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Trace metal transfer in a soil - plant - snail microcosm field experiment and biomarker responses in snails

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Capsule: Long term metal transfer and biomarker response in caged snails.

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Abstract

A microcosm experiment was performed to investigate temporal (up to 16 weeks) and spatial variation in metal transfer in a soil - food (nettle) - snail (*Cepaea nemoralis*) food chain and biomarker responses in the digestive gland of the same snails. Adult snails were sampled from an uncontaminated site and transferred to five sites located 0.5, 2.5, 3, 5, and 10 km from a historically polluted point source. All sites were park areas where grasslands interfered with patches of deciduous forest. Soil physicochemical properties (pH, clay %, OC %) significantly explained the uptake of metals by nettle. Concentrations of metals in the digestive gland (DG) of snails were significantly related to those in nettle, but rarely to soil physicochemical properties. In general metal concentrations in the DG fluctuated while As, Ni, Pb and Zn showed a site dependent increase with time. Despite the long term exposure, biomarker concentrations (lipid, glycogen, proteins, glutathione S-transferases), and shell morphology, were not related to DG metal concentrations. Our investigation emphasizes the need for controlled long-term studies on the transfer and effects of metals in food chains since short term studies might only show temporary physiological changes due to experimental acclimation.

Highlights:

- ►Significant transfer of trace metals in a soil–food–snail food chain.
- ►Metal levels in snails are well–explained by levels in the food plant.
- ►An increase of trace metals (As, Ni, Pb and Zn) in the digestive gland was observed.
- ►Substantial spatio–temporal variation in metal concentration in nettle and snails.
- ►No biomarker effects were observed after long term (16 weeks) trace metal exposure.

1. Introduction

Elevated concentrations of trace metals can adversely affect soil health and may present an ecological and human health risk if they enter the food chain (Robinson et al., 2006). The bioavailable soil metal fraction can be taken up by plants and/or animals and subsequently transferred to higher levels of the food chain (Dallinger et al., 2001; Vermeulen et al., 2009b). The study of the behaviour of trace metals in food chains is therefore essential to quantify the risk of trace metal consumption by higher organisms.

Often no correlation can be found between the outcomes of field and laboratory based food chain studies. This is because laboratory studies tend to over-simplify the complexity and variability of exposure (Spurgeon and Hopkin, 2000). To partly account for the high complexity of exposure to pollutants, soil scientists proposed to actively biomonitor sites using stationary and restricted "microcosms" or cages which permit to study sentinel species *in situ*, under realistic field exposure scenarios (e.g. Moore et al., 1996; Solomon and Sibly, 2002). The use of microcosms has several advantages; they are simple and cheap (Scheifler et al, 2006), they have proved successful in detecting metal related changes when used in ecological risk assessment studies (Weyers et al., 2004), and they allow replication, even though replication in the true sense in field situations is impossible (Giesy and Odum, 1980). Moreover, they reduce the avoidance of contaminated food by the sentinel species and may partly allow control for the patchy distribution of contaminants.

Various land snail species (Gastropoda, Pulmonata) have become popular in microcosm studies because they accumulate high concentrations of certain trace metals via oral, dermal and respiratory routes of exposure (Regoli et al., 2006; Scheifler et al., 2006). Trace metals tend to accumulate in their digestive gland to such an extent that they are considered as appropriate sentinels of metal pollution (e.g. Boshoff et al., 2013; Gomot and Pihan, 1997; Gomot de Vaufleury and Pihan, 2000). Appropriate sentinel species should be easy to collect and to manipulate, should be abundant to allow proper statistical analysis, and should be large

79 enough to measure several potential biomarkers of metal stress (Beeby, 2001; Van Gestel and
80 Van Brummelen, 1996). Biomarkers are useful descriptors or endpoints of field situations
81 which may allow the identification of chemical stressors and their potential ecological risk.
82 They give additional information that cannot be obtained from chemical analysis of pollutant
83 concentrations alone, and they may integrate effects of mixtures of chemicals over long
84 exposure periods (Beeby, 2001; Van Gestel and Van Brummelen, 1996).
85 Microcosm studies using helicid land snails showed an increase of trace metals in soft
86 tissues of the snails with increasing soil metal concentrations and accumulation of trace
87 metals with time (e.g. Fritsch et al., 2011; Scheifler et al., 2003). However, the effects on the
88 snail's condition through the quantification of biomarkers have hardly been investigated.
89 Metal exposure did not negatively affect snail condition (body weight, shell size and shell
90 weight) in one study (Fritsch et al., 2011) but this may be because the time of exposure (up to
91 seven weeks as in the majority of microcosm studies) may be too short to have affected the
92 snail's condition (Fritsch et al., 2011). Indeed, longer exposure periods (up to 16 weeks) may
93 improve the determination of actual concentrations available for transfer in the food chain
94 (Scheifler et al., 2006). Hence, there is a need for long-term microcosm studies that would
95 allow a multidisciplinary and integrative approach in which soil physicochemical properties,
96 accumulation patterns, and biochemical, or cellular, responses to pollutants in the sentinel
97 organism are investigated simultaneously (Shugart et al., 1992).
98 In the present study we used such a multidisciplinary microcosm approach to (1) test if soil
99 physicochemical properties, as well as soil and nettle (*Urtica dioica*) metal concentrations,
100 affect trace metal accumulation in the digestive gland of the snail *Cepaea nemoralis* (2)
101 determine if metal concentrations in the digestive gland of snails affect biomarker responses
102 (3) investigate the effect of exposure time, site and metal type on metal accumulation and
103 biomarker response in snails.
104

105 **2. Material and Methods**

106 *2.1 Study sites and species*

107 The city of Antwerp (North of Flanders, Belgium) is a densely populated urban area with a
108 history of industrial activity and metal pollution. From the mid 1950's till the 70's, non-
109 ferrous smelting activities were prominent in this area. Even though emission is nowadays
110 strictly controlled the persistent character of metals ensures their long term presence in
111 contaminated soils (OVAM, 2008). Accumulation of pollutants by (mostly) vertebrates has
112 been well-studied in the area (e.g.; Rogival et al., 2007; Tersago et al., 2004; Vermeulen et al.,
113 2009 a,b). Five sites in the Antwerp region were selected of which the "Umicore Precious
114 Metal Refinery" (UPMR) in Hoboken (UMI) is a historically polluted point source and a still
115 active smelter. The other sites were "Fort 7" (F7), "Hobokense Polder" (HOP), "Fort 6" (F6),
116 and "Kontich-Kazerne" (KK) which were at distances of respectively 0.1, 2.5, 3, 5, and 10 km
117 from UMI. All sites were park areas where grasslands interfered with patches of deciduous
118 forest; the UMI site itself is located in a similar landscape.

119 For this study we used the common European land snail species *Cepaea nemoralis*
120 (Linnaeus, 1758) (family Helicidae). This species prefers fresh *Urtica dioica* leaves as diet
121 (Wolda et al., 1971) and is easy to collect in high numbers. Both *C. nemoralis* and *U. dioica*
122 are common at the study sites.

123

124 *2.2 Experimental setup*

125 In September 2011, 375 adult *C. nemoralis* snails were collected from an uncontaminated
126 site situated in Eke (Belgium), (49°38'8.4" N, 4°37'32" E). In the lab each snail was
127 described in terms of banding and colouring and consequently coded to allow later
128 identification (Murray, 1975) and to make sure that no autochthonous snails accidentally
129 entered the microcosms (none did). Thereafter wet weight of each individual (animal and
130 shell) was determined with an electronic balance (Kern and Sohn GmbH, Germany) to the

nearest 0.0001 g. Shell height and width were taken with digital calipers (573-121-10 NTD12-15C, Japan) to the nearest 0.01 mm.

Snails were exposed in cylindrical, stainless-steel microcosms (thickness 1 mm, diameter and height 25 cm) (Fritsch et al., 2011). Five microcosms per site, each with 15 snails, were pushed 10 cm deep into the soil (therefore from soil surface to top the microcosms were 15cm high) at a distances of 1-5 m over an area with abundant natural nettle coverage (no nettle leaves were added to the microcosms throughout the experiment). After placing the microcosms all signs of autochthonous snails were removed. The top of each microcosm was securely covered with an iron grid.

Snails were able to feed on soil, litter and vegetation and were exposed via dermal, respiratory and digestive routes. During the exposure period no mortality was observed. Over a period of 16 weeks three snails were removed (week 0 = unexposed snails) after 1, 2, 4, 8 and 16 weeks (WE1 to WE16) from each microcosm, and weighed as described above. All snails were then frozen. Three times between weeks 4 and 8, five *U. dioica* leaves were randomly sampled in close proximity to each of the microcosms. Nettle leafs were pooled and considered as one sample. Surface soils which included the litter layer were sampled after 16 weeks from the exact patch where each microcosm stood.

