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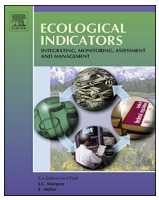

Multi-model inference analysis of toxicological responses and levels of heavy metals in soft tissue of land snail *Cornu aspersum* caged in proximity to an industrial setting

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ABSTRACT

The use of indicator species has become a standard monitoring approach in environmental risk assessment; however, pollutant bioavailability and its geographical distribution as well as species-sensitiveness could be affected by different factors. The present study investigated the potential use of the land snail *Cornu aspersum* as bioindicator of industrial air pollution by using cytological and biochemical responses and trace element levels in individuals caged in proximity to an industrial setting. Eleven sites were selected based on wind direction and distance. Snails were placed in holed plastic cages, transplanted to each site and maintained for 30 days fed *at libitum* and under constant humidity. Several oxidative stress responses including catalase (CAT) and glutathione-s-transferase (GST) activities, levels of malondialdehyde (MDA) and total metallothioneins (MTs) content were determined in snail hepatopancreas while lysosomal membrane stability (LMS) and loss of DNA integrity through micronuclei frequency (MN) were assessed in haemocytes. Trace elements were also analysed in snail whole soft tissues. Multi-model inference used to predict snail's biological responses in relation to trace elements levels and distance from the industrial setting was able to disentangle the relative contributions of different influencing predictors and could be successfully applied in environmental risk assessment by using land snail as bioindicator species.

1. Introduction

Air pollution remains one of the most significant anthropogenic sources of health disorders for both humans and wildlife. Monitoring tools consist primarily of atmospheric deposition analysis using technical instrumentation for mapping and exposure assessment; however, such instrumentation does not allow for addressing pollutant bioavailability and, more importantly, ecological impacts to terrestrial biota (De Temmerman et al., 2004). The use of organisms as bioindicators is now considered an effective alternative and well-established tool in pollution monitoring programs, as they can detect and predict environmental concentrations of pollutants and related effects across time and spatial extent (Massini et al., 2019).

Bioconcentration can describe the extent and intensity of pollution in small-scale surveys as well. Through biomonitoring studies, pollution sources and impacts produced by multiple sources that generate small-scale patterns within an irregular landscape, including wind dynamics that affect dispersion of pollutants, can be identified (Abirel et al., 2014).

Bioindicators respond effectively to different levels of pollution. Understanding the extent of biological responses in living organisms exposed to contaminated matrices is fundamental for their use in monitoring and assessing ecological impact at the ecosystem level (Connon et al., 2012). Biomonitoring of air pollution has been conducted mainly with lichens and mosses by virtue of their low cost in comparison to chemical analyses and the effect-related information that can be obtained (Conti and Cecchetti, 2001). However, lichens could be less sensitive to accumulation processes upon transplanting when small particle concentrations in the atmosphere are particularly high, probably due to stress response (Massini et al., 2019).

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More recently, terrestrial gastropods have been studied as potential bioindicators for air pollution monitoring and assessment.

Land snails play an important role in soil ecosystems as a component of herbivorous and detritivores fauna (Russell-Hunter, 1983) and are important prey for mammals, birds and large invertebrates (Symondson and Lidell, 1993). The species *Cornu aspersum* (often indicated *Helix aspersa* or *Cantareus aspersus*) has a wide distribution in Europe and in various Mediterranean countries. It is common in urban areas and its responsiveness to environmental changes including chemical contamination has been widely documented (see Balbi et al., 2018 and references within).

Several studies reported the marked capacity of gastropods to bioconcentrate elements from the surrounding environment (Gomot-de Vaufleury and Phian, 2000; Beeby and Richmond 2002; El-Shenawy et al., 2012). Dallinger (1993) defines gastropods as macroconcentrators of several heavy metals. Land snails accumulate heavy metals via different routes as food, from contact with soil and also by inhalation; they are, therefore, successfully used as bioindicator species of soil contamination (Viard et al., 2004a; Viardet al., 2004b; De Vaufleury 2015; Pauget and de Vaufleury, 2015). In our previous study, vaporized $CdCl_2$ in controlled laboratory conditions resulted in cadmium accumulation in various tissue of the land snail *C. aspersum* (hepatopacreas > whole body > foot), as well as significant increases in cytotoxicity, oxidative stress response (catalase activity) and metallothionein content (gene and proteins) (Sturba et al., 2018). Growth inhibition and impairment of reproductive capacity have been also reported in gastropods exposed to heavy metals both in laboratory-controlled conditions and in the field (Gomot-de Vaufleury and Kerhoas, 2000; Schmielau et al., 2019).

