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RESEARCH ARTICLE

On the use of *Arion ater* to biomonitor environmental pollution by Cd, Cu, Fe, Mn and Zn, with a special insight into the population variability

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Abstract The suitability of *Arion ater* as a biomonitor of Cd, Cu, Fe, Mn and Zn was assessed. Individual specimens were collected from 22 sampling sites. Slugs from 3 of the sites were analysed individually, whereas the slugs from the other sites were pooled to make a composite sample for each site. The tissue burdens did not differ between individuals from contaminated and uncontaminated sites, and there was no gradient of bioaccumulation of any of the elements in the surroundings of the smelter. Analysis of the individual specimens from the 3 sites revealed very high coefficients of variation for the metal concentrations. As a result of the high level of variation, large numbers of slugs are required to produce a low error in characterizing the mean concentration at each site. Furthermore, as a consequence of the similar mean concentrations and high variability, large numbers of samples are needed to detect significant differences between pairs of sites.

Keywords Heavy metals · Slugs · *Arion ater* · Intrapopulation variability · Smelter

Introduction

Interest in the potential use of terrestrial invertebrates as biomonitors has increased in the past few decades (see for instance Laskowski and Hopkin 1996; Hamers et al. 2004; Nica et al. 2013; Staunton et al. 2014). Slugs are one of the most commonly used types of gastropods because of their wide distribution and generally low mobility, and they have been used to characterize levels of contamination in terrestrial environments (Popham and D'Auria 1980). The genus *Arion* is considered to be potentially suitable for biomonitoring for the following reasons: (i) it is easily identified and taxonomically well characterized; (ii) its relatively large size makes is easy to sample and handle; (iii) its life cycle is known; and (iv) it has a limited ability to excrete pollutants (see for instance Ireland 1981, for Cd).

The use of biomonitors is often hindered by high levels of intrapopulation variability in the concentrations of contaminants. This problem has been described in relation to the use of certain species of plants (Aboal et al. 2001, 2006) and animals (González et al. 2006; Lamas et al. 2007) as biomonitors. Previous studies on slugs have reported a high degree of variability in tissue burdens of contaminants (see, e.g. Ireland 1981; Kalinowska 1984). The implications with regard to the use of these animals as biomonitors have not been studied thoroughly. A high degree of intrapopulation variability negatively affects the potential usefulness of a species for estimating the representative concentration of a contaminant for a population and for detecting differences in the concentrations representing different populations. In addition, the possibility of detecting such differences will depend on the number of specimens sampled in each population (Zar 1984). The effect of the intrapopulation variability on the sample size of slugs required for biomonitoring contamination has not previously been investigated. Thus, in the literature consulted, the sample sizes varied between 6 (Popham and D'Auria 1980) and 20 (Kalinowska 1984) individuals.

The aim of the present study was to evaluate the use of the slug *Arion ater* as a biomonitor of heavy metal pollution, by

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establishing the following: (i) the difference in tissue burdens of metals between individual specimens sampled in contaminated and uncontaminated zones; (ii) the existence of gradients of bioaccumulation in the surroundings of an industrial source of contamination; and (iii) the intrapopulation variability in tissue burdens of metals, to enable calculation of the sample required to estimate the mean concentrations of metals at a certain error level and to establish significant differences between sampling sites.

Material and methods

Sampling and processing

Adult specimens of *Arion ater* (Linnaeus, 1758) were collected from different sampling sites (SS) in Galicia (NW Spain). The species feeds on faeces, green and decaying plant matter, fungi and carrion. The median weight of the sampled slugs was 11.27 g d.w., ranged between 2.96 and 52.2 g d.w. The sampling sites (22 in total; Fig. 1) comprised the following: (i) 11 SS (B1-B11) located within large areas of natural forest, dominated by *Quercus robur*, distant from urban centres and industries; and (ii) 11 SS (P1-P11) located in grasslands, including some shrubs (*Ulex europaeus*), around a Fe-Si smelter. The selection of the smelter scenario was based on high levels of contamination (e.g. As, Co, Cr, Cu, Fe, Ni, Pb, V and Zn) previously detected by use of the moss biomonitoring technique (Varela et al. 2014). The number of slugs collected at each SS is shown in Table 1. Slugs were sampled at sunrise, during 5 consecutive days at each site, between September 2002 and September 2003, and they were then transferred to a polythene container and transported as quickly as possible to the laboratory (<8 h). Once in the laboratory, adhered soil and mucus were carefully removed from the slugs. The gut contents were cleared daily from the container, to minimize coprophagy, and after 4 days, the slugs were sacrificed by freezing. For SS B1-B9 and P1, P3-P10, all slugs collected at each site were homogenized together, whereas for the other SS (B10, B11 and P2), the slugs were homogenized individually. In all cases, the slugs were homogenized in a laboratory blender.

