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

[1,3]Lucija Šerić Jelaska, [2]Jasna Jurasović, [3]David S. Brown, [3]Ian P. Vaughan, [3]William O. C. Symondson

[1]Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia

[2]Institute for Medical Research and Occupational Health, Ksaverska cesta 2, 10000 Zagreb, Croatia

[3]Cardiff School of Biosciences, Cardiff University, Sir Martin Evans Building, Museum Avenue, Cardiff, CF10 3AX, UK

Key words: carabid beetles, earthworms, metal bioaccumulation, molecular gut content analyses, prey choice, slugs

Correspondence:

Lucija Šerić Jelaska, Fax: + 385 (0)1 4826260;

E-mail: slucija@biol.pmf.hr

Running title:

Molecular diet analyses and metal transfer

Abstract

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

Introduction

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

The main biological factors controlling metal accumulation in terrestrial invertebrates are diet and the structure and physiology of the digestive system (Hopkin 1989). After ingesting toxic metals, some organisms may avoid harmful effects by sequestering them in certain tissues and immobilising them in a form that cannot interfere with vital biological processes (Hopkin 1989). When organisms assimilate large amounts of heavy metals and do not excrete them, biomagnification up the food chain can occur. By the process of biomagnification, even low concentrations of some toxins (e.g. DDT, PCBs, organic mercury) can end up in living organisms at higher concentrations and can be disseminated to distant places through migration. Biomagnification of some toxic compounds occurs in both aquatic and terrestrial ecosystems affecting many organisms, such as raptors and otters (e.g. Castro *et al*. 2011; Mayack 2012), and can be harmful to human health. After mercury discharge in Japan in 1956, mercury contamination of fish and shellfish severely affected humans and animal populations for decades (e.g. Harada 1995). Although biomagnification of metals is less 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*
95 *al.* 1996; Holland 2002; Symondson *et al*. 2000, 2006; Eskelson *et al*. 2011; Hatteland *et al*.
96 2011; Boreau de Roincé *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.

Materials and methods

1. Field collection

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

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

For metal analyses, 8-16 top-soil samples (3L) per site were taken twice, in late spring and in autumn. Samples were taken randomly from the top 10 cm across the area at each site. Earthworms, slugs and carabids were collected from the same layer of soil at the same sites by digging and hand sorting, during three visits per season. Adult carabids were also collected by pitfall trapping to get substantial number of individuals for PCR analysis of their gut contents and heavy metal analyses. Five empty traps (0.5 L plastic cups) were left at each site for two weeks during each season. Traps were emptied every morning and beetles used for PCR analysis of gut content were placed individually in plastic tubes, transported to the 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 µL)
186 contained the following: 1 µL of template DNA, 0.625 U *Taq* polymerase (Invitrogen), 10
187 µM of each primer, 0.8 µL 2.5 mM dNTPs, 10x PCR buffer, 50 mM $MgCl_2$ (Invitrogen), 10
188 µg bovine serum albumin (New England Biolabs) and dH_2O (Sigma Aldrich). PCRs were
189 carried out in a GeneAmp 9700 thermal cycler (Applied Biosystems) using the following
190 conditions: 94 ºC for 3 min; 45 cycles of 94 ºC for 30 sec, 47 ºC for 1 min, 72 ºC for 1 min;
191 and finally 72 ºC 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*

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

Primer design for Limax cinereoniger

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

DNA from field-collected *L. cinereoniger* and negative control samples were amplified using the species-specific primers in a 10 μL reaction containing ca. 200 ng of total genomic DNA, 0.625 U *Taq* polymerase (Invitrogen), 10 μM of each primer, 0.8 μL 2.5 mM dNTPs, 10x PCR buffer, 50 mM $MgCl_2$ (Invitrogen), 10 μg bovine serum albumin (New England Biolabs) diluted up to volume with sterile water (Sigma Aldrich). Thermal cycling conditions were as follows: 95 ºC for 3 min; 35 cycles of 94 ºC for 30 s, 61 ºC for 45 s, 72 ºC for 45 s; and finally 72 ºC for 7 min. This novel primer pair proved to be species-specific under the optimised PCR cycling conditions with no evidence of cross-amplification, even when tested with related slug species such as *Limax maximus*.

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 µL) contained 1 µL of template DNA, 0.625 U *Taq* polymerase
235 (Invitrogen), 10 µM of each primer, 0.8 µL 2.5 mM dNTPs, 10x PCR buffer, 50 mM $MgCl_2$
236 (Invitrogen), 10 µg 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 ºC for 3 min; 35 cycles
239 of 94 ºC for 30 s, 65 ºC for 45 s, 72 ºC for 45 s and finally 72 ºC for 10 min. Cycling
240 conditions for *L. cinereoniger* DNA samples were as follows: 95 ºC for 3 min; 35 cycles of
241 94 ºC for 30 s, 61 ºC for 45 s, 72 ºC for 45 s; and finally 72 ºC 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 µL), for amplifying *D. reticulatum* and *Arion sp.* DNA,
246 contained 1.2 µL of extracted DNA, 5 µL of Multiplex PCR Master Mix (Qiagen), 0.2 µM
247 each primer, 10 µg bovine serum albumin (New England Biolabs), and sterile distilled water
248 (Qiagen). After initial denaturing step at 95 ºC for 15 min, amplification proceeded for 35
249 cycles at 94 ºC for 30s, 53 ºC for 1 min 30 s, 72 ºC for 1 min 30 s and a final extension at 72
250 ºC 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 µg/mL).
255

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

3. Metal analyses

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

4. Statistical analyses

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

Results

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

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 predating 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

Metal concentrations in invertebrates

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

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

Seasonal and species variations in metal concentrations

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

Node diagrams (Fig. 4) show the links between mean metal concentrations in carabid species and the main prey, presented by the size of nodes and predation events in both seasons. Significant differences were found in metal concentrations between carabid species (Kruskal-Wallis ANOVA, $P<0.05$), (Table S4). *N. brevicollis* accumulated more lead and cadmium (Fig. 4, Table S4) than did the two *Abax* species (lead, $U=718.000$, $Z=5.31836$; cadmium, $U=996.000$, $Z=3.83615$; $P<0.001$). *A. parallelepipedus* accumulated more mercury than did *Nebria* species ($U=393.000$, $Z=-7.05115$, $P<0.001$). *Abax* species were dominant at the highly mercury-polluted sites.