Various species of land snails have been used as sentinels in biomonitoring studies conducted in urban areas impacted by heavy metals (Regoli et al., 2006; Radwan et al 2010a; El-Shenawy et al., 2012; Abdel-Halim et al., 2013; Filippi et al., 2018). The translocation of healthy caged snails to a contaminated site allowed for recognition of exposure-related biological responses and for identification of contaminant sources (Gomot-de Vaufleury and Phian, 2000). Use of local specimens provides more information on heavy metals accumulation and distribution on a time-scale basis; however, it could underestimate actual levels of contamination due to induction of individual adaptive responses and/or selection of more resistant genotypes over time (Voua Otomo and Reinecke, 2010; Notten et al. 2006). During lifetime, the interaction with soil as for instance during egg-laying and embryo development, sheltering and hibernation could speed adaptation even in the presence of high levels of contamination (Rota et al., 2016). Filippi et al. (2018) reported that micronucleus frequencies were reduced by one-half in wild specimens of land snails compared to caged ones (13 days) despite levels of heavy metals in whole soft tissue were significantly higher in wild snails. Thus, the use of healthy caged snails in a biomonitoring program for heavy metals deposition appears to be an ideal means for achieving relevant and accurate information about the biological relevance of air pollution. This includes determination of the magnitude of physiological imbalances caused by air pollution and adaptive defence mechanisms by local populations.

The present study represents the first attempt to use the land snail *C. aspersum* as a bioindicator of air pollution in an industrialized area using an integrated approach based on cytological, biochemical, geochemical and statistical analysis. Snails were caged and situated at different distances from an industrial source to determine correlations with the biological sub-lethal effects observed. Concentrations of six heavy metals, selected on the basis of a previous study in the area, were detected in various tissues of snails 30 days after translocation. Several oxidative stress responses including catalase (CAT) and glutathione-s-transferase (GST) activities, malondialdehyde (MDA) levels and total metallotioneins (MTs) content were analysed in snail hepatopancreas as well as lysosomal membrane stability (LMS) and loss of DNA integrity in snail haemocytes. We used multi-model inference to predict biological responses measured in snails in relation to trace element concentrations in their whole soft tissue and distance from the industrial setting.

2. Materials & methods

2.1. Materials and reagents

The following chemicals were purchased from Sigma-Aldrich: neutral red (NR) dye, K_2HPO_4, KH_2PO_4, dithiothreitol (DTT), phenylmethanesulfonylfluoride (PMSF), Leupeptin, Pepstatin A, Aprotein, 5,5′−1-chloro-2,4-di-nitrobenzene (CDNB), NaH_2PO_4, Na_2HPO_4, reduced glutathione (GSH), dimethyl sulfoxide (DMSO), chloroform, H_2O_2, Triton × 100, trichloroacetic acid (TCA) and 2-thiobarbituric acid (TBA). Hydrochloric acid (HCl), sodium chloride (NaCl), methanol, calcium chloride ($CaCl_2$), and nitric acid (HNO_3) were purchased from Panreac. Ethylenediamine tetraacetic acid (EDTA), β-mercaptoethanol, and Coomassie blue were purchased from Biorad. Sucrose and Giemsa were purchased from J.T. Baker and KCl from Carlo Erba.