2.3 Soil physicochemical characteristics

Per site, in the same patch of each microcosm surface soil samples 3-4 cm (i.e. a mixture of litter and soil) were collected.

Soil pH was determined on 5.0 g of fresh soil samples in a 1:5 v/v suspension of soil in 1M KCl with a glass electrode (744 Metrohm, Switzerland) (Vanhoof et al., 2007). The organic matter content of the soil samples was determined by loss on ignition (LOI) (Schumacher, 2002) following Nelson and Sommers (1996) and Heiri et al. (2001). Particle size distribution was analyzed on 1.0 g dry soil using laser diffraction (Malvern Mastersizer

157 S., UK) (Queralt et al., 1999). Soil texture was expressed as a percentage based on particle
158 size (i.e. clay < 2 µm; silt < 50 µm; sand 50 µm-2 mm) and described using the GRADISTAT
159 program (Blott and Pye, 2001). We used only the clay percentage for our analysis since clay
160 fractions < 2 µm are generally known to adsorb higher metal concentrations than any other
161 soil fraction. Soil types (i.e. sandy loam or loamy sand) were classified according to the
162 proportions of the different soil fractions (clay %, loam %, sand %) and the Belgian soil
163 triangle (Blott and Pye, 2001).
164
165 *2.4 Metal concentration analysis of soil, nettle and snail digestive gland*
166 Soil samples were dried for 64 h at 60 °C and cooled in a desiccator. The aqua-regia
167 extractable metal contents have sometimes been called the pseudo total contents, and those
168 metals not soluble in aqua regia are considered to be mostly bound to silicate minerals and
169 unimportant for estimating the mobility and behavior of elements (Chen and Ma, 2001). The
170 pseudo total metal concentration was measured following Rogival et al. (2007). After cooling,
171 the samples were diluted to 40 ml with ultrapure water (MilliQ®, USA) and stored at 10 °C
172 until metal analysis. Soil concentrations were compared to the Flemish soil quality standards.
173 The standards include a correction for organic matter and clay content, allowing a comparison
174 between different soil types (VLAREM, 2012).
175 Sampled nettle leaves were not washed but placed directly into brown paper bags for oven
176 drying at 60 °C for 96 h. After drying, nettles were ground and weighed to approximately 0.1
177 - 0.2 g into 50 ml polypropylene tubes to which a mixture of hydrochloric acid (HCl; 37%)
178 and nitric acid (HNO_3; 69 %) in a ratio 3:1 v/v were added. Samples were left for four weeks
179 to soak in the acid after which they were digested in an open heat bock (Environmental
180 express 54 Hotblock™ SC154, USA) for two hours (30 min at 60 °C and 90 min at 135 °C)
181 (Agemian et al., 1980). After cooling, the samples were diluted to 40 ml with ultrapure water

(MilliQ®, USA) and stored at 10 °C until metal analysis. Per site the average metal concentration in *U. dioica* leaves and soil per 5 microcosms was calculated.

Upon arrival in the lab, snails (animal and shell) were weighed to the nearest 0.0001 mg using an electronic balance (Mettler AT261 - Mettler Toledo, Belgium) after which they were stored at -80 °C. As such, each snail was frozen within three hours after collection.

Before dissection, snails were separated from their shells and the shells were weighed separately (to the nearest 0.0001 mg). The digestive gland (DG) of snails was isolated from the body and divided in two; one part was dried for metal concentration determination, while the other part was used for the biomarker analyses. The digestive gland is the major site for metal accumulation in terrestrial snails (e.g. Boshoff et al., 2013) and plays a crucial role in metal detoxification and processing (e.g. Kammenga et al., 2000). The part of the DG used to determine trace metal concentrations was dried at 60 °C for 24 h and cooled in a desiccator after which the dry weight (0.02-0.03 g), was weighed to the nearest 0.0001 g. They were subsequently digested in nitric acid (HNO_3; 69 %) and hydrogen peroxide acid (H_2O_2; 30 %) in a ratio 20:1 v/v using a microwave (Blust et al. (1988). After cooling, the samples were diluted to 12 ml with ultrapure water (MilliQ®, USA) and stored at 10 °C until metal analysis.

The metal concentrations of As, Cd, Cu, Zn, Ni and Pb were measured in soil, nettle, and snail DG samples by means of an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Varian Ultra Mass 700, Australia). Blanks and standard certified reference materials (CRM) were prepared in the same manner as the samples and analyzed for quality assurance. Certified reference material (Institute for Reference Materials and Measurements (IRMM), Geel, Belgium) to verify recoveries were: light and sandy soil (BCR 142) and sewage sludge (BCR 144) for soil, hay powder (BCR 129) and white clover (BCR 402) for nettle, and mussel tissue (BCR-CRM 668) and bovine liver (BCR-CRM 185R) for snails. All concentrations are reported on a dry-weight basis. The detection limits for all metals were 0.1

207 µg/L except for As 0.2 µg/L. For all metals in the digestive gland, nettle and soil the
208 recoveries were equal to 90-110 % of the certified values in the CRM reference material used.
209
210 *2.5 Biomarker analysis*
211 Biomarkers in the digestive gland were measured in 50 snails which were collected in
212 week 16. Therefore, two snails per microcosm were sampled from each of the five
213 microcosms placed at the five sites.
214 *2.5.1 Measurement of shell strength and thickness*
215 Shell strength of snails was measured with an isometric Kistler force transducer (type
216 9203, Kistler Inc., Switzerland) connected to a Kistler charged amplifier (see Jordaens et al.
217 (2006)).
218 *2.5.2 Energy reserves and glutathione s-transferases measured in the digestive gland*
219 The other part of the DG was used for biomarker analysis and was homogenized in a 0.5
220 ml phosphate buffer (0.1 M Na_2HPO_4 and KH_2PO_4, pH 7.4) (Habig, 1974) using an electrical
221 homogenizer (65518 Motorhandstück MHX/E, Xenox, Germany). Samples were further
222 centrifuged for one minute at 4 °C and 5.9 *g* (Eppendorf Centrifuge 5415 R, Germany). The
223 supernatant was divided over four vials of 100 µl, 50 µl, 200µl and 100 µl to measure energy
224 reserve concentrations, more specifically lipid, protein and glycogen content and Glutathione
225 S-transferases concentration, respectively.
226 The expression of the lipid content was analysed using the Bligh and Dyer (1959) method.
227 Protein concentrations were measured with the Bradford method (Bradford, 1976) while
228 glycogen content was determined using the method of Roe and Dailey (1966). Lipid, protein
229 and glycogen content were expressed in mg/g.
230 Total energy reserves (kilojoules per gram (kJ/g)) were calculated according to Bowen et
231 al. (1995): 1 g protein, lipid or glycogen equals 17.3, 38.9 and 16.9 kJ energy, respectively.

232 Glutathione S-transferases specific activity was measured according to the method of
233 Habig et al. (1974). Activity was expressed as ml mol/min/mg of proteins.
234
235 *2.6 Statistics*
236 The statistical distribution of the data was checked with the Shapiro-Wilk test. Data was
237 $\log_{10}(x+1)$ transformed to approach normality (i.e. except pH which is already in a log
238 format). Data are presented as mean ± S.D. (standard deviation). Comparison between sites
239 for soil physicochemical properties, metal concentrations (in plants and soil) and for
240 biomarkers over time was done by ANOVA at a 5 % level of significance. Significant
241 difference between two means was measured using Bonferonni's multiple comparison test
242 (Abdi, 2007).
243 The effects of "site", and "time" and their interaction on metal accumulation in the DG
244 were analyzed by two-way ANOVA. Furthermore, the effect of "site", "time" and "metal" on
245 biomarker response was analyzed by three-way ANOVA.
246 Two principle component analysis (PCA) were conducted, a first to explore the
247 associations between soil physicochemical properties, metal concentrations in soil and metal
248 concentrations in *U. dioica*, and a second to explore the relationship between metal
249 concentrations in the DG of snails and biomarker responses. In both cases the gradient length
250 is the amount of variation explained by the combination of environmental variables (principal
251 components; PC) and is expressed as SD. Since a short gradient length (SD < 3) was
252 determined using a preliminary Correspondence Analysis (CA), a linear response model was
253 used. Scores were divided by SD and log transformed. Scaling was focused on inter species
254 distance.
255 Stepwise multiple linear regression (MLR) analyses was used to identify the dominant or
256 best contributing variables (soil physicochemical properties, soil metal concentrations, and *U.*
257 *dioica* concentrations) influencing metal accumulations in *U. dioica* and in the snail DG.