Discussion

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

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

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

Diet composition and heavy metal tissue burden

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

Metal transfer has been measured *in situ* indirectly by measuring metal concentrations in different food chain compartments (e.g. Notten *et al*. 2005, Roodbergen *et al*. 2008). We used a similar approach in our study to measure metal concentrations in different food web compartments (soil, earthworms that ingest soil substrate, slugs as herbivores and carabids as carnivorous), in order to explore the relationship between the relative proportion of each prey 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, predating 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-primer 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

505 **Acknowledgements**
506 This work was supported by postdoctoral grant of the Croatian Science Foundation (Project
507 No. 02.03/88), awarded to LSJ and by the Croatian Ministry of Science, Education and
508 Sports (Grants No. 022-0222148-2135 and 119-1193080-1206). The Project was hosted by
509 WOCS at Cardiff University. We would like to thank numerous colleagues (S. Jelaska, I.
510 Russo, J.K. Bluemel, S. Mondol, M. Temunović, V. San Andrés, A. Sekovanić, A. Sulimanec
511 Grgec, T. Orct, S. Mataušić, K. Vlahoviček, D. Franjević) for all their help in the field and in
512 the laboratory and valuable advices on data analyses. Great thank to J. Morgan and R.
513 Donnelly for their help in choosing the study sites and for providing valuable data on soil
514 conditions.
515

516 **References**

517

518 Boreau de Roincé C, Lavigne C, Ricard J, Franck P, Bouvier J, Garcin A, Symondson WOC
519 (2012) Predation by generalist predators on the codling moth versus a closely-related
520 emerging pest the oriental fruit moth: a molecular analysis. *Agricultural and Forest*
521 *Entomology,* **14**, 260–269.

522

523 Butovsky RO (2011) Heavy metals in carabids (Coleoptera, Carabidae). *Zookeys*, **100**, 215–
524 222.

525

526 Cabana G, Rasmussen JB (1994) Modelling food chain structure and contaminant
527 bioaccumulation using stable nitrogen isotopes. *Nature (London),* **372**, 255–257.

528

529 Cameron RAD, Jackson N, Eversham B (1983) A field key to the slugs of the British Isles
530 Taunton: Field Studies Council.

531

532 Castro I, Aboal JR , Fernandez JA, Carballeira A (2011) Use of raptors for biomonitoring of
533 heavy metals: gender, age and tissue selection. *Bulletin of Environmental Contamination and*
534 *Toxicology*, **86**, 347-351.

535

536 Chen Y, Giles KL, Payton ME, Greenstone MH (2000) Identifying key cereal aphid
537 predators by molecular gut analysis. *Molecular Ecology*, **9**, 1887–1898.

538

539 Dallinger R (1993) Strategies of metal detoxification in terrestrial invertebrates. In: Dallinger,
540 R., Rainbow, P.S. (Eds.), Ecotoxicology of Metals in Invertebrates. Lewis Publishers,
541 London,pp. 245–280.
542
543 Davey JS, Vaughan IP, King RA, Bell JR, Bohan DA, Bruford MW, Holland JM,
544 Symondson WOC (2013) Intraguild predation in winter wheat: prey choice by a common
545 epigeal carabid consuming spiders. *Journal of Applied Ecology*, **50**, 271–279.
546
547 Dodd, CS (2004) Development and optimisation of PCR-based techniques in predator gut
548 analysis. PhD thesis, Cardiff University, Cardiff, UK.
549
550 European Environment Agency, Joint Research Centre (2010) The European environment —
551 state and outlook 2010, Soil. EEA and JCR, Luxemburg, Publications Office of the European
552 Union.
553
554 Eskelson MJ, Chapman EG, Archbold DD, Obrycki JJ, Harwood JD (2011) Molecular
555 identification of predation by carabid beetles on exotic and native slugs in a strawberry
556 agroecosystem, *Biological Control*, **56**, 245-253.
557
558 Freude H, Harde KW, Lohse GA, Klausnitzer B (2004) Die Käfer Mitteleuropas, Band 2,
559 Spectrum, Elsevier, München, 521 pp.
560
561 Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of
562 mitochondrial cytochrome coxidase subunit I from diverse metazoan invertebrates.
563 *Molecular Marine Biology and Biotechnology*, **3**, 294–299.

564

565 Foltan P (2004) Influence of slug defence mechanisms on the prey preferences of the carabid
566 predator *Pterostichus melanarius* (Coleoptera: Carabidae). *European Journal of Entomology,*
567 **101**, 359–364.

568

569 Georgii HW (1986) Atmospheric Pollutants in Forest Areas: Their Deposition and
570 Interception. Kluwer Academic Publishers 289 pp.

571

572 Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis
573 program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

574

575 Harada (1995) Minamata disease: methylmercury poisoning in Japan caused by
576 environmental pollution. *Critical Reviews in Toxicology*, 25/1: 1-24.

577

578 Harper GL, King RA, Dodd CS, Harwood JD, Glen DM, Bruford MW, Symondson WOC
579 (2005) Rapid screening of invertebrate predators for multiple prey DNA targets. Molecular
580 Ecology, **14**, 819–827.

581

582 Hatteland BA, Symondson WOC, King RA, Skage M, Schander C, Solhøy T (2011)
583 Molecular analysis of predation by carabid beetles (Carabidae) on the invasive Iberian slug
584 *Arion lusitanicus*. *Bulletin of Entomological Research* 101, 675-686.

585

586 Hendrickx F, Maelfait J, Langenbick F (2003) Absence of cadmium excretion and high
587 assimilation result in cadmium biomagnification in a wolf spider. *Ecotoxicology and*
588 *Environmental Safety*, **55**, 287–292.

589

590 Hengeveld R (1980) Polyphagy, oligophagy and food specialization in ground beetles
591 (Coleoptera, Carabidae). *Netherlands Journal of Zoology*, **30**, 564-584.

592

593 Holland J (ed.) (2002) *The Agroecology of Carabid Beetles*. Intercept Ltd., 356 pp.

594

595 Hopkin SP (1989) Ecophysiology of Metals in Terrestrial Invertebrates. Elsevier Applied
596 Science, London, 366pp.

597

598 Ireland MP (1981) Uptake and distribution of cadmium in the terrestrial slug *Arion ater* (L.).
599 *Comparative Biochemistry and Physiology,* **68A**, 37 – 41.

600

601 Janssen MPM, Bruins A, De Vries TH, Van Straalen NM (1991) Comparison of cadmium
602 kinetics in four soil arthropod species. *Archives of Environmental Contamination Toxicology*
603 **20**, 305-312.

604

605 Janssen MPM, Ma WC, Van Straalen NM (1993) Biomagnification of metals in terrestrial
606 ecosystem. *Science of Total Environment* Suppl., 511-524.

607

608 Jarman SN, Redd KS, Gales NJ (2006) Group-specific primers for amplifying DNA
609 sequences that identify Amphipoda, Cephalopoda, Echinodermata, Gastropoda, Isopoda,
610 Ostracoda and Thoracica. *Molecular Ecology Notes*, **6**, 268–271.