2.2. Translocation study

The study was carried out in October 2018 in a heavily industrialized area in Italy, which was divided into three sub-areas designated as of high-, medium- and low-risk, based on the distance from the main industrial sources. The high-risk sub-area includes sites located between 0 and 3 km from the main industrial settings; the medium-risk sub-area includes sites located from 3 km to 7 km and the low-risk sub-area includes sites located over 7 km from the sources. A total of eleven sites were selected in order to embrace various levels of risk (high, medium and low) based on distance from main industrial settings, wind direction (main winds are north-northwest/south-southeast) and proximity to air pollution monitoring stations, located at various distances inside the city center. Sites 1, 2, 4, 5, 6 and 8 were located south of the main industrial setting and risk levels include high, medium and low. Sites 3 and 9 were located southeast and sites 10, 7 and 11 were located north of the main industrial setting, covering risk levels from medium to low (Fig. 1). Morphology and geology of the study area was also considered as well as previous data on heavy metals

High risk: 0-3 Km	Medium risk: 3-7 Km	Low risk: > 7 Km
Site 1: 2.3 Km	Site 10: 6.3 Km	Site 4: 8.5 Km
Site 2: 2.5 Km	Site 3: 6.8 Km	Site 8: 9.2 Km
Site 5: 2.6 Km	Site 6: 4.7 Km	Site 9: 15 Km
	Site 7: 7 Km	Site 11: 40 Km

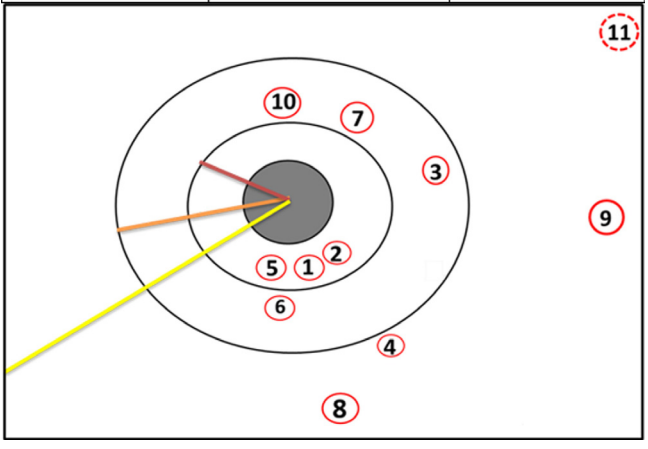

Fig. 1. Sites divided according to level of risk based on distance from the main industrial settings and graphical representation of distribution of the eleven sites around the industrial setting and in the sub-area of risk. Site 11 is dashed. Distances are expressed in km.

and PM_{10} levels in the air.

Adult land snails (*C. aspersum*) (3 ± 0.1 cm mean shell length; 2.3 ± 0.1 cm mean shell width; 9.5 ± 0.8 g mean total weight), were provided by a commercial organic farm located in a rural location in Italy, far from any city center or industrial settings.

Snails were maintained under standard laboratory conditions for acclimation from their dormancy state (for details see Sturba et al., 2018). They were further divided into groups of 50 individuals which were placed into transparent plexiglass cages (20 × 35 × 60 cm) perforated with holes of 1 cm of diameter to allow the passage and circulation of air during field exposure.

In order to guarantee optimal conditions for snail health during *in situ* experiments before translocation, a preliminary experiment was conducted to assess the effects of environmental and biological factors on snail physiological responses under controlled laboratory conditions (see supporting information). Biological responses as well as temperature and humidity, were recorded inside each cage and found to be in accordance with previous study where external and internal temperatures did not differ. Humidity was found to be higher inside the box than the outside (supporting information Figs. S2 and S4). Temperature and humidity of the study area, during the exposure experiment (ranging respectively 22 °C to 15 °C and 91 to 56%) have been reported in the supporting information (Fig. S4).

In each site, two separate cages were placed at regular distances approximately 5 m apart. Cages were maintained *in situ* for 30 days and snails were constantly (twice a week) hydrated using distilled water and fed with lettuce (*Lactuca sativa*) and carrot (*Daucus carota*) *ad libitum*. Excreta and uneaten food were removed before hydration and feeding. A small container (10 × 8 cm) was placed inside each cage and filled with water to maintain constant humidity and to avoid dormancy state. The last feeding was 4–5 days before sacrifice. At the end of 30 days, cages were removed from the 11 sites, shipped to the laboratory and, after recording mortality, surviving animals were processed for the analysis. Ten animals from each site were stored at −80 °C for trace element analysis in whole soft tissue. Thirty animals were dissected and hepatopancreas immersed in liquid nitrogen and then stored at −80 °C for cytological and biochemical assays. A control site was located far away from any source of contamination and snails were maintained in the same way of the other sites of translocation study. At the end of the exposure period trace element analysis of whole soft tissue of snails was performed.