Similarly, stepwise MLR analyses were applied to investigate which metal concentrations in the DG of snails contribute significantly to explain biomarker responses. Statistical analyses were based on replicate measurements of biomarkers in the digestive gland, soil and vegetation per microcosm and site.

The data were analyzed with the statistical packages SPSS (IBM SPSS Software, Inc.), SigmaPlot 11.0 (Systat Software, Inc.) and GraphPad Prism 6 (GraphPad Software, Inc.) while ordination models were calculated using CANOCO version 4.5. (ter Braak and Smilauer, 1998).

3. Results

3.1. Soil physicochemical properties and metal concentrations

Table 1 show for each site the soil type and the mean, standard deviation (SD) and range of pH, clay % and OC %. ANOVA indicated significant differences in soil physicochemical properties among all sites (Table 2) while Bonferonni's multiple comparison tests indicated that KK and F6 shared similar soil characteristics.

Figure 1 gives an overview of the mean ± SD and significant differences in pseudo total metal concentrations in the soil. ANOVA showed significant among site variation for all measured trace metals in the soil; i.e. for As: $F = 142.70$, $p < 0.0001$, Cd: $F = 56.12$, $p < 0.0001$, Cu: $F = 118.70$, $p < 0.0001$, Ni: $F = 21.52$, $p < 0.0001$, Pb: $F = 53.64$, $p < 0.0001$, and Zn: $F = 20.25$, $p < 0.0001$ ($df = 4, 25$ in all cases). In general, HOP showed the highest mean concentrations, followed by UMI and F7, while the sites situated the furthest away from the point source, F6 and KK, showed the lowest mean metal concentrations.

The Flemish soil quality standards mg/kg (dw) were exceeded for As at HOP (86.36 ± 22.19) and UMI (29.71 ± 11.02), for Cd at HOP (20.85 ± 8.41), F7 (8.80 ± 2.38), and UMI (15.56 ± 8.73), for Cu at HOP (1007.40 ± 266.20) and UMI (261.39 ± 179.35), for Pb at HOP (1426.16 ± 270.79) and UMI (1073.77 ± 814.01), for Ni at HOP (36.53 ± 17.45) and UMI

(19.22 ± 9.50) and for Zn at all sites KK (c) (319 ± 14.43), F6 (104.52 ± 75.82), F7 (220.20 ± 71.10), HOP (799.10 ± 254.20) and UMI (394.29 ± 160.81) (VLAREM, 2012).

ANOVA showed significant among site variation for all measured trace metals in the soil; i.e. for As: $F = 142.70$, $p < 0.0001$, Cd: $F = 56.12$, $p < 0.0001$, Cu: $F = 118.70$, $p < 0.0001$, Ni: $F = 21.52$, $p < 0.0001$, Pb: $F = 53.64$, $p < 0.0001$, and Zn: $F = 20.25$, $p < 0.0001$ ($df = 4, 25$ in all cases). In general, HOP showed the highest mean concentrations, followed by UMI and F7, while the sites situated the furthest away from the point source, F6 and KK, showed the lowest mean metal concentrations.

3.2 Urtica dioica *metal concentrations*

On average, As, Cd and Pb concentrations in *U. dioica* sometimes exceeded the European Commission maximum tolerant levels for animal food such as grasses, which could pose a potential risk to herbivores such as snails or grazers and small mammals higher up the food web (Directive 2002/32/EC, 2002 measured in mg/kg with a moisture level of 12%)". This was the case for As at UMI (tolerant level: 4 mg/kg), Cd at HOP and UMI (tolerant level: 1 mg/kg) and Pb at HOP and UMI (tolerant level: 10 mg/kg).

A paired t-test was used to evaluate the difference in mean leaf metal concentrations and mean soil metal concentrations per metal and per site. Mean leaf metal concentrations were significantly lower in *U. dioica* compared to mean metal concentrations measured in soils from the same location. Leaf metal concentrations in *U. dioica* differed significantly among sites for As: $F = 121.90$, $p < 0.0001$, Cd: $F = 27.74$, $p < 0.0001$, Cu: $F = 43.92$, $p < 0.0001$, Ni: $F = 44.00$, $p < 0.0001$, Pb: $F = 69.32$, $p < 0.0001$, and Zn: $F = 17.82$, $p < 0.0001$ ($df = 4, 25$ in all cases) (Figure 1). More specifically, the highest mean leaf metal concentrations were measured at UMI, followed by HOP and F7. At F6 and KK comparable, but lower, leaf metal concentrations were measured than at UMI, HOP and F7 (Figure 1).

3.3. Cepaea nemoralis *digestive gland metal concentrations*

Figure 2 shows the mean ± SD and significant differences in metal accumulation in the DG between sites within each sampling period. The highest metal concentrations in the DG were measured at UMI > HOP > F7 while the lowest concentrations were measured at F6 and KK. Metal concentrations in the DG follow the same trend as metal concentrations in *U. dioica*.

In general metal concentrations in the DG fluctuated between sites and within each sampling period except in the case of Cu (WE 1-2), Cd (WE 1-8) and Zn (WE 1-16). However, when we compare metal concentrations in the DG within the same site over time a significant site dependent increase is observed for As, Ni, Pb and Zn (Figure 2).

Figure 3 shows the PCA results based on soil properties, and metal concentrations in the DG, soil and nettle. The first (81.4%) and second (11.5%) PC together explained 92.9% of the total variation. All metal concentrations (except Cd and Zn in the DG) in soil, nettle and the DG correlated positively (all $R > 0.70$) with PC 1 while pH correlated negatively with PC 1. Cadmium and Zn in the DG of snails correlated positively with PC 2, while clay and OC % correlated negatively with PC 2. The sites KK, F6 and F7 are characterized by higher pH-values than HOP and UMI, but with low levels of metals in the soil, nettle and DG of snails. HOP is characterized by high clay % and OC % and with the highest metal concentrations in the soil. The UMI site is associated with the lowest levels of clay and OC %, but with the highest metal levels measured in the DG of the snails.

ANOVA showed that there was a significant “Site x Time” interaction for As, Ni and Pb (Table 3). These results are visualized in Figure 2 where As, Ni and Pb concentrations gradually increase over time and per site. Although a significant effect of time and site was observed for Cu no interaction was observed. There was neither a significant difference in accumulation of Cd in the DG among sites or with time while Zn in the DG increased with time with no difference in accumulation among sites.

335 Stepwise MLR showed that metal concentrations in soil significantly contributed to metal
336 concentrations in nettle (Table 4). Also clay % contributed significantly to As in nettle, OC %
337 and pH to Cd in nettle and lastly pH to Ni and Zn in nettle. Furthermore a second MLR
338 showed that DG metal concentrations (i.e. except for Zn), were significantly related to the
339 concentrations found in the nettle leaves (Table 5). Cadmium concentrations in the DG were
340 also determined by the OC % of the soil. The Zn DG concentration could not be explained by
341 any of the variables.
342
343 *3.4. Shell morphology, energy reserves and Glutation-S-transferase*
344 Figure 4 gives an overview of the mean ± SD and significant differences in biomarker
345 responses as a function of site and time. No consistent variation in morphological or
346 physiological biomarker response was observed at any of the study sites. The highest
347 concentrations of metals were measured in the DG of snails sampled at UMI but no
348 significant relationships with biomarker responses were found.
349 Figure 5 shows the results of the PCA analysis on the biomarker responses and the metal
350 concentrations measured in the DG of snails. The first (87.7%) and second (6.4%) PC
351 together explained 94.1% of the variation. All measured metal concentrations in the DG
352 correlated positively (all $R > 0.70$) with PC 1. Shell strength, protein, glycogen and total
353 energy content correlated (all $R > 0.70$) negatively to PC 2, while GST content correlated
354 positively with PC 2.
355 The ANOVA, within the exposure period of 0-16 weeks, per site, revealed separate
356 significant effects of exposure “Site”, “Time” and “Metal” on all biomarker responses (Table
357 6). However, the combined effect of “Site x Time x Metal” was not significant for any of the
358 biomarkers. In the case of shell thickness, protein content, GST and total energy the biggest
359 proportion of variance (largest F values) were explained by the effect of time. For example, in

Figure 4 a significant decrease in lipid concentration is seen over time while for GST a slight increase is observed until week 8 after which concentrations decreases again (Table 6).

Table 7 summarizes the results of the stepwise MLR; Ni concentrations contributed to shell strength, Ni and Zn to shell thickness, Cd to protein content and lastly Zn to lipid and glycogen levels.