611

612 King RA, Vaughan IP, Bell JR, Bohan DA, Symondson WOC (2010) Prey choice by carabid
613 beetles feeding on an earthworm community analysed using species- and lineage-specific
614 PCR primers. *Molecular Ecology*, **19**, 1721–1732.
615
616 Kramarz P (1999) Dynamics of accumulation and decontamination of cadmium and zinc in
617 carnivorous invertebrates. 1. The Ground Beetle, *Poecilus cupreus* L. *Bulletin of*
618 *Environmental Contamination and Toxicology***, 63**, 531–537.
619
620 Kuusk AK, Agustí N (2008) Group-specific primers for DNA-based detection of springtails
621 (Hexapoda: Collembola) within predator gut contents. *Molecular Ecology Resources*, **8**, 678–
622 681.
623
624 Lindquist L, Block M, Tjälve H (1995) Distribution and excretion of Cd, Hg, methyl-Hg and
625 Zn int he predatory beetle *Pterostichus niger* (Coleoptera: Carabidae). Environmental
626 *Toxicology and Chemistry***, 14**, 1195-1201.
627
628 Luff ML (2007) The Carabidae (ground beetles) of Britain and Ireland. Carl Hildebrand
629 Lindroth, Royal Entomological Society of London, 247 pp.
630
631 Mayack DT (2012) Hepatic mercury, cadmium, and lead in mink and otter from New York
632 State: monitoring environmental contamination. *Envrironmental Monitoring and Assessment*,
633 **184**, 2497-2516.
634

635 McKemey AR, Symondson WOC, Glen DM (2003) Predation and prey size choice by the
636 carabid beetle *Pterostichus melanarius* (Coleoptera: Carabidae): the dangers of extrapolating
637 from laboratory to field. *Bulletin of Entomological Research*, **93**, 227-234.
638
639 Morgan JE, Morgan AJ (1998) The distribution and intracellular compartmentation of metals
640 in the endogeic earthworm *Aporrectodea caliginosa* sampled from an unpolluted and a metal-
641 contaminated site. *Environmental pollution*, **99/2**, 67-175.
642
643 Mršić N (1997) Živali naših tal.Uvod v pedozoologijo—sistematika in ekologija s splošnim
644 pregledom talnih živali. Tehniška založba Slovenije, Ljubljana, 416 pp.
645
646 Nahmani J, Hodson ME, Black S (2007) A review of studies performed to assess metal
647 uptake by earthworms. *Environmental Pollution*, **145**, 402-424.
648
649 Nash MA, Thomson LJ, Horne PA, Hoffman AA (2008) *Notomus gravis* (Chaudoir)
650 (Coleoptera: Carabidae) predation of *Deroceras reticulatum* Müller (Gastropoda:
651 Agrolimacidae), an example of fortuitous biological control. *Biological Control* **47**, 328–334.
652
653 Notten M, Oosthoek A, Rozema J, Aerts R (2005) Heavy metal concentrations in a soil–
654 plant–snail food chain along a terrestrial soil pollution gradient. *Environmental Pollution*,
655 **138**, 178–190.
656
657 Paill W (2000) Slugs as a prey for larvae and imagines of *Carabus violaceus* L. (Coleoptera,
658 Carabidae) pp. 221-222. In Brandmaye P, Lovei GL, Brandmayr TZ, Casale A and Vigna

659 Taglianti A (Eds.), Natural history and applied ecology of carabid beetles. Sofia, Bulgaria,
660 Pensoft Publishers.
661
662 Purchart L, Kula E (2007) Content of heavy metals in bodies of field ground beetles
663 (Coleoptera, Carabidae) with respect to selected ecological factor. *Polish Journal of Ecology*
664 **55**, 305-314.
665
666 Roodbergen M, Klok C, van der Hout A (2008) Transfer of heavy metals in the food chain
667 earthworm Black-tailed godwit (*Limosa limosa*): comparison of a polluted and a reference
668 site in The Netherlands. *Science of Total Environment*, **406**, 407-412.
669
670 Sims RW, Gerard BM (1985) Earthworms: Keys and Notes for the Identification and Study
671 of the Species. Linnean Society of London, Estuarine and Brackish-Water Sciences
672 Association, Brill Archive, 171 pp.
673
674 Stone D, Jepson P, Laskowski R (2002) Trends in detoxification enzymes and heavy metal
675 accumulation in ground beetles (Coleoptera: Carabidae) inhabiting a gradient of pollution.
676 *Comparative Biochemistry and Physiology. Part* C **132**, 105-112.
677
678 Sunderland KD (1975) The diet of some predatory arthropods in cereal crops. *Journal of*
679 *Applied Ecology*, **12**, 389–396.
680
681 Sunderland, KD, Sutton SL (1980) A serological study of arthropod predation on terrestrial
682 isopods in a dune grassland ecosystem. *Journal of Animal Ecology*. **49/3**, 987-1004.
683

684 Symondson WOC (1997) Does *Tandonia budapestensis* (Mollusca: Pulmonata) contain
685 toxins? Evidence from feeding trials with the slug predator *Pterostichus melanarius*
686 (Coleoptera: Carabidae). *Journal of Molluscan Studies* **63**: 541–545.
687
688 Symondson WOC, Cesarini S, Dodd PW, Harper GL, Bruford MW , Glen DM, Wiltshire
689 CW, Harwood JD (2006) Biodiversity vs. biocontrol: positive and negative effects of
690 alternative prey on control of slugs by carabid beetles. *Bulletin of Entomological Research,*
691 **96**, 637–645.
692
693 Symondson WOC, Glen DM, Erickson ML, Liddell JE, Langdon CJ (2000) Do earthworms
694 help to sustain the slug predator *Pterostichus melanarius* (Coleoptera: Carabidae) within
695 crops? Investigations using monoclonal antibodies. *Molecular Ecology*, **9**, 1279–1292.
696
697 Symondson, W.O.C., Glen, D.M., Wiltshire, C.W, Langdon, C.J. & Liddell, J.E. (1996)
698 Effects of cultivation techniques and methods of straw disposal on predation by *Pterostichus*
699 *melanarius* (Coleoptera: Carabidae) upon slugs (Gastropoda: Pulmonata) in an arable field.
700 *Journal of Applied Ecology* 33, 741-753.
701
702 Šerić Jelaska L, Blanusa M, Durbesic P, Jelaska SD (2007) Heavy metal concentrations in
703 ground beetles, leaf litter, and soil of a forest ecosystem. *Ecotoxicology and Environmenal*
704 *Safety*, **66**, 74-81.
705
706 Thiele HU (1977) Carabid beetles in their environments. Springer Verlag, Berlin, Heidelberg,
707 New York, 369 pp.
708

709 Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of
710 progressive multiple sequence alignment through sequence weighting, position-specific gap
711 penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673-4680.
712
713 Traugott M, Bell JR, Raso L, Sint D, Symondson WOC (2012) Generalist predators disrupt
714 parasitoid aphid control by direct and coincidental intraguild predation. *Bulletin of*
715 *Entomological Research,* **102***,* 239-247.
716
717 Van Straalen NM, Ernst WHO (1991) Metal biomagnification may endanger species in
718 critical pathways. *Oikos*, **62**, 255–256.
719
720 Van Straalen NM, van Wensem J. (1986) Heavy metal content of forest litter arthropods as
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 ±SE and whisker
742 ±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 ± SE, m±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.