Chemical analysis

Prior to analyses, samples were dried to constant weight (45 °C) in a forced air oven, and an aliquot (0.6 g d.w.) of each sample was digested with HNO_3 (65 %) and H_2O_2 in a microwave oven (CEM MDS2100). The concentrations of Cd, Cu, Fe, Mn and Zn were determined by flame absorption spectrophotometry (Perkin Elmer 2100), using air-acetylene flame. The operating parameters (i.e. wavelengths) employed were those recommended by the supplier: 228.9 nm for Cu, 324.8 nm for Cu, 248.3 nm for Fe, 279.5 nm for Mn and 213.9 nm for Zn.

To monitor the processes of extraction and determination of the metal contents, certified reference material (BCR N° 278R, *Mytilus edulis*) was analysed (1 reference sample every

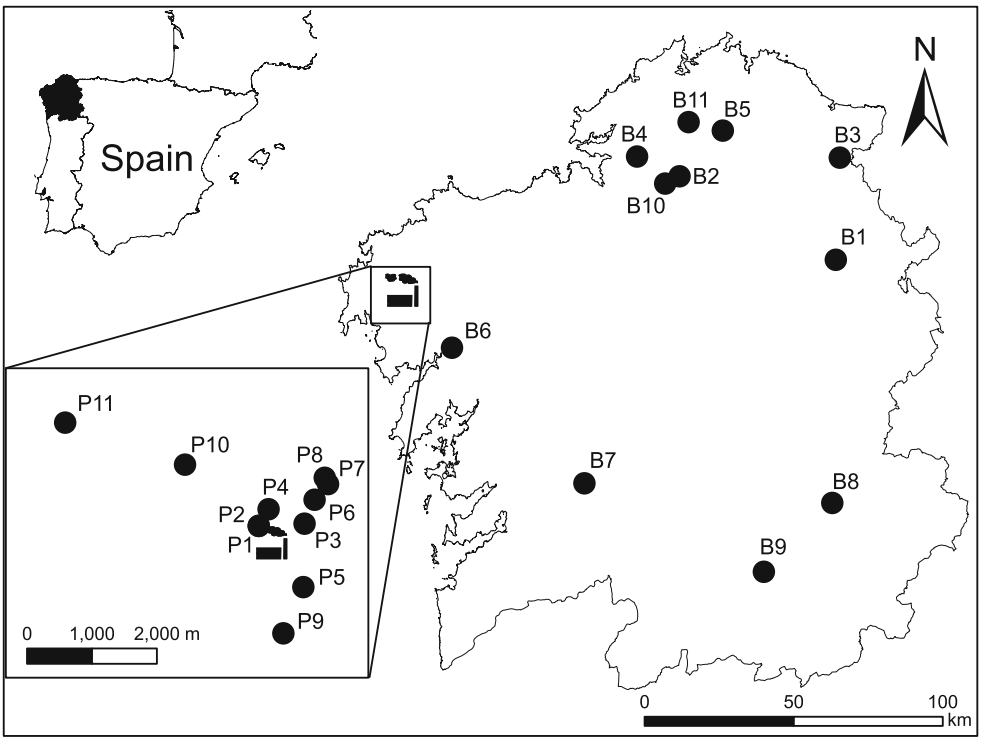

Fig. 1 Map showing the location of the *Arion ater* sampling sites in Galicia (NW Spain). The enlarged area shows the location of the sampling sites around the studied smelter

Table 1 Concentrations of heavy metals (μg g^{-1}) determined in composite samples of *Arion ater* collected from uncontaminated sites (B1-B11) and from the surroundings of an industrial source of emission of these metals (P1-P11)