4. Discussion

4.1. The role of soil physicochemical properties on accumulation

According to the Flemish soil remediation standards the sites F6 (Zn), HOP (As, Cd, Cu, Ni, Pb, Zn), F7 (Cd, Pb, Zn) and UMI (As, Cd, Cu, Ni, Pb, Zn) could be classified as polluted. Soil metal concentrations at F6, F7, HOP and UMI were similar to those reported in earlier studies on the same sites (Rogival et al., 2007, Vermeulen et al., 2009b) and in the same range as polluted soils outside of our study area that focused on accumulation in snails (Pihan and Vaufleury, 2000; Scheifler et al., 2006).

Neutral pH values and lower accumulation of metals in the DG were observed at KK, F6 and F7. Most probably this is because trace metal adsorption and precipitation increases (especially Cd, Pb and Zn) under neutral to alkaline soils (i.e. Bakircioglu et al., 2011; Morel, 1997). Also the combination of a neutral pH and high OC content at KK and F6 could further explain the decrease in trace metal bioaccessibility. In contrast Hobokense Polder and UMI showed slightly acid soil pH levels. As pH decreases a higher percentage of metals will be present in its ionic form. These metal ions must compete with additional cations such hydrogen (H^+), calcium (Ca^{2+}), aluminium (Al^{3+}), iron (Fe^{2+}) and magnesium (Mg^{2+}) to successfully bind to exchange sites (Bakircioglu et al., 2011). Therefore, at lower pH levels metals may become more bioaccessible for uptake by plants (Bakircioglu et al., 2011).

Interestingly, HOP (situated 3 km from the active smelter) showed higher pseudo total soil metal concentrations than UMI (situated next to the active smelter). Nevertheless, *U. dioica*

and the DG of the snails sampled from UMI contained higher mean metal concentrations than HOP. Currently the output of metal dust from the still active factory at UMI is negligible. Therefore, the distance to the source of pollution should be ruled out. The most plausible explanation for the increase in tissue metal concentrations could be to assume an increase in metal bioaccessibility at UMI compared to HOP. Indeed, clay % and OC % levels are more than double at HOP compared to UMI also and both are known to reduce the bioaccessibility of trace metals (Bitton and Dragan, 2005, Bakircioglu et al., 2011). The above explained relations are clearly visualized in the PCA.

Pauget et al. (2012) observed a significant correlation between soil physicochemical properties (i.e. pH and CEC) and the accumulation of metals in the snail species *Cantareus aspersus*. However, based on the MLR we could not conclude with certainty that soil metal and physicochemical properties did contribute to metal accumulation in the DG. Yet, soil physicochemical properties determined the accumulation of metals in *U. dioica* and since snails are mainly exposed via their digestive route (Coeurdassier et al., 2002; Scheifler et al., 2006) soil physicochemical properties indirectly may have affected the accumulation of metals in *C. nemoralis*. Furthermore, the PCA analysis showed strong relations between soil and nettle metal concentrations. Hence, our findings corroborate those of Notten et al. (2006) who found metal concentrations (i.e. As, Ni, Pb and Zn) in the DG of *C. nemoralis* to be significantly predicted by metal levels in nettle.

Concentrations measured in *U. dioica* from our sites can potentially be harmful to snails. Notten et al. (2006) measured lower Cu (7.5 ± 0.7 mg/kg) and Pb (4.0 ± 0.5 mg/kg) but similar Zn (531 ± 56 mg/kg) concentrations in *U. dioica* than what was measured at HOP and UMI. *Cepaea nemoralis* snails that fed on these leaves showed significantly lower consumption rates and egg laying decreased. The observed effects were explained by a combination of decreased consumption and an increased demand of energy for the accumulation and detoxification of metals. Because of the higher energy demand less energy

would then be available to invest in reproduction.

4.2. Local and temporal effects of metal accumulation in the digestive gland of snails

Arsenic and Ni concentrations in the DG increased until WE4, then slightly decreased until WE16 but was still higher than in WE0, 1 and 2. Lead and Zn concentrations gradually increased until WE16. As, Ni and Pb concentrations depended on exposure time in combination with site (i.e. metal concentrations measured at the site and the site-specific bioaccessible metal fraction controlled by soil properties). While Cd concentrations in the DG was not affected by any of the factors, Cu concentrations was affected by the effect of time and site separately and Zn concentrations only by exposure time.

The uptake of non-essential elements (As, Cd and Pb) are likely not regulated to a specific level, or regulated less efficiently than essential elements (Mann et al., 2011). Studies on As accumulation and effects in snails are scare, yet, our DG concentrations were in accordance with those (0.41-2.24 mg/kg dw) measured in terrestrial snails sampled at a polluted industrial site in Taiwan (Hsu et al., 2006) and an industrial waste site in France (Auzon) (0.18 - 4 mg/kg dw) (Pauget et al., 2013). In a previous study on differential organ metal concentrations in *C. nemoralis* Cd levels of 33.93-148.40 mg/kg were reported by Boshoff et al. (2013) and 94 µg/g by Notten et al. (2006). In our study a similar range of Cd concentrations were measured in the DG (60-150 mg/kg). Even higher concentrations were observed in the snail *Helix aspersa* fed on a Cd rich diet (Scheifler et al., 2002). Snails are known to accumulate Cd at higher levels without severe affects by binding to metallothionein-like proteins present in the DG (Dallinger et al., 2004; Nica et al., 2012; Rabitsch, 1996; Scheifler et al., 2006). In our study Pb levels gradually increased and were in accordance with those (mean ± SD) 239.2 ± 57.3 mg/kg (dw) measured in *H. aspersa* exposed to polluted soils and vegetation in microcosms (Scheifler et al., 2006). Gimbert et al. (2008) observed high levels of accumulated Pb bound to the granular fraction of *H. aspersa*. Snails were able to

excrete this fraction leading to a steady state in internal Pb body burdens. However, significant levels of Pb, were retrieved at the end of the depuration phase and retained in the cell debris fraction. Swaileh and Ezzughayyar, (2001) found *H. engaddensis* to be tolerant to Pb since snails on a Pb-contaminated diet still had the ability to feed while their growth rate appeared normal.

Copper, Ni and Zn are essential for snail physiological functioning and therefore uptake is regulated until a threshold level is exceeded (Mann et al., 2011). Snails need elevated amounts of Cu specifically as a constituent of hemocyanin (Dallinger et al., 2005) which is transformed and assimilated after accumulation (Dallinger and Wieser, 1984). Information on the accumulation and effects of Ni in terrestrial snails is scarce but Nowakowska et al. (2012) did not observe an effect on DG lipid peroxidation when concentrations of 6 mg/kg (dw) were measured in the DG. Because our Ni concentrations were in the same range as this study, we believe that they were too low to have a detrimental effect on physiological functioning. At low environmental Zn concentrations, snails are able to retain Zn in tissues for essential functions. However, in our study Zn levels gradually increased and did not reach a steady state by the end of our experiment. Cytosolic Zn-binding ligands has already been identified (Cd-MT and non-metallothionein components) which might explain the high level of Zn in the DG (Mourier et al., 2011; Pauget et al., 2012). At very high concentrations (100mg/kg (dw) in diet) Zn can affect feeding and growth (Swaileh and Ezzughayyar, 2001). In our study Zn concentrations in *U. dioica* reached levels as high as 200 mg/kg (dw).