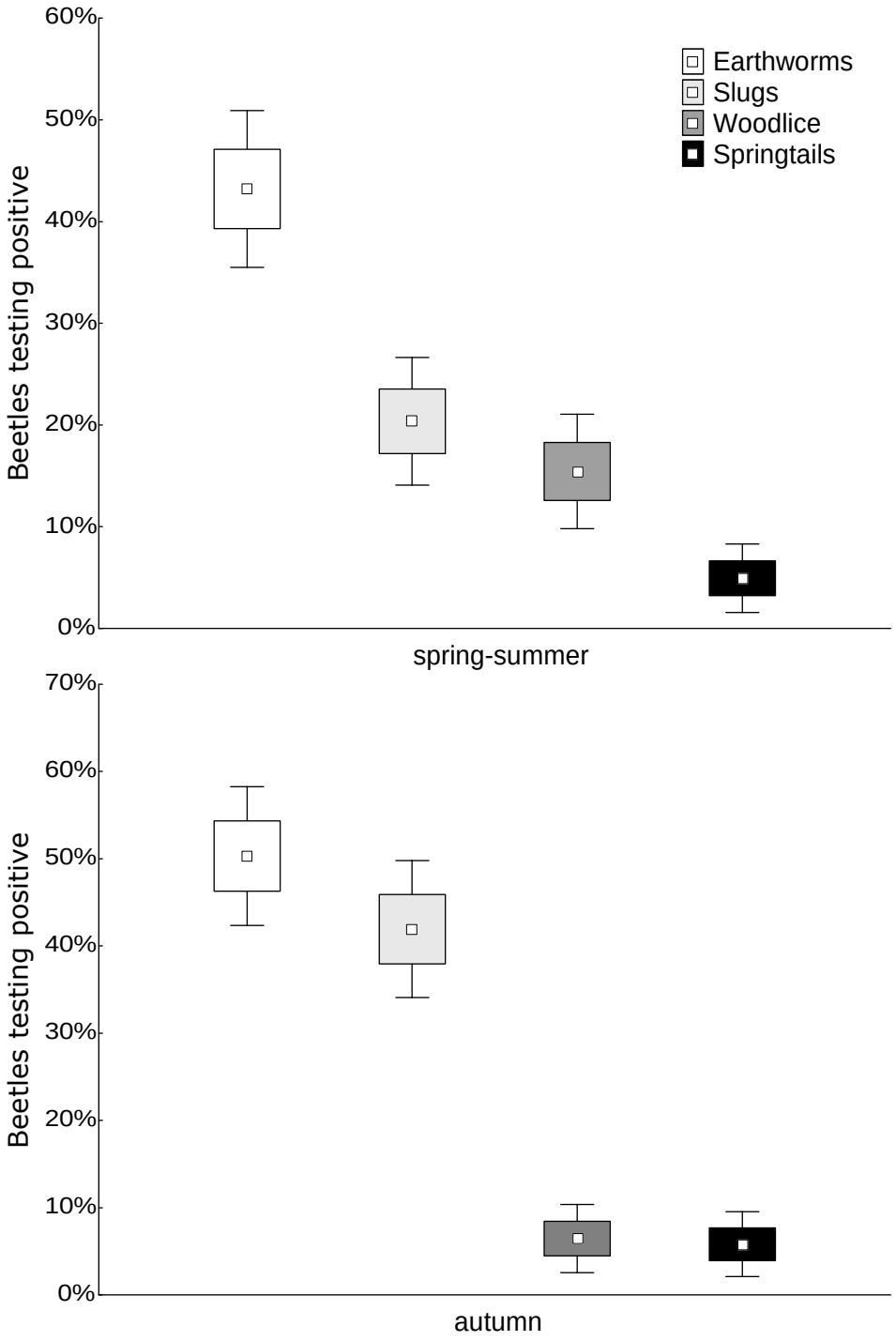
754

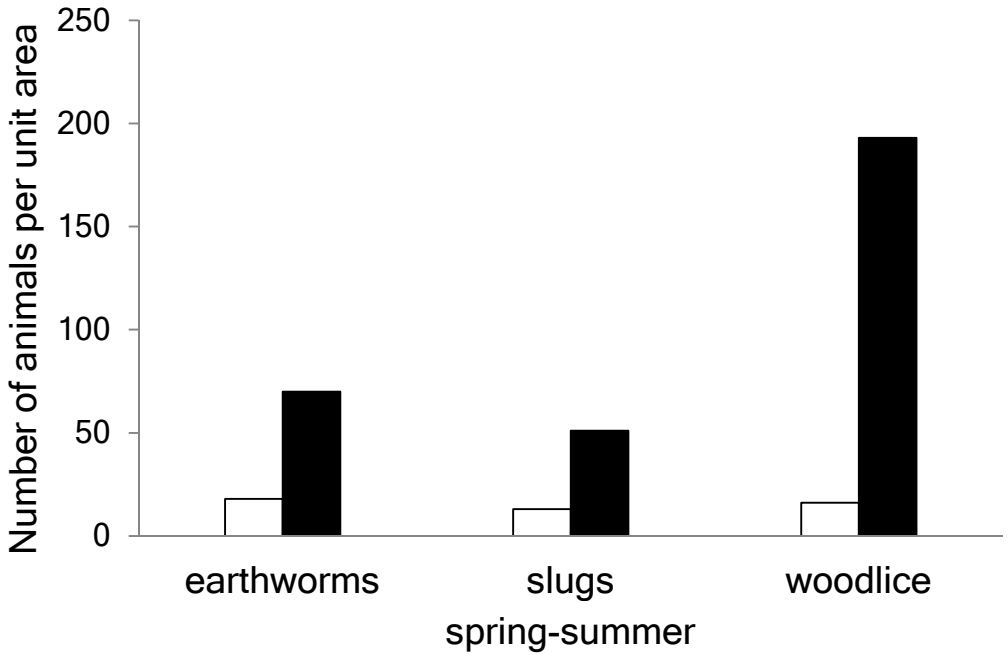
755 Figure 4. Node diagrams of observed trophic interactions between prey groups and predator
756 species with measured metal concentrations in the tissue. Size of the nodes reflects mean
757 concentrations of lead (a), cadmium (b), and mercury (c) in earthworms, slugs and carabids.
758 Yellow circles 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