SS	*n*	Distance (m)	Cd	Cu	Fe	Mn	Zn
B1	41	–	3.53	101	51.0	3740	343
B2	43	–	3.06	99.7	43.4	800	234
B3	52	–	6.77	90.4	52.5	3740	280
B4	11	–	5.26	69.7	84.8	5920	450
B5	49	–	9.40	88.9	70.0	1210	394
B6	12	–	6.16	67.0	240	636	410
B7	7	–	1.21	79.8	48.5	1170	192
B8	23	–	2.76	68.7	70.0	1350	212
B9	16	–	2.56	84.8	65.0	2360	192
B10	37	–	2.72	52.3	94.0	1020	318
B11	23	–	2.84	40.6	303	1793	531
P1	10	373	6.76	107	108	9200	475
P2	42	380	2.51	80.9	112	3325	249
P3	10	501	9.60	192	374	8000	1160
P4	10	514	4.14	126	174	6260	656
P5	10	810	10.2	129	248	11,800	1130
P6	10	862	3.69	131	404	8890	1100
P7	10	1181	5.60	120	448	8200	1180
P8	10	1224	6.20	121	576	4780	1060
P9	10	1420	3.20	84.0	204	8180	870
P10	10	1850	3.60	116	146	4500	545
P11	10	3748	4.34	99.0	238	5600	890

For samples P1-P10, the distance from the focal point of emission is indicated

n number of slugs sampled, *SS* sampling site

9 samples). The recoveries were satisfactory: 77 % for Cd, 86 % for Cu, 93 % for Mn and 83 % for Zn (Fe was not certified in this CRM). Furthermore, these data were used to calculate the variability associated with the extraction process and sample analysis. The variability, expressed as the coefficient of variation, ranged between 5 and 15 %. The existence of contaminating material during processing and extraction was controlled by including analytical blanks (1 every 9 samples). The quantification limits (μg g^{-1}) were 0.016, 0.027, 0.872, 0.500 and 0.330 for Cd, Cu, Fe, Mn and Zn, respectively.

Statistical analysis

The number of samples required to characterize the contamination in populations B10, B11 and P2 was calculated as recommended by Zar (1984): $N=t^2_{(0.05;\ n\text{-}1)}\cdot\sigma 2/D^2\ \mu^2$, where t is the value of the Student's t statistic for a probability of 0.05 and n-1 degrees of freedom; σ^2 is the sampling variance; D is the desired error at which the levels of contamination in the population are estimated and μ is the population sampling mean. Data normality was confirmed in all cases by Lilliefor's modification of the Kolmogorov-Smirnov test.

A power test to calculate the sample size, recommended by Zar (1984), was used. This test allows to know how many specimens are needed to differentiate between two SS. Assuming each subsample is from a normal population, the minimum sample size required to achieve the desired test characteristics can be estimated as follows:

$$n \geq \frac{2\sigma_p^2}{\delta^2}\left(t_{a,v} + t_{\beta(1),v}\right)^2$$

where n is the sample size; δ is the minimum detectable difference between population means; t is the value of the Student's t statistic; α is the significance level (α=0.05); 1-β is the power of the test (β=0.1); ν is the number of degrees of freedom (2 * (n-1)); and σ^2_p is the population variance.

When the populations were not-normal distributed, a power test proposed by Aboal et al. (2006) (called U test) was used. For this, Box-Cox transformations were applied (Legendre and Legendre 1998), with the Minitab 1.4 statistical package, to normalize the data prior to application of this test (for details of the procedure, see Aboal et al. 2006).

Results

The concentrations of the heavy metals (Cd, Cu, Fe, Mn and Zn) in the composite samples of slugs from each SS are shown in Table 1. For SS P2, B10 and B11, the values shown in the table correspond to the weight-adjusted mean value of each individual determination, which is equivalent to values for composite samples. For background areas, the concentrations (μg g^{-1}) ranged as follows: 1.1–9.40 for Cd; 40.6–101 for Cu; 43.4–303 for Fe; 636–5920 for Mn; and 192–531 for Zn. For samples from the polluted area, the ranges of values were as follows: 2.51–10.2 for Cd; 80.9–192 for Cu; 112–576 for Fe; 3325–11,800 for Mn; and 249–1180 for Zn.