4.3. Morphological characteristics, energy reserves and GST response

Invertebrates that live in areas that are chronically polluted with metals often show morphological and/or physiological changes due to increased energy expenses associated with detoxification and excretion processes (Holmstrup et al., 2011; Jones and Hopkin, 1998; Radwan et al., 2010; Regoli et al., 2006; Smolders et al., 2004). However, in *C. nemoralis* we

464 did not find clear morphological changes in shell strength, and thickness that could be related
465 to the interaction between site and time even when exposed to polluted soil and *U. dioica* for
466 a longer period of 16 weeks instead of 4-7 weeks as in earlier studies (i.e. with the exception
467 of Ni and Zn in the MLR). Similarly, Jordaens et al. (2006) and Fritsch et al. (2011) detected
468 no effects of metal exposure (Cd and Pb) on shell size or weight of first generation *C.*
469 *nemoralis* snails exposed within microcosms for a period of 28 days.
470 In our study the MLR indicated that Ni (DG) and Zn (DG) contributed significantly to
471 changes in shell morphology. However, no consistent changes in shell morphology were
472 observed since shell strength slightly increases with time at F7 but slightly decreases at HOP.
473 Even though adult snails have a fully developed shell, variability in the shell morphology of
474 wild populations can occur (Gomot de Vaufleury and Pihan, 2000). A possible hypothesis to
475 explain the decrease in shell thickness could be that when Ni and Zn accumulation in the DG
476 exceeds a certain limit, they are bound and exported to the shell which in turn hampers the
477 shell structure. Also Jordaens et al. (2006) could not clarify the negative relationship between
478 Zn concentrations in the shell and shell strength.
479 More interesting is the relation between Zn (DG) and energy levels (lipid and glycogen)
480 which, did in the case of lipid content, decreased significantly over time at all sites. Seasonal
481 changes in temperature have been described to change the composition of lipids in terrestrial
482 snails (Kotsakiozi et al., 2012). In contrast, Horst and Zandee, (1973) observed that lipid
483 content and composition in *C. nemoralis* snails sampled at unpolluted sites remained
484 remarkably constant during an annual cycle which included periods of hibernation/aestivation.
485 Therefore, the decrease in lipid content in our study could indicate increased energy needs for
486 metal detoxification processes.
487 Mean protein, glycogen, GST and total energy levels measured in the DG showed variable
488 changes which often depended on a specific site, the exposure time and metal. However when
489 investigating the parameters site, time and metal together no long term effect of interaction on

any of the measured biomarkers was observed. Nevertheless short term biomarker response was observed that corresponds to earlier findings. Grara et al. (2012) exposed (i.e. 28 days) laboratory reared *H. aspersa* to metal rich (Cu, Zn, Ni, Pb) dust and found a significant decrease in lipid and carbohydrate levels and a significant increase in total protein concentration. Furthermore, Dallinger et al. (2004) and Kammenga et al. (2000) observed a significant increase in protein content (Cd-metallothionein) in the DG of *H. aspersa* after Cd exposure (i.e. 2 weeks) in a controlled laboratory environment. In our study, Cd concentrations in the DG significantly explained protein content, yet, no consistent increase in protein levels were observed. Regoli et al. (2006) tested a wide array of biochemical protein responses in the DG of cultured *H. aspersa* snails exposed (i.e. 4 weeks) in microcosms to metal polluted sites. In the same study GST concentrations fluctuated within and between sites and could not be linked directly to metal pollution. In contrast Radwan et al. (2010) observed a significant increase in GST activity in nursery reared *Theba pisana* after exposure (i.e. 48h) to sub lethal Pb doses in a controlled laboratory environment. Furthermore, Cu, Pb and Zn concentrations resulted in a significant increase in the level of lipid peroxidase while glutathione content decreased. Patil et al. (2011) found glycogen levels in the foot of the freshwater snail *Indoplanorbis exustus* to increase significantly after one day of exposure to Zn sulphate and depleted significantly after two weeks of exposure. From these observations it was suggested that glycogen was the main source of energy for counteracting metal stress, while the proteins and lipids were spared. In our study, glycogen showed no consistent increase or decrease in time, even though it was related to increasing Zn levels in the DG. It is important to stress that the above studies were all conducted under controlled laboratory environments over a period of 4 weeks or less and are therefore an observation of short term effects. Similarly, short term effects were also observed in our study (i.e. decrease in lipid concentrations and an increase in GST) however these effects ceased with time and were in most cases not correlated to metal concentrations in the DG.

516 Snails have mechanisms to metabolize, bind, export, store and excrete metals (Drexler et
517 al., 2003). These mechanisms are activated by a process called acclimatization. Therefore,
518 accumulated metals will reach a steady state after a certain period of exposure and they will
519 not be able to interfere with biochemical reactions (Rainbow, 2007; Vijver et al., 2004). For
520 this reason we hypothesize that low biomarker response observed in our study may be the
521 result of acclimatization expressed in tolerance traits (i.e. excretion, compartmentalization)
522 while the minor temporal changes that we observed within sites (e.g. Zn levels in DG
523 affecting lipid levels) could be an acclimation response (i.e. physiological changes that occur
524 through experimentally induces stressors, transfer to new environment or stress response due
525 to the new restricted microcosm environments) (see also Fritsch et al. (2011) and Shehzad
526 (2008)).
527
528 **5. Conclusions**
529 Microcosms used in the present study were an efficient tool for studying the *in situ* transfer
530 and effects of pollutants on snails at specific sites and over temporal time intervals. Metal
531 concentrations in the snails digestive gland were best explained by the trace metal(loid)
532 concentrations in their main food source. Similar to previous studies short term biomarker
533 responses were observed. However, on long term these effects ceased and could not be related
534 to metal concentrations in the digestive gland. Our study emphasizes the need for controlled
535 long-term studies (i.e. even longer than 16 weeks) on the transfer and effects of metals in food
536 chains. Moreover, additional endpoints have to be investigated that might be more responsive
537 of metal pollution that the ones investigated in the present study.
538
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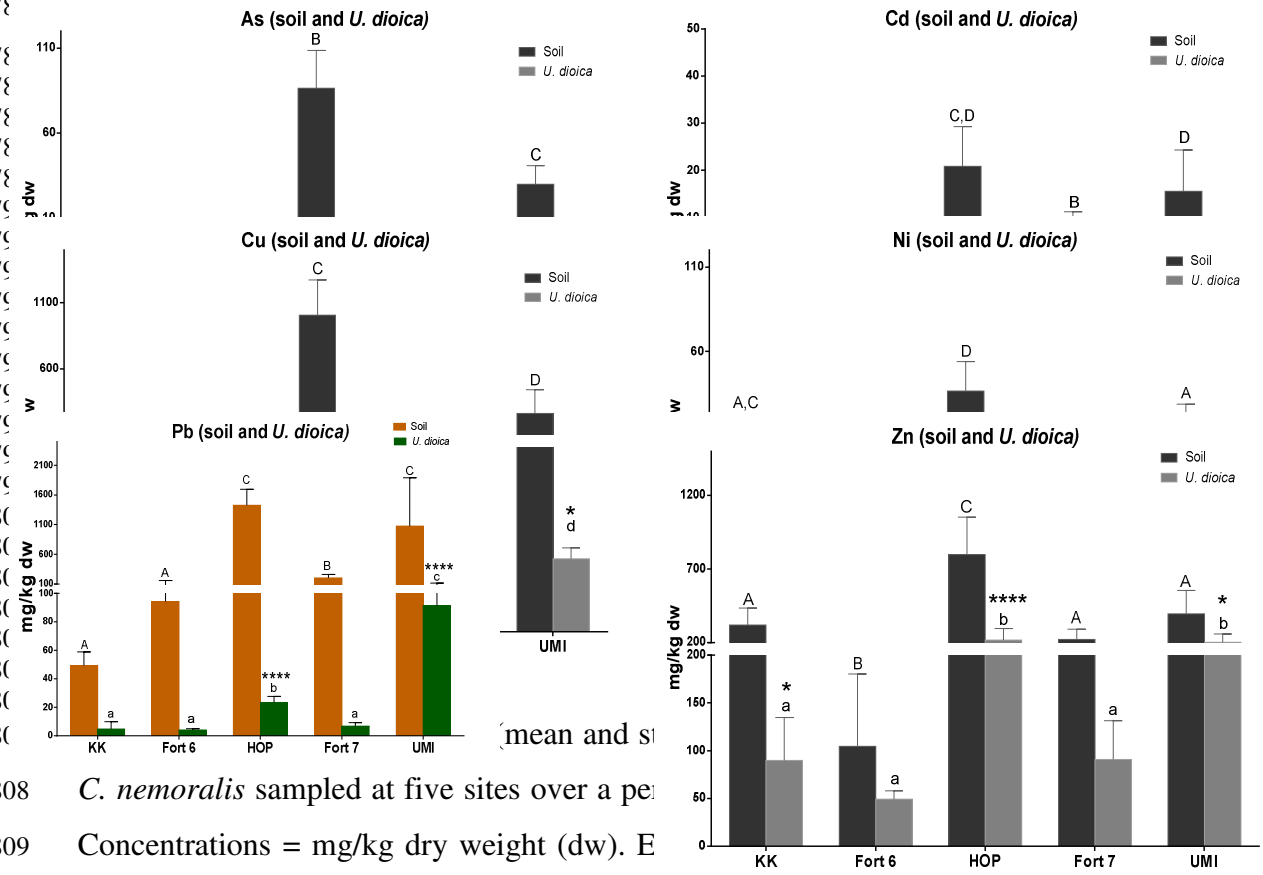
Figure 1: Metal concentrations in soil and *U. dioica* (mean and standard deviation) measured at KK = Kontich-kazerne, F6 = Fort 6, HOP = Hobokense Polder, F7 = Fort 7, UMI = Umicore. Different letters indicate significant differences ($p < 0.05$) among sites (upper case for soil and lower case for *U. dioica*). Stars indicate significant differences between soil and *U. dioica* per site (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).