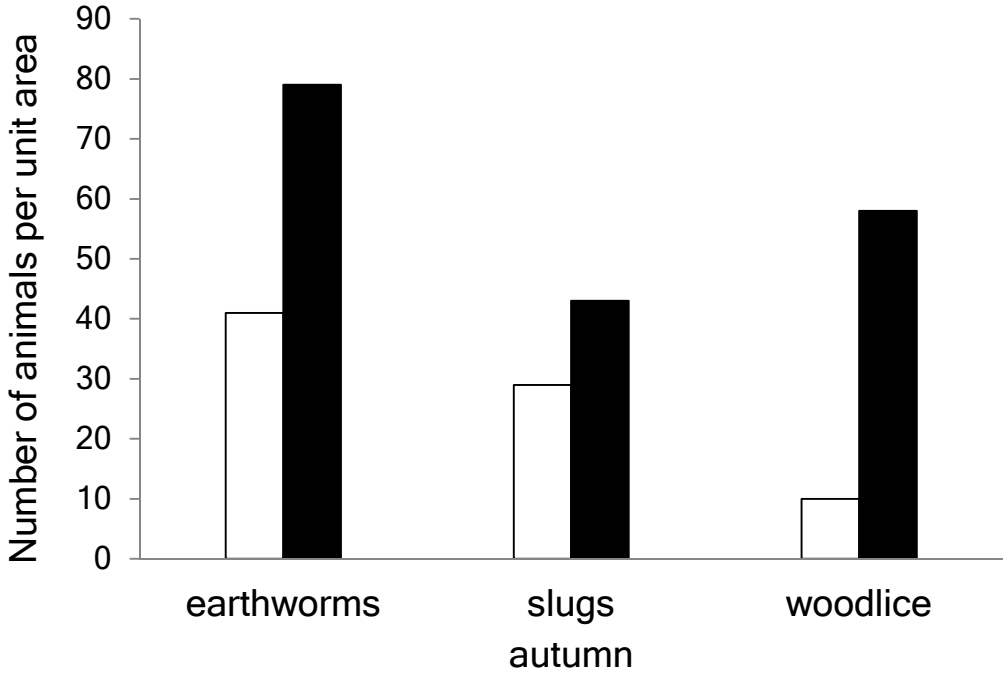
763 for Pb (0.08-623.1 mg/kg), Cd (0.1-56.5 mg/kg) and Hg (0.035-0.666 mg/kg) concentrations
764 measured in carabids. The same range is chosen for arrow thickness, representing the
765 percentage (from 0.1 to 1) of PCR positive carabid species tested for each prey within the
766 season.
767
768 Tables

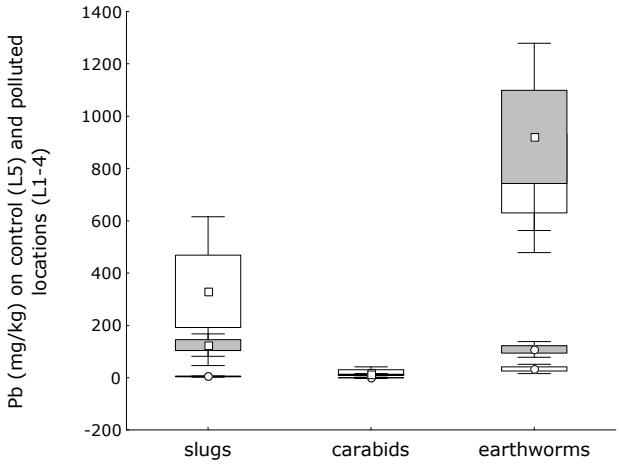
769 Table 1. Sequences of primers (5'-3') used for multiplex (M) and singleplex (S) PCR analyses.

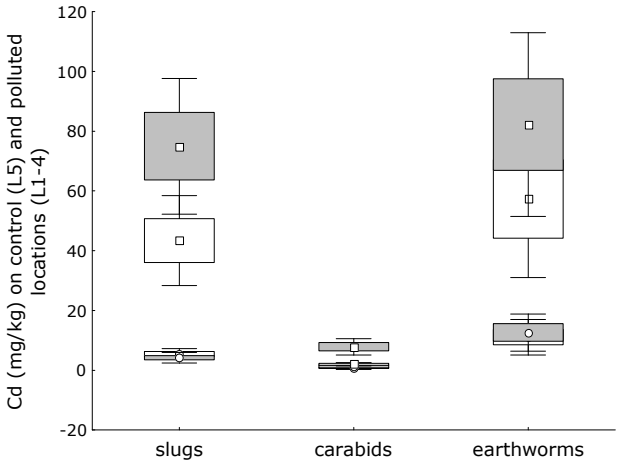
Species	PCR	Primer name	Primer sequence	Amplicon size (bp)	Reference
Earthworms	S	185F	TGTGTACTGCCGTCGTAAGCA	225-236	Harper *et al*. 2005
		14233R	AAGAGCGACGGGCGATGTGT		
Deroceras reticulatum	M	Dr11F	CTATACACAATTTTTAAATAAG	109	Dodd 2004, Harper *et al*. 2005
		DRF29RC	GCTTCTGGTTTATCTATTATTTGGT		
Arion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

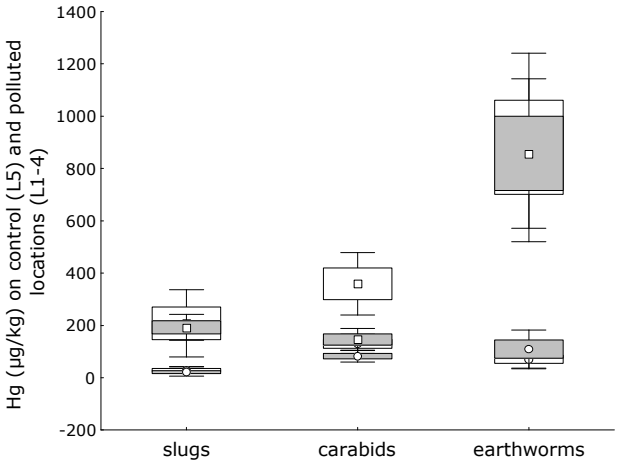

Earthworms
Slugs
Woodlice
Springtails
Beetles testing positive
60%
50%
40%
30%
20%
10%
0%
spring-summer
Beetles testing positive
70%
60%
50%
40%
30%
20%
10%
0%
autumn