The distribution of metal concentrations in slugs from the SS in which individual samples were analysed (i.e. P2, B10 and B11) are shown in Fig. 2. With the exception of Cu, which was the only element that was normally distributed, the distributions were skewed to the right, although samples from contaminated (P2) and presumably uncontaminated sites (B10 and B11) showed very similar mean concentrations of metals. The largest difference was in the concentrations of Mn, although the difference was less than fourfold. The coefficients of variation were very high, and in more than 66 % of cases, they were higher than 50 %. As a result of this high degree of variability, large numbers of slugs were required to characterize the mean concentrations of metals at each SS with a low

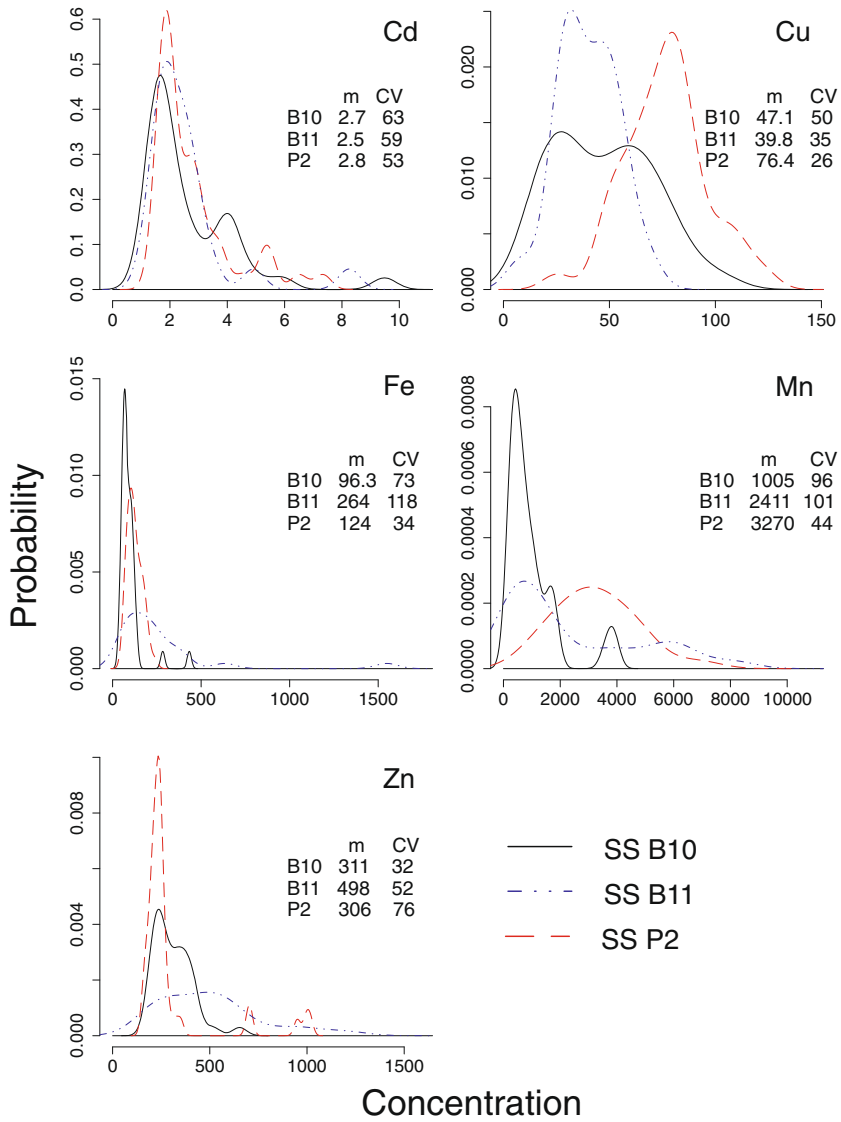

Fig. 2 Distributions of the concentrations (μg g^{-1}) of the metals in *Arion ater* estimated by kernel smoothing. For each metal and sampling site, the mean concentration (m), expressed in μg g^{-1}, and coefficient of variance (CV), expressed as a percentage, are shown

error level (Table 2). For the maximum number of slugs collected at one SS (i.e. 52 in B3, Table 1), the error level was always below 20 %, except for Fe and Mn. However, the same error level was not achieved for the minimum number of slugs sampled (i.e. 7 at B7, Table 1). Finally, as a consequence of similar means and high variability, the minimum number of samples required to detect significant differences between pairs of SS was very high (Table 3).