C. nemoralis sampled at five sites over a pe
Concentrations = mg/kg dry weight (dw). E
Kontich-kazerne, Fort 6, HOP = Hobokense Polder, Fort 7, UMI = Umicore. Different letters indicate significant differences among sites within each sample occasion (WE1-16) ($p < 0.05$).

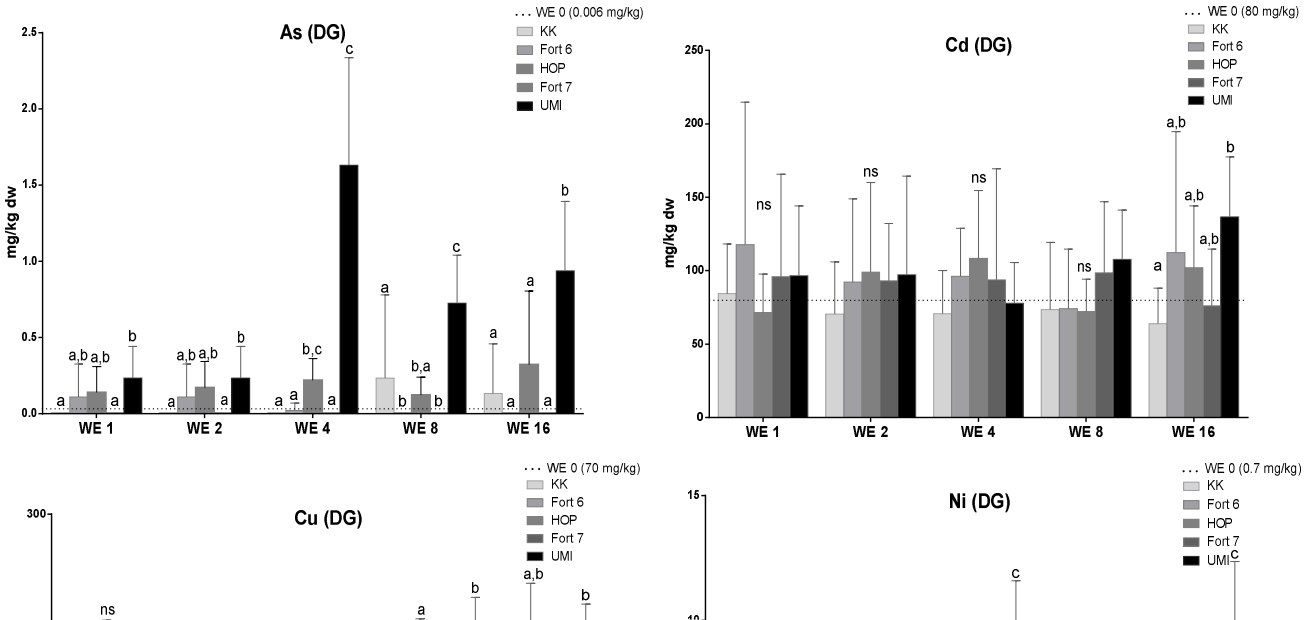
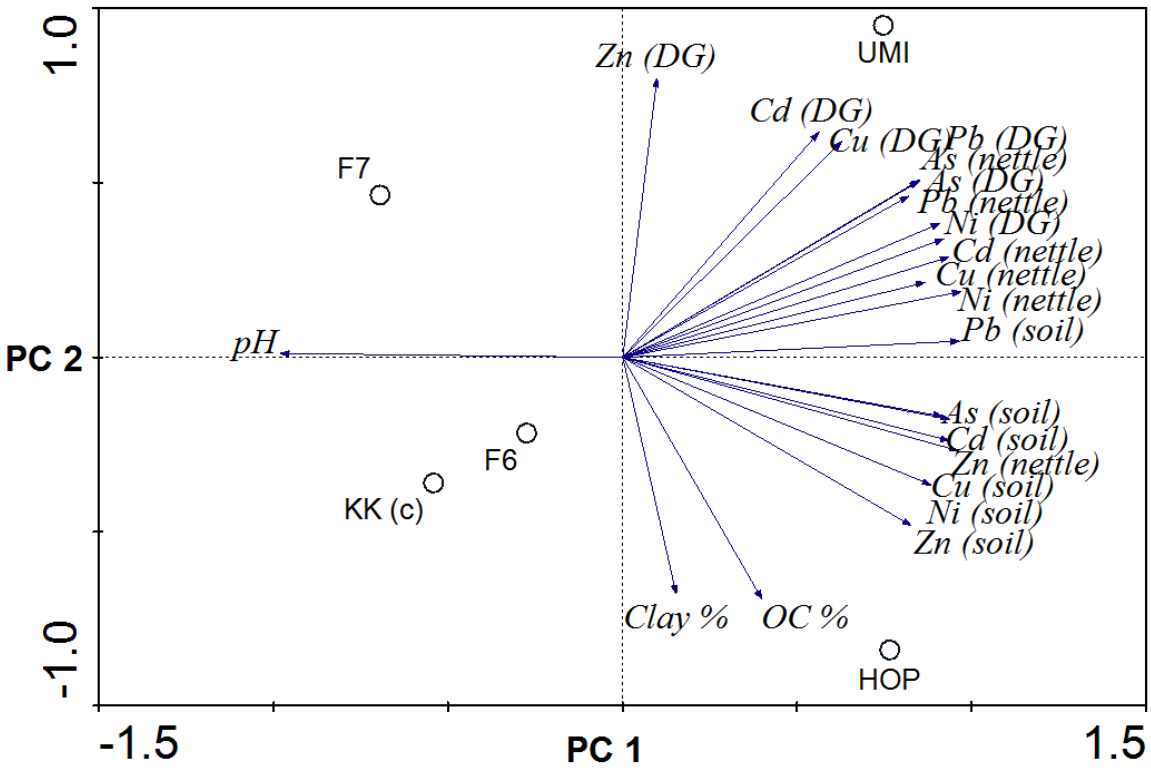


Figure 3: Results of the PCA based on soil properties, and metal concentrations in the digestive gland, soil and nettle (n = 5). The first (81.4%) and second (11.5%) principle components (PC) together explained 92.9% of the total variation. All correlations with PC 1 and 2 (R > 0.70) are indicated in the table below the figure.



PC 1 (81.4 %)		PC 2 (11.5 %)	
As (DG)	0.79	*Cd (DG)*	0.70
Ni (DG)	0.86	*Zn (DG)*	0.80
As (nettle)	0.74	*Clay %*	-0.71
Cd (nettle)	0.88	*OC %*	-0.83
Cu (nettle)	0.86		
Ni (nettle)	0.94		
Pb (nettle)	0.74		
Zn (nettle)	0.97		
As (soil)	0.81		
Cd (soil)	0.96		
Cu (soil)	0.78		
Ni (soil)	0.85		
Pb (soil)	0.97		
Zn (soil)	0.80		
pH	-0.98		

