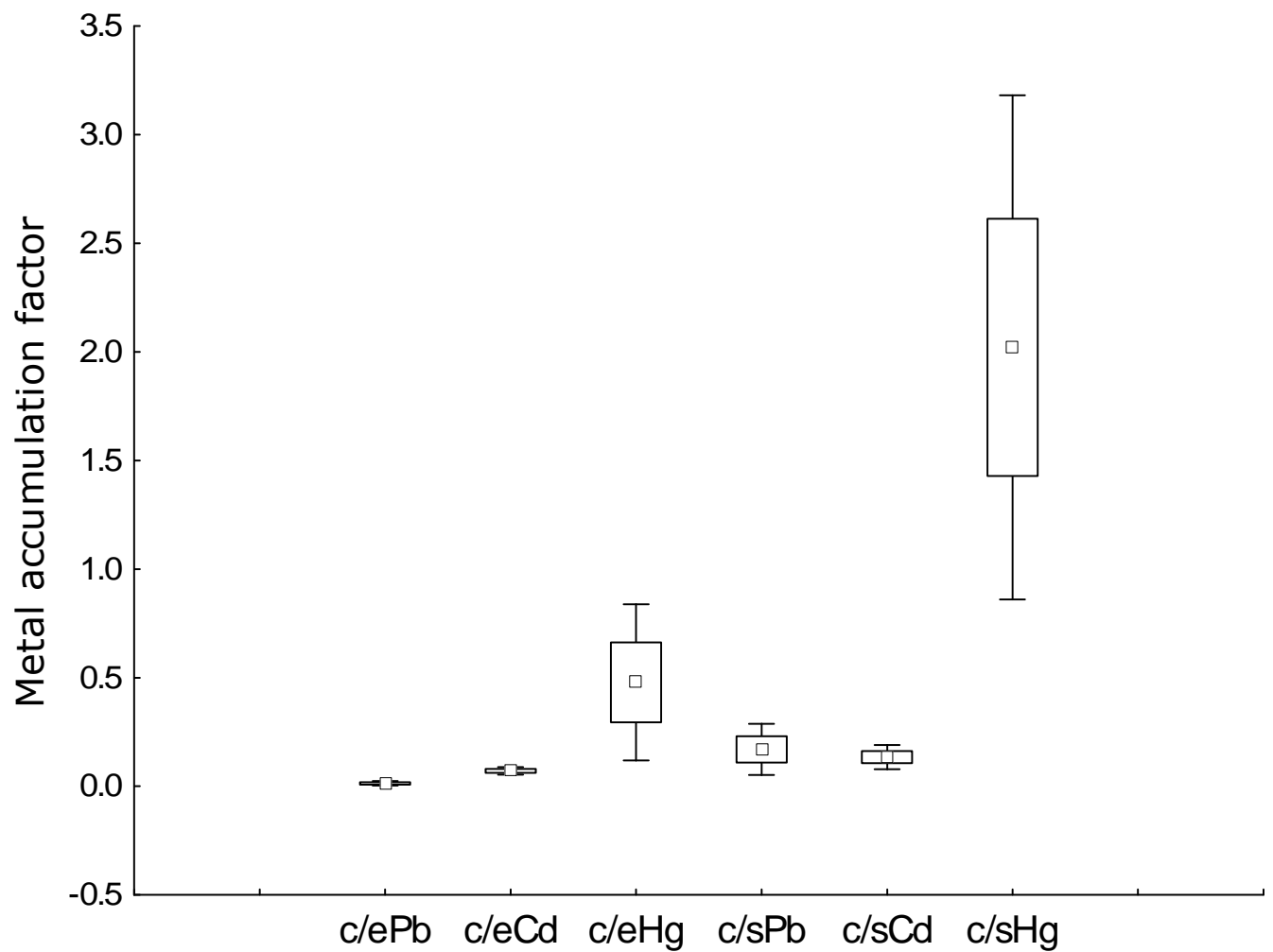


Figure S1. Mean ( $\pm$ SE and  $1.96*SE$ ) accumulation factors (AFs) for lead, cadmium and mercury in carabids (c) and their prey (e, earthworms; s, slugs).