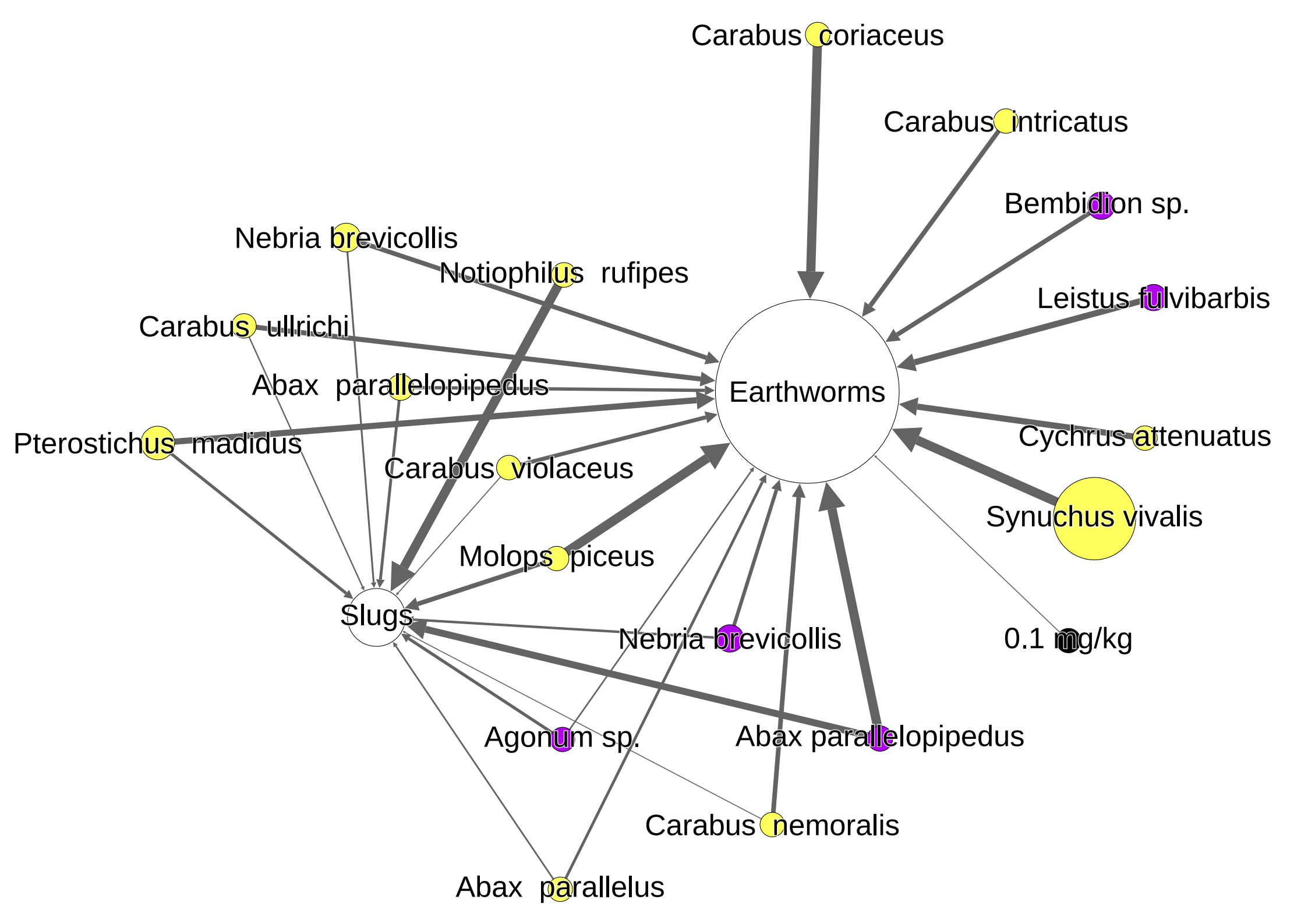

Number of animals per unit area
250
200
150
100
50
0
earthworms
slugs
woodlice
spring-summer

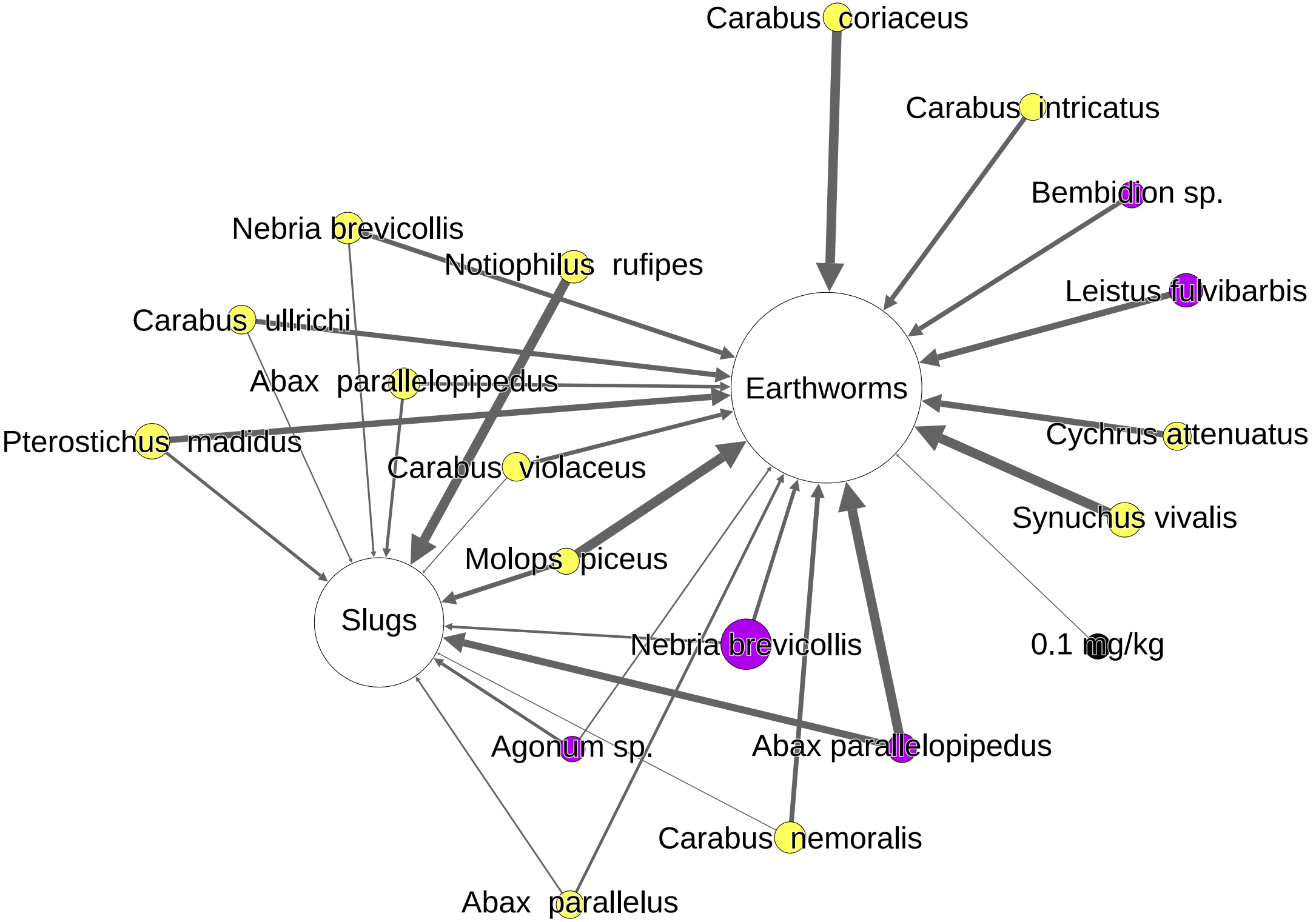

Number of animals per unit area
90
80
70
60
50
40
30
20
10
0
earthworms
slugs
woodlice
autumn