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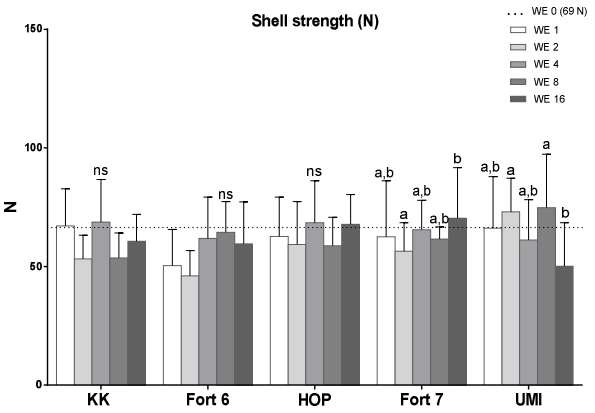
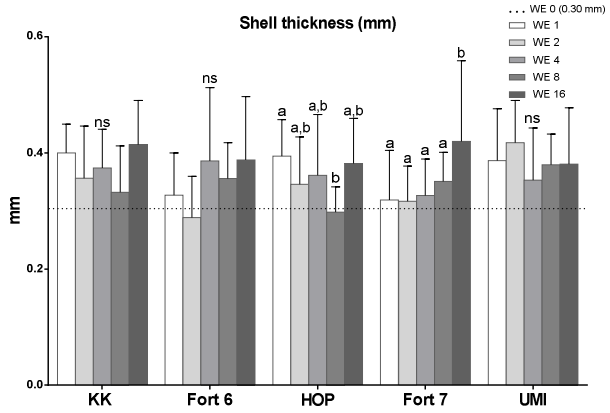
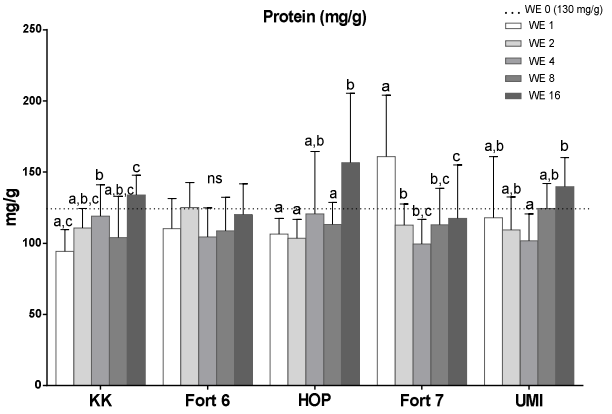
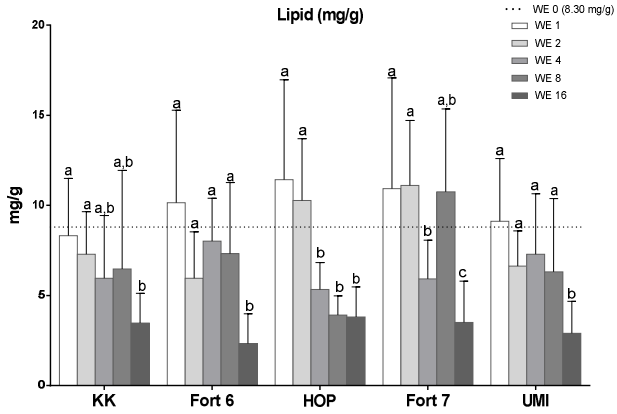
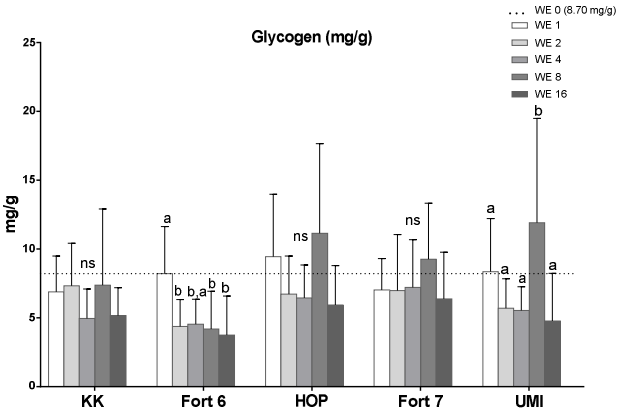
836 **Figure 4:** Biomarkers (mean and standard deviation) measured per week (WE 0 = dotted line
837 and WE 1-WE 16) at each site. Sites; KK = Kontich-kazerne, Fort 6, HOP = Hobokense
838 Polder, Fort 7, UMI = Umicore. Significant differences ($p < 0.05$) between weeks measured
839 per site are indicated at the top of each column (ns = not significantly different).

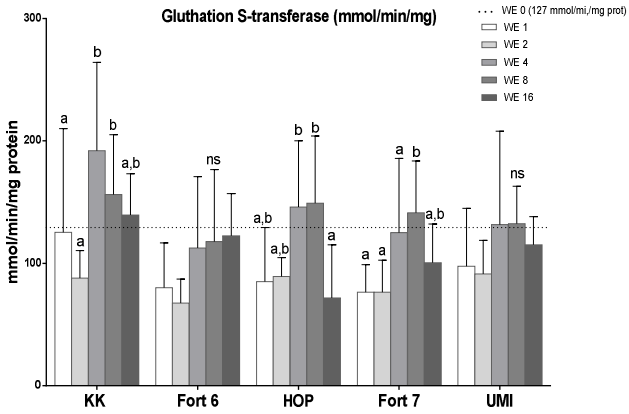

Shell strength (N)
WE 0 (69 N)
WE 1
WE 2
WE 4
WE 8
WE 16
N
KK
Fort 6
HOP
Fort 7
UMI

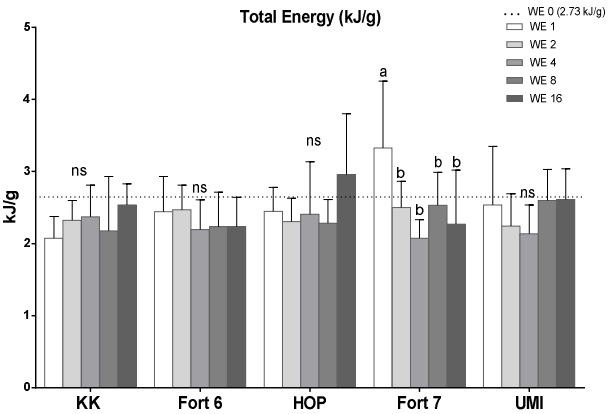

Shell thickness (mm)
WE 0 (0.30 mm)
WE 1
WE 2
WE 4
WE 8
WE 16
mm
KK
Fort 6
HOP
Fort 7
UMI

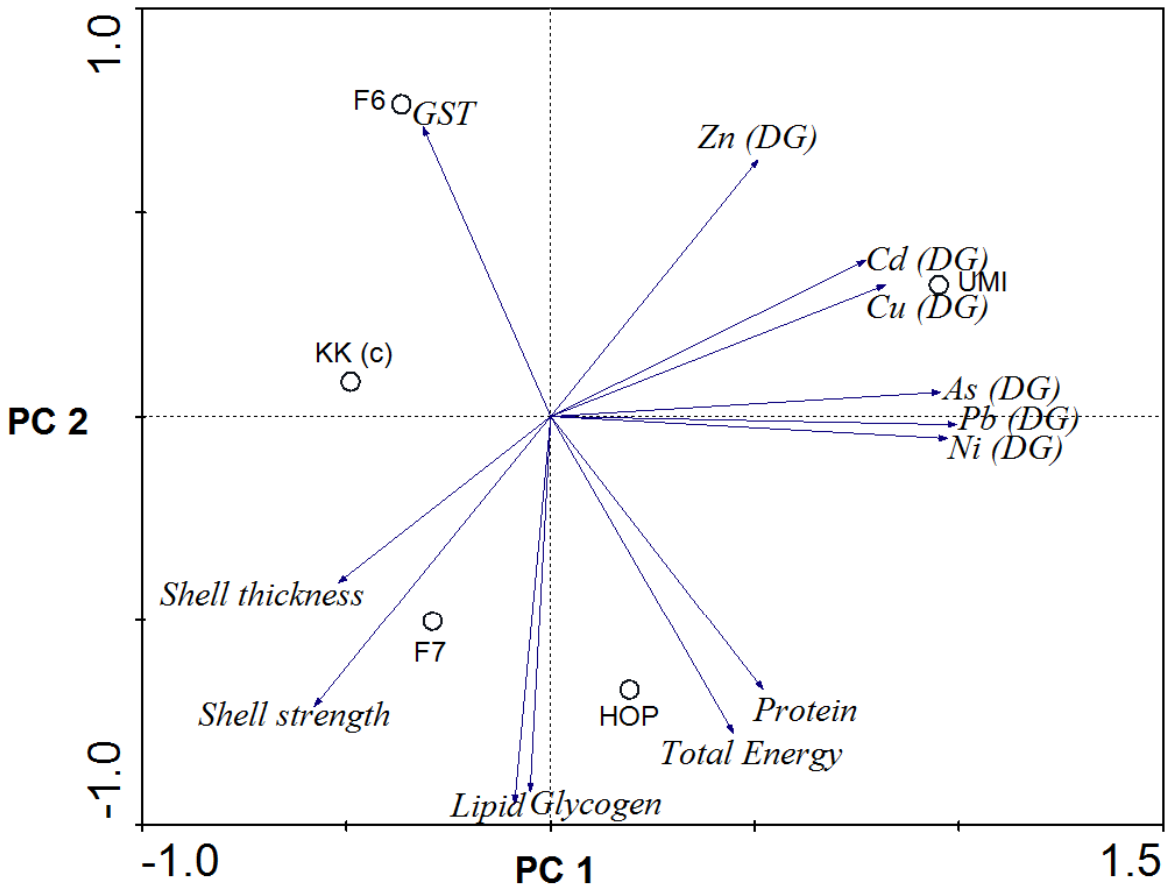

Protein (mg/g)
WE 0 (130 mg/g)
WE 1
WE 2
WE 4
WE 8
WE 16
mg/g
KK
Fort 6
HOP
Fort 7
UMI