2.3. Biological response

Cytotoxicity and genotoxicity were investigated in snail haemocytes isolated from haemolymph collected using a syringe without needle from a small hole made at the top of the shell as described by Snyman et al. (2000). The Neutral Red Retention Time assay (NRRT) was performed according to the method Lowe et al. (1995). A total of 200 µL of haemolymph was placed on a coverslip and the adhered cells incubated for 15 min with 200 µL of a freshly prepared neutral red (NR) dye working solution (5 µL of 20 mg neutral red powder dissolved in 1 mL DMSO added to 995 µL snail Ringer solution). Haemocytes were washed two times with Ringer solution (NaCl 5 g/l; KCl 0.08 g/L; $CaCl_2$ 0.6 g/L pH 7.3) and observed at 15 min intervals under an Olympus BX51 light microscope. Images were taken with a DP50 camera at 80X using Olympus DP-software. The NRRT was calculated as the time at which ≥50% of counted haemocytes showed red cytosols after leakage of the NR dye from lysosomes.

The micronucleus assay (MN) was performed by a modified method of da Silva et al. (2013). The haemolymph was diluted (1:1, v/v) in freshly prepared methanol:acetic acid (1:1, v/v) solution. After 10 min on ice, the solution was placed on a slide for 1 h and the adhered cells fixed in methanol for 10 min at −20 °C. The slides were air-dried and stained for 10 min in a Giemsa 6% solution. Slides were observed under a light microscope at 100X with the immersion technique on an Olympus BX51 microscope. The test was expressed as the ‰ micronucleus.

Oxidative stress enzyme activity and lipid peroxidation were carried out in ten composite pools (20 individuals) of the S9 fraction obtained from land snail midgut glands. Tissues were homogenized (1:4 w/v) in a buffer (50 Mm K_2HPO_4, 0.75 M sucrose, 1 mM EDTA, 0.5 mM DTT, 400 µM PMSF, 10 µM Leupeptin, 1 µM Pepstatin A, 1 mg/L Aprotein, pH 7.5) and centrifuged at 12000 × *g* for 20 min at 4 °C according to the method of Regoli et al. (2006). The resulting supernatants (S9) were used to analyse soluble proteins, catalase (CAT) and glutathione-S-transferase (GST) enzyme activity and malondialdehyde (MDA) content.

CAT activity (EC 1.11.1.6) was measured by recording decrease in absorbance at 240 nm due to H_2O_2 consumption (Aebi, 1984). An aliquot of S9 fraction was added to 100 mM phosphate buffer (pH 7) and the reaction started by addition of the H_2O_2 (150 mM in 100 mM phosphate buffer) at 30 °C. Enzyme kinetic was recorded for 1 min using a Shimadzu UV-160A spectrometer and CAT activity was calculated as µmol H_2O_2 consumed min^{-1} mg^{-1} protein.

GST activity was assessed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate according to the method of Habig et al. (1974). GST assay conditions in the reaction mixture (final volume 220 µL) were as follows: pH 7.4, 18 °C in a microplate containing 1.5 mM 1-chloro-2,4-dinitrobenzene (CDNB, extinction coefficient 9.6 mM^{-1} cm^{-1}) in 0.1 M NaH_2PO_4/Na_2HPO_4 buffer, 1.5 mM reduced glutathione (GSH) and 20 µL diluted S9 fractions. The increase in absorbance of the reaction product was measured at 340 nm, before and after 2 min incubation at 18 °C using a Victor 3 1420 Multilabel Counter (Wallak). Activity was expressed as µmol GS-DNB conjugate min^{-1} mg^{