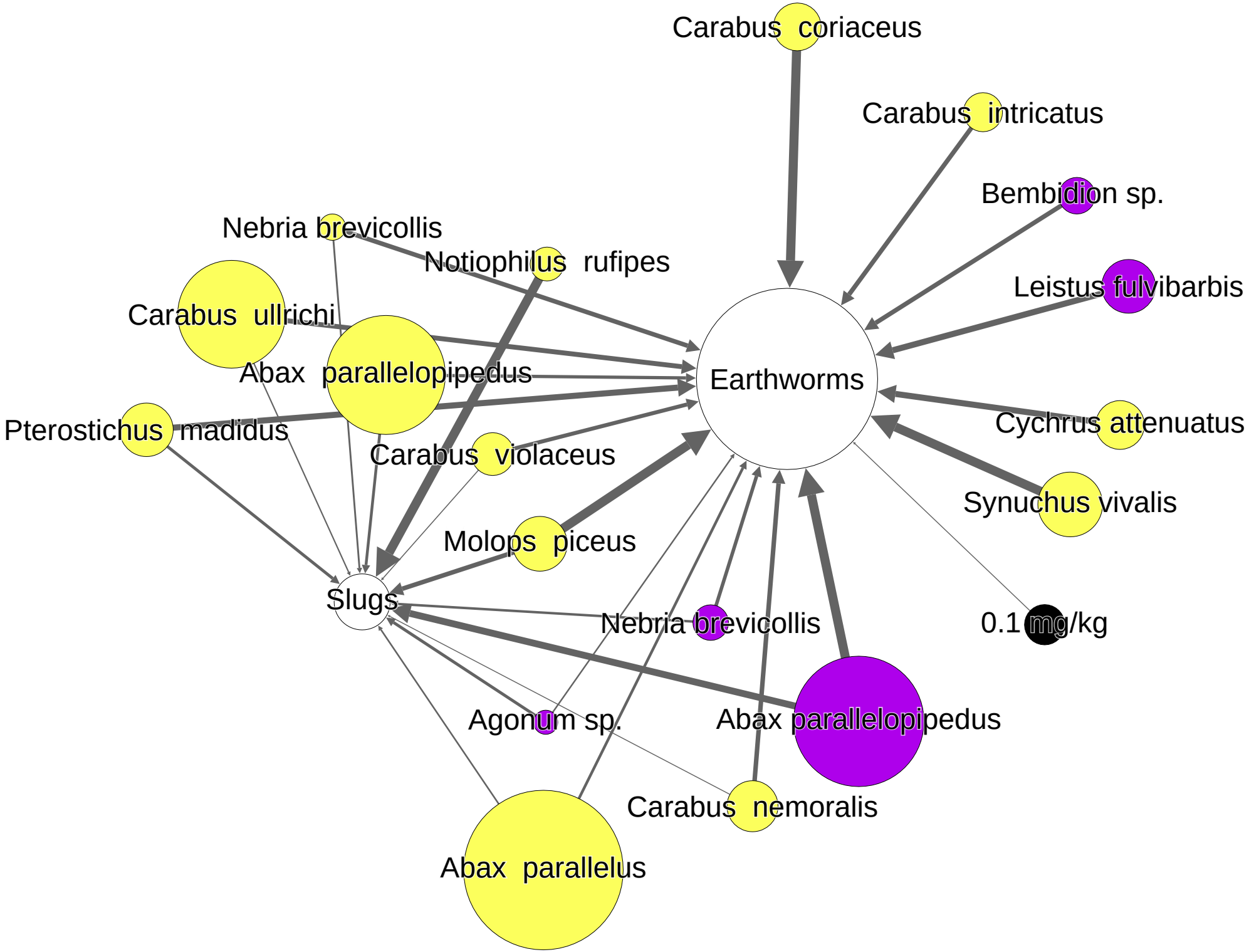

Pb (mg/kg) on control (L5) and polluted locations (L1-4)
1400
1200
1000
800
600
400
200
0
-200
slugs
carabids
earthworms


Cd (mg/kg) on control (L5) and polluted locations (L1-4)
120
100
80
60
40
20
0
-20
slugs
carabids
earthworms


Hg (µg/kg) on control (L5) and polluted locations (L1-4)
1400
1200
1000
800
600
400
200
0
-200
slugs
carabids
earthworms


Carabus coriaceus
Carabus intricatus
Bembidion sp.
Leistus fulvibarbis
Cychrus attenuatus
Synuchus vivalis
0.1 mg/kg
Nebria brevicollis
Notiophilus rufipes
Carabus ullrichi
Abax parallelopipedus
Pterostichus madidus
Carabus violaceus
Molops piceus
Earthworms
Slugs
Nebria brevicollis
Agonum sp.
Abax parallelopipedus
Carabus nemoralis
Abax parallelus


Carabus coriaceus
Carabus intricatus
Bembidion sp.
Leistus fulvibarbis
Nebria brevicollis
Notiophilus rufipes
Carabus ullrichi
Abax parallelopipedus
Pterostichus madidus
Carabus violaceus
Earthworms
Cychrus attenuatus
Synuchus vivalis
Molops piceus
Slugs
Nebria brevicollis
0.1 mg/kg
Agonum sp.
Abax parallelopipedus
Carabus nemoralis
Abax parallelus


Carabus coriaceus
Carabus intricatus
Bembidion sp.
Leistus fulvibarbis
Nebria brevicollis
Notiophilus rufipes
Carabus ullrichi
Abax parallelopipedus
Earthworms
Pterostichus madidus
Carabus violaceus
Cychrus attenuatus
Synuchus vivalis
Molops piceus
Slugs
Nebria brevicollis
0.1 mg/kg
Agonum sp.
Abax parallelopipedus
Carabus nemoralis
Abax parallelus

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 MΩ 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_3 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 ^{114}Cd, ^{202}Hg and ^{208}Pb were used for element quantification, while ^{103}Rh, ^{153}Tb and ^{193}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.