Table S1. List of invertebrates used for non-target primer tests.

Carabid beetles

*Carabus nemoralis*

*Abax parallelepipedus*

*Calathus fuscipes*

*Notiophilus biguttatus*

*Bembidion* sp.

Other beetles

Rove beetles - *Ocypus olens*

Dor beetle - *Geotrupes* sp.

Hymenoptera

Ant (sp 1)

Ant (sp 2)

Ant (sp3)

Diptera

Crane fly larvae (sp 1)

Butterflies and Moths

*Pieris brassicae*

*Cydia pomonella*

*Grapholita molesta*

Earwings (sp 1)

Springtails (sp 1)

Spiders

*Pardosa* sp.

*Erigone atra*

Woodlice

*Philoscia muscorum*

*Oniscus asellus*

*Porcellio scaber*

Earthworms

*Aporrectodea caliginosa*

*Aporrectodea smaragdina*

*Eisenia foetida*

*Lumbricus castanea*

*Lumbricus rubellus*

*Lumbricus terrestris*

*Deandrobaena octaedra*

Slugs

*Deroceras reticulatum*

*Limax maximus*

*Lehmannia marginata*

*Tandonia budapestensis*

Snails

*Cepea nemoralis*

*Helix aspersa*

Nematodes (sp 1)



Table S2. Screening of field-caught carabid species for four prey groups. Below are the numbers of individuals of each species tested and percentage of beetles testing positive for each prey group.

Species	Locations where species occurred	No. ind. tested for diet (overall abundances)	Beetles testing positive (%)			
			Earthworms	Slugs	Woodlice	Springtails
<i>Nebria brevicollis</i>	3,4,5	87 (165)	40.23	26.44	10.3	6.9
<i>Abax parallelus</i>	1,2	68 (92)	78.95	84.21	1.5	10.3
<i>A. parallelepipedus</i>	1,2,3,4,5	63 (107)	39.68	33.33	34.9	1.6
<i>Carabus nemoralis</i>	1,2	18 (28)	72.22	38.89	0.0	0.0
<i>C. ullrichi</i>	2	13 (19)	53.85	7.69	0.0	0.0
<i>C. violaceus</i>	1,2,4,5	9 (19)	44.44	22.22	0.0	0.0
<i>Cychrus attenuates</i>	1,2	7 (10)	71.43	14.29	0.0	0.0
<i>Pterostichus madidus</i>	3,4,5	7 (16)	71.43	28.57	14.3	0.0
<i>Agonum sp</i>	5	6 (9)	16.67	33.33	0.0	0.0

<i>C. convexus</i>	2	6 (6)	83.33	0.0	0.0	0.0
<i>C. coriaceus</i>	2	6 (8)	83.33	33.33	0.0	0.0
<i>C. intricatus</i>	1,2	6 (7)	50.00	0.0	0.0	0.0
<i>Leistus fulvibarbis</i>	3,4	3 (6)	66.67	0.0	0.0	66.7
<i>Bembidion nigricorne</i>	3	2 (5)	100	0.0	0.0	0.0
<i>C. problematicus</i>	4	2 (2)	0.0	50.00	50.0	0.0
<i>Molops piceus</i>	2	2 (4)	100	50.00	0.0	0.0
<i>P. melanarius</i>	3,4	2 (2)	50.00	50.00	0.0	0.0
<i>P. transversalis</i>	1,2	2 (2)	50.00	50.00	0.0	0.0
<i>Synuchus vivalis</i>	3,4	2 (7)	100	0.0	50.0	0.0
<i>Aptinus bombardia</i>	1	1 (1)	100	0.0	0.0	100
<i>Notiophilus rufipes</i>	2	1 (2)	0.0	100	0.0	0.0