Discussion

Although the metal concentrations in the animals should ideally reflect environmental pollution levels, only small differences were observed between the tissue burdens of slugs collected from the area exposed to very high levels of atmospheric contamination (Varela et al. 2014) and those collected from areas distant from focal points of contamination (Table 1). In

Table 2 Number of individual specimens of *Arion ater* required to characterize the mean concentrations of different metals at a sampling site, for different error levels (20, 10 and 5 %)

	Cd			Cu			Fe			Mn			Zn		
	B10	B11	P2	B10	B11	P2	B10	B11	P2	B10	B11	P2	B10	B11	P2
$N_{20\%}$	39	25	36	25	7	13	53	11	144	91	19	105	10	51	28
$N_{10\%}$	157	101	142	100	26	51	212	44	574	365	76	420	41	205	112
$N_{5\%}$	629	405	568	400	105	203	849	174	2297	1462	302	1681	163	821	447

Table 3 Minimum number of samples required to differentiate the mean concentrations of metals at the different sampling sites (SS), determined by the method for normal populations or the *U* test (see text for further details)

SS pairs	Cd	Cu	Fe	Mn	Zn
B10-B11	>200	12	46	30	>200
B10-P2	>200	170	58	67	36
B11-P2	>200	5	71	>200	124

general, the magnitude of the ranges of concentrations obtained for background and contaminated sites were similar and overlapped, with the exception of the concentrations of Mn, which were clearly enriched in the contaminated area. Furthermore, there was no relation between concentration and the distance from the focal point of contamination in the contaminated area (P1-P11, Table 1).

The tissue burdens of metals may be subjected to homeostatic regulation, which would minimize any differences in the levels of metals in slugs sampled in contaminated and uncontaminated zones. Snails have been shown to exert a strong degree of homeostatic regulation of Cu (Dallinger et al. 2005; Nica et al. 2013), which is consistent with the normal distributions of Cu concentrations observed in the present study (Fig. 2). Nevertheless, other elements, such as Cd and Zn, are subject to a lower degree of homeostatic regulation (Ardestani et al. 2014).

The high level of intrapopulation variability in the metal concentrations explains the lack of differences in the metal burdens in slugs from contaminated and uncontaminated zones and the lack of a gradient of accumulation in the surroundings of the focal point of contamination. If the SS are not appropriately characterized because of an insufficient number of samples, differences and gradients will not be detected; nevertheless, if the difference is very small, it makes no sense to further increase the sample size. The intrapopulation variability may be caused by many factors such as nutritional, physiological and reproductive status, the sex and the age of animals (see, e.g. Ireland 1984; Greville and Morgan 1989; Menta and Parisi 2001; Oehlmann and Schulte-Oehlmann 2003). The intrapopulation variability in the metal concentrations in the slugs in the present study was extremely high (Fig. 2), as observed in previous studies (e.g. Mn in Popham and D'Auria 1980). Therefore, very high numbers of slugs are required to characterize and differentiate the SS. Thus, to characterize some of the metals at SS B10, B11 and P2 with an error of 20 %, the number of individuals required would be 91, 51 and 144, respectively (Table 2). Fewer specimens are required to characterize the mean concentrations when the variability in the distributions of the metal concentrations in the slugs from a SS is low (e.g. for Cu only 7 slugs were required at B11; Table 2). The number of specimens required to differentiate populations is also very high (i.e. in almost half of the comparisons, it would be necessary to use more than 120 slugs). Cadmium is an extreme case, in which more than 200 slugs would be required to differentiate the distributions of the metal concentrations at the SS compared. Obviously, this number of samples would be difficult to obtain and is also ethically unacceptable. Assuming that the levels of variability found in this study are representative of the species studied, the number of slugs that are usually collected (i.e. 6–20 individuals) would not enable a SS to be adequately characterized or different SS to be differentiated. On the other hand, the variability in the concentrations of the essential elements was lower than those of the nonessential elements, as the former are subject to homeostatic regulation, whereas the latter are accumulated (Laskowski and Hopkin 1996). Thus, the concentrations of Cd were less variable than those of elements such as Fe and Mn (Fig. 2).

One way of decreasing the variability may be to select the tissue in which the variability in metal concentrations is lower than in the whole samples. The tissue would also have to contain high levels of metals and be of sufficiently high biomass to enable the analytical determinations to be carried out. The hepatopancreas accumulates large amounts of metals, and analytical errors are lower than for whole samples; however, the organ is relatively small and the concentrations of metals are highly variable (see, e.g. Ireland 1979; 1981), so the analysis of the hepatopancreas does not appear to be a valid alternative to the analysis of whole slugs.

Conclusions

Although *A. ater* possesses several characteristics that appear to make it suitable as a biomonitor, the use of the species to monitor contamination by Cd, Cu, Fe, Mn and Zn in terrestrial ecosystems is not recommended. The species is not suitable as a biomonitor in this case because of the high intrapopulation variability in the concentrations of the metals studied, and because for Cu, Fe and Zn, no differences were detected in the tissue concentrations of metals between slugs from uncontaminated and contaminated sites.

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