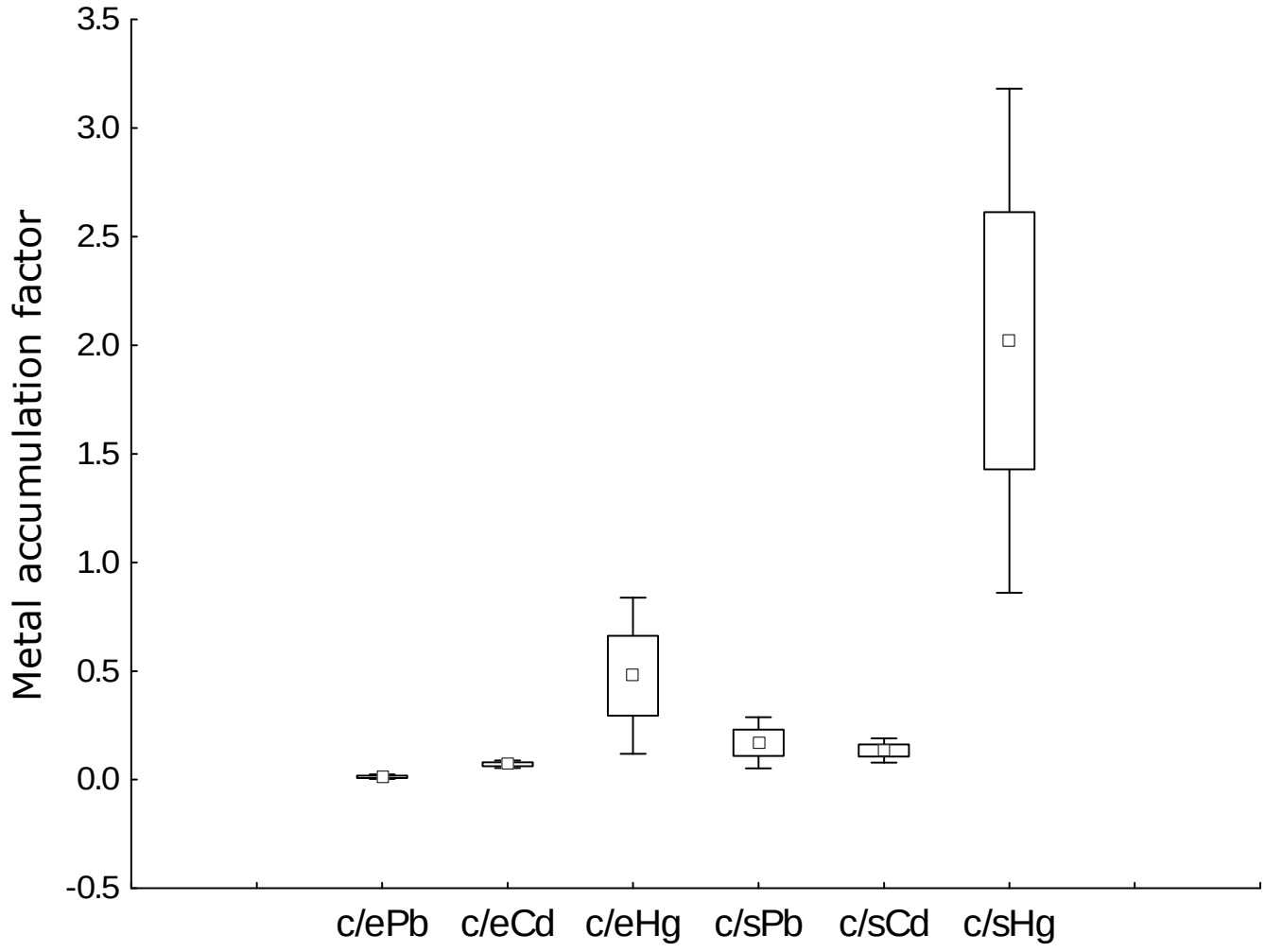


Figure S1. Mean (±SE and 1.96*SE) accumulation factors (AFs) for lead, cadmium and mercury in carabids (c) and their prey (e, earthworms; s, slugs).

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
Nebria brevicollis	3,4,5	87 (165)	40.23	26.44	10.3	6.9
Abax parallelus	1,2	68 (92)	78.95	84.21	1.5	10.3
A. parallelepipedus	1,2,3,4,5	63 (107)	39.68	33.33	34.9	1.6
Carabus nemoralis	1,2	18 (28)	72.22	38.89	0.0	0.0
C. ullrichi	2	13 (19)	53.85	7.69	0.0	0.0
C. violaceus	1,2,4,5	9 (19)	44.44	22.22	0.0	0.0
Cychrus attenuates	1,2	7 (10)	71.43	14.29	0.0	0.0
Pterostichus madidus	3,4,5	7 (16)	71.43	28.57	14.3	0.0
Agonum sp	5	6 (9)	16.67	33.33	0.0	0.0

C. convexus	2	6 (6)	83.33	0.0	0.0	0.0
C. coriaceus	2	6 (8)	83.33	33.33	0.0	0.0
C. intricatus	1,2	6 (7)	50.00	0.0	0.0	0.0
Leistus fulvibarbis	3,4	3 (6)	66.67	0.0	0.0	66.7
Bembidion nigricorne	3	2 (5)	100	0.0	0.0	0.0
C. problematicus	4	2 (2)	0.0	50.00	50.0	0.0
Molops piceus	2	2 (4)	100	50.00	0.0	0.0
P. melanarius	3,4	2 (2)	50.00	50.00	0.0	0.0
P. transversalis	1,2	2 (2)	50.00	50.00	0.0	0.0
Synuchus vivalis	3,4	2 (7)	100	0.0	50.0	0.0
Aptinus bombarda	1	1 (1)	100	0.0	0.0	100
Notiophilus rufipes	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	*N* 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^{1a} ±244.9
	s	s	6		0.748±1.38	6.662±7.74	57.88^{1b} ±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
L3 (UK)	c	a	22		26.17[2a] ±25.17	9.480±18.21	123.2±59.48
	c	s	15		114.8[2b] ±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[3a] ±149.7
	s	s	9		940.7±1092	84.98±38.11	478.3[3b] ±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
L4 (UK)	c	a	45		8.020±7.18	8.989±9.97[4a]	58.40±26.91
	c	s	12		4.628±3.15	2.752±3.27[4b]	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[5a] ±17.52	53.27±29.60	118.3±61.92
	s	s	10		73.33[5b] ±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[6a] ±0.09	1.346±1.78	82.72±37.21
	c	s	25		0.457[6b] ±0.56	0.776±0.64	129.7±87.91
	e	a	18		107.9[7a] ±60.10	12.62±12.46	109.8±146.9
	e	s	9		33.63[7b] ±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[8a] ±0.28	76.2[9a] ±17.6
	soil	s	16	4.12±0.10	56.05±12.7	0.466[8b] ±0.15	170.2[9b] ±43.9

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	*N*	Body weight (g dry weight)	Pb mg/kg	Cd mg/kg	Hg μg/kg
Abax ater	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
A. parallelus	L1 (Cro)	3	0.133	0.385	1.146	155.6
	L2 (Cro)	21	0.069	0.305	0.825	639.3
Agonum sp.	L5 (UK)	3	0.019	0.226	0.164	35.17
Bembidion quadrimaculatum	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

Carabus cancellatus	L4 (UK)	1	0.144	6.783	0.168	34.35
C. coriaceus	L2 (Cro)	2	0.128	1.780	0.823	0.252
C. intricatus	L1 (Cro)	1	0.336	0.399	0.591	95.15
C.nemoralis	L1 (Cro)	1	0.288	1.459	0.474	131.9
	L2 (Cro)	9	0.275	0.266	2.335	143.8
C. ullrichi	L2 (Cro)	6	0.293	0.084	1.245	370.9
C. violaceus	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
Cychrus attenuatus	L1 (Cro)	1	0.075	0.200	0.054	83.67
	L2 (Cro)	2	0.088	0.486	1.531	159.0
Cy. caraboides	L5 (UK)	1	0.151	0.166	0.084	105.9
Leistus fulvibarbis	L4 (UK)	1	0.006	7.852	2.883	153.6
Molops piceus	L2 (Cro)	2	0.040	0.122	0.402	157.6
Nebria brevicollis	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
Pterostichus fasciatopunctatus	L1 (Cro)	1	0.064	0.284	0.238	43.42
P. madidus	L3 (UK)	4	0.041	79.69	7.510	162.6
	L5 (UK)	5	0.044	1.138	0.448	145.6
Synuchus vivalis	L3 (UK)	3	0.004	374.5	4.306	317.5
	L4 (UK)	2	0.006	5.823	1.652	18.02