Lipid (mg/g)
WE 0 (8.30 mg/g)
WE 1
WE 2
WE 4
WE 8
WE 16
mg/g
KK
Fort 6
HOP
Fort 7
UMI


Glycogen (mg/g)
WE 0 (8.70 mg/g)
WE 1
WE 2
WE 4
WE 8
WE 16
mg/g
KK
Fort 6
HOP
Fort 7
UMI


Gluthation S-transferase (mmol/min/mg)
WE 0 (127 mmol/mi,/mg prot)
WE 1
WE 2
WE 4
WE 8
WE 16
mmol/min/mg protein
KK
Fort 6
HOP
Fort 7
UMI


Total Energy (kJ/g)
WE 0 (2.73 kJ/g)
WE 1
WE 2
WE 4
WE 8
WE 16
kJ/g
KK
Fort 6
HOP
Fort 7
UMI



PC 1 (87.7 %)		PC 2 (6.4 %)	
As (DG)	0.96	*Shell strength*	-0.72
Cd (DG)	0.81	*Protein*	-0.67
Cu (DG)	0.85	*Lipid*	-0.96
Ni (DG)	0.99	*Glycogen*	-0.90
Pb (DG)	0.95	*GST*	0.70
		Total Energy	-0.77

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869 **Tables 1:** Soil type and soil physicochemical properties (pH, clay %, organic carbon OC %);

870 mean ± SD and range (minimum-maximum) of the five studied sites.

***Site*[a]**	***n***	***pH-KCl***	***Clay %***	***OC %***	***Soil type***
KK	5	6.41 (0.43) 5.55 - 6.66	4.93 (0.62) 4.07 - 5.80	23.24 (7.67) 9.88 - 32.78	*Loamy Sand*
F6	5	6.07 (0.55) 5.39 - 6.70	2.87 (1.00) 1.95 - 4.72	19.33 (5.61) 10.08 - 27.36	*Light Sandy Loam*
HOP	5	5.55 (0.42) 4.99 - 5.90	8.74 (3.30) 4.95 - 14.64	26.23 (6.47) 17.60 - 32.90	*Heavy Sandy Loam*
F7	5	6.62 (0.40) 6.06 - 7.06	4.87 (0.47) 4.25 - 5.50	3.42 (0.52) 2.43 - 3.96	*Loamy Sand*
UMI	5	5.62 (0.88) 4.88 - 6.09	2.48 (1.28) 1.04 - 4.40	13.19 (5.53) 5.40 - 18.56	*Loamy Sand*

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872 [a] = KK = Kontich-kazerne, F6 = Fort 6, HOP = Hobokense Polder, F7 = Fort 7, UMI = Umicore.
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Tables 2: Analysis of variance of soil physicochemical properties measured in soil from each site.

		pH-KCl			*Clay %*			*OC %*	
Source of variation	df	SS	MS	F	SS	MS	F	SS	MS
Between sites	4	5.46	1.36	**6.54****	0.77	0.19	**16.24****	2.38	0.59
Risidual	25	5.21	0.21		0.30	0.01		0.52	0.02
Total	29	10.67			1.07			2.89	

[a] Levels of significance: p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***, p < 0.0001 = ****

Tables 3: Analysis of variance for the level of metal accumulation in the digestive gland of *Cepaea nemoralis* as affected by site, time and site x time interaction.

		As			*Cd*			*Cu*			*Ni*			
Source of variation	df	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	S
Sites (S)	4	1.53	0.38	**80.30**** [(a)]	0.55	0.14	2.38	1.07	0.27	**5.71*****	8.50	2.13	**141.60****	32
Time (T)	4	0.16	0.04	**8.33****	0.11	0.03	0.47	0.46	0.11	**2.46***	1.97	0.49	**32.74****	9
S x T	16	0.64	0.04	**8.43****	0.97	0.06	1.05	0.78	0.05	1.05	8.50	2.13	**8.12****	2
Residual	223	1.06	0.00		12.91	0.06		10.40	0.05		3.35	0.02		9

[a] Levels of significance: p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***, p < 0.0001 = ****

Table 4: Results of the stepwise multiple regression to explain accumulated metal concentrations in *Urtica dioica*. Variables included metal concentrations in soil and soil physicochemical properties. Empty cells indicate excluded values that showed signs of covariability and/or did not contribute significantly to explaining metal concentrations in *U. dioica*.

***Urtica dioica* (n = 50)**	***Regression***			***Y = a + [Soil]* (X_1) *+ pH* (X_2) *+ Clay %* (X_3) *+ OC %***		
Metal	F	p	a	[Soil]	pH	Clay %
As	29.06	**<0.0001**	0.35	**0.45****** [(a)]		**-0.74******
Cd	31.64	**<0.0001**	0.73	**0.37******	**-0.09***	
Cu	39.32	**<0.0001**	0.81	**0.24******		
Ni	20.99	**<0.0001**	1.08	**0.32***	**-0.15***	
Pb	59.56	**<0.0001**	-0.48	**0.66******		
Zn	76.66	**<0.0001**	1.99	**0.51******	**-0.20******	

[a] Levels of significance: p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***, p < 0.0001 = ****

Table 5: Results of the stepwise multiple regression to explain accumulated metal concentrations in *Cepaea nemoralis.* Variables included metal concentrations in *U. dioica*, soil and soil physicochemical properties. Empty cells indicate excluded variables that showed signs of covariability and/or did not contribute significantly to explaining metal concentrations in the digestive gland.

Digestive gland (n = 50)	***Regression***			***Y = a + [U. dioica]*** **(X_1) *+ [Soil]*** **(X_2) *+ pH*** **(X_3) *+ Clay %*** **(X_4) *+***			
Metal	F	p	a	[*U. dioica*]	[Soil]	pH	Cla
As	74.93	**<0.0001**	-0.23	**0.34****** [(a)]			
Cd	6.79	**0.003**	2.09	**0.45****			
Cu	6.77	**0.01**	1.60	**0.33***			
Ni	117.53	**<0.0001**	0.07	**0.93******			
Pb	195.99	**<0.0001**	0.66	**0.81******			
Zn	1.93	*ns*					

[a] Levels of significance: p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***, p < 0.0001 = ****

Table 6: Analysis of variance of variance for the biomarker responses in *Cepaea nemoralis* as affected by site, time and metal concentration along with the interactions.

		Shell Strength			***Shell Thickness***			***Proteine***			***Lipid***			***Glycogen***		
Source of variation	df	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F	SS	MS	F
Sites (S)	4	0.88	0.22	**16.46****** [a]	0.03	0.01	**12.45******	0.26	0.07	**8.88******	2.07	0.52	**16.31******	6.25	1.56	**38.29**
Time (T)	4	0.50	0.12	**9.27******	0.07	0.02	**26.32******	1.77	0.44	**60.40******	24.92	6.23	**195.93******	5.58	1.40	**34.22**
S x T	16	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.0
Metal (M)	5	3.37	0.21	**15.78******	0.13	0.01	**12.08******	2.49	0.16	**21.27******	10.05	0.63	**19.76******	5.37	0.34	**8.23**
S x M	20	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.1	0.00	0.02	0.01	0.00	0.0
T x M	20	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.1	0.00	0.02	0.01	0.00	0.0
S x T x M	80	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.5	0.00	0.02	0.02	0.00	0.0
Error	1350	18.04	0.01		0.88	0.00		9.88			42.92	0.03		55.07	0.04	
Total	1500	4783.22			27.92			6395.96			1166.86			1143.55		

[a] Levels of significance: p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***, p < 0.0001 = ****

Table 7: Results of the stepwise multiple regression to explain biomarker responses in the digestive gland of *Cepaea nemoralis*. Empty cells indicate excluded variables that showed signs of covariability and/or did not contribute significantly to explaining biomarker responses.

nse	Regression		Y = a + *As (DG)* (X_1) + *Cd (DG)* (X_2) + *Cu (DG)* (X_3) + *Ni (DG)* (X_4) + *Zn (DG)* (X_5)						
	F	p	a	As (DG)	Cd (DG)	Cu (DG)	Ni (DG)	Pb (DG)	Zn (DG)
l)	8.77	**0.04**	75.41				**-23.03****		
mm)	5.23	**0.01**	-0.13				**0.16***		**-0.12***
	4.31	**0.04**	1.91		**0.05***				
	5.90	**0.02**	1.59						**-0.28***
	16.29	**< 0.0001**	2.55						**-0.50******
nsferases (mmol/min/mg)	1.18	*ns*							
/g)	1.02	*ns*							

[a] Levels of significance: p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***, p < 0.0001 = ****