Table S3. Metal concentrations and pH values (mean±SD) in soil, earthworms (e), slugs (s) and carabids (c) at five locations. Concentrations are reported as mg/kg dry weight for lead and cadmium, and as µg/kg for mercury. Significant seasonal differences in metal concentrations at each study site are indicated with different superscripts; the same number with different letters denotes significantly different concentrations at the 0.05 level (Mann-Whitney U test). Abbreviations: Spring-summer (s), autumn (a)

Location	Groups	Season	<i>N</i> ind.	pH	Pb (mg/kg)	Cd (mg/kg)	Hg (µg/kg)
L1 (Cro)	c	s	10		0.455±0.40	0.784±0.81	105.1±76.97
	e	a	9		180.2±120.9	7.889±4.97	413.3±335
	e	s	22		168.9±92.52	8.182±3.54	361.5±169
	s	a	13		0.883±0.93	7.841±7.45	217.6 <sup>1a</sup> ±244.9
	s	s	6		0.748±1.38	6.662±7.74	57.88 <sup>1b</sup> ±103.6
	soil	a	11	3.75±0.14	67.91±15.3	0.565±0.208	310.9±152.4
	soil	s	11	3.80± 0.16	73.54±12.6	0.465±0.15	234.2±74.6
L2 (Cro)	c	a	13		0.406±0.41	1.194±0.79	491.7±264.6
	c	s	53		0.289±0.20	1.321±1.32	538.7±688.3
	e	a	16		75.45±74.98	15.13±9.20	1515±1420
	e	s	13		116±48.92	24.58±13.91	1075±686

	s	a	2		1.689±2.27	6.42±4.54	314.7±27.14
	s	s	3		16.78±28.07	33.75±22.91	115±35.30
	soil	a	11	4.96±0.17	127.2±71.9	1.557±0.62	442.2±422.9
	soil	s	11	4.96±0.13	139.7±60.06	1.369±0.53	617.8±415.2
<hr/>							
L3 (UK)	c	a	22		26.17 <sup>2a</sup> ±25.17	9.480±18.21	123.2±59.48
	c	s	15		114.8 <sup>2b</sup> ±240.7	4.692±4.21	146.1±126.9
	e	a	17		2441±1154	174.3±132.4	708.5±681.9
	e	s	15		2446±1025	162.7±146.9	1790±2310
	s	a	25		227.5±138.2	122.3±79.71	193.8 <sup>3a</sup> ±149.7
	s	s	9		940.7±1092	84.98±38.11	478.3 <sup>3b</sup> ±490.9
	soil	a	12	5.73±0.10	8204±2406	24.27±13.25	590.2±311.2
	soil	s	14	5.86±0.15	8829±2830	24.84±15.2	669±290.7
<hr/>							
L4 (UK)	c	a	45		8.020±7.18	8.989±9.97 <sup>4a</sup>	58.40±26.91
	c	s	12		4.628±3.15	2.752±3.27 <sup>4b</sup>	44.69±40.47
	e	a	9		294.1±132.2	101.7±76.13	414.1±511.4
	e	s	7		367.3±344.6	46.57±41.00	203.5±187

	s	a	8		36.31 <sup>5a</sup> ±17.52	53.27±29.60	118.3±61.92
	s	s	10		73.33 <sup>5b</sup> ±40.71	30.95±15.29	82.99±34.77
	soil	a	15	5.64±0.18	2525±2426	21.48±9.18	191.8±46.7
	soil	s	14	5.55±0.19	2630±1054	23.57±6.15	217.9±53
L5 (UK)	c	a	13		0.210 <sup>6a</sup> ±0.09	1.346±1.78	82.72±37.21
	c	s	25		0.457 <sup>6b</sup> ±0.56	0.776±0.64	129.7±87.91
	e	a	18		107.9 <sup>7a</sup> ±60.10	12.62±12.46	109.8±146.9
	e	s	9		33.63 <sup>7b</sup> ±22.6	11.08±7.73	70.46±47.06
	s	a	6		4.375±1.85	4.202±1.70	21.55±14.26
	s	s	14		5.092±2.92	5.524±3.03	30.15±21.61
	soil	a	8	4.52±0.10	69.11±18.8	0.713 <sup>8a</sup> ±0.28	76.2 <sup>9a</sup> ±17.6
	soil	s	16	4.12±0.10	56.05±12.7	0.466 <sup>8b</sup> ±0.15	170.2 <sup>9b</sup> ±43.9

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Means at different study sites bearing a superscript with the same number but different letters differ significantly from each other at the 0.05

level. For example: 1a differs significantly from 1b. Values carrying different superscripted numbers do not differ significantly.

Table S4. Mean metal concentrations in ground beetles at five locations. Concentrations are expressed as mg/kg dry weight for lead and cadmium, and as µg/kg dry weight for mercury. *N*, the number of beetles used in the metal analysis.

Species	Site	<i>N</i>	Body weight (g dry weight)	Pb mg/kg	Cd mg/kg	Hg µg/kg
<i>Abax ater</i>	L2 (Cro)	23	0.132	0.379	1.382	718.1
	L3 (UK)	6	0.118	35.02	4.317	91.62
	L4 (UK)	3	0.123	5.517	3.559	84.27
	L5 (UK)	12	0.110	0.249	0.998	136.9
<i>A. parallelus</i>	L1 (Cro)	3	0.133	0.385	1.146	155.6
	L2 (Cro)	21	0.069	0.305	0.825	639.3
<i>Agonum sp.</i>	L5 (UK)	3	0.019	0.226	0.164	35.17
<i>Bembidion quadrimaculatum</i>	L3 (UK)	2	0.003	10.28	0.474	119.7
	L4 (UK)	1	0.004	15.55	0.144	16.43
	L5 (UK)	1	0.002	0.660	0.205	417.2

<i>Carabus cancellatus</i>	L4 (UK)	1	0.144	6.783	0.168	34.35
<i>C. coriaceus</i>	L2 (Cro)	2	0.128	1.780	0.823	0.252
<i>C. intricatus</i>	L1 (Cro)	1	0.336	0.399	0.591	95.15
<i>C. nemoralis</i>	L1 (Cro)	1	0.288	1.459	0.474	131.9
	L2 (Cro)	9	0.275	0.266	2.335	143.8
<i>C. ullrichi</i>	L2 (Cro)	6	0.293	0.084	1.245	370.9
<i>C. violaceus</i>	L1 (Cro)	3	0.247	0.351	1.016	76.65
	L2 (Cro)	1	0.299	0.053	1.831	590.2
	L4 (UK)	1	0.314	1.708	2.786	11.87
	L5 (UK)	5	0.256	0.279	0.862	54.03
<i>Cychrus attenuatus</i>	L1 (Cro)	1	0.075	0.200	0.054	83.67
	L2 (Cro)	2	0.088	0.486	1.531	159.0
<i>Cy. caraboides</i>	L5 (UK)	1	0.151	0.166	0.084	105.9
<i>Leistus fulvibarbis</i>	L4 (UK)	1	0.006	7.852	2.883	153.6
<i>Molops piceus</i>	L2 (Cro)	2	0.040	0.122	0.402	157.6
<i>Nebria brevicollis</i>	L3 (UK)	22	0.022	28.39	9.505	114.1

	L4 (UK)	46	0.019	7.455	8.672	55.62
	L5 (UK)	11	0.023	0.236	1.598	95.18
<i>Pterostichus</i>						
<i>fasciatopunctatus</i>	L1 (Cro)	1	0.064	0.284	0.238	43.42
<i>P. madidus</i>	L3 (UK)	4	0.041	79.69	7.510	162.6
	L5 (UK)	5	0.044	1.138	0.448	145.6
<i>Synuchus vivalis</i>	L3 (UK)	3	0.004	374.5	4.306	317.5
	L4 (UK)	2	0.006	5.823	1.652	18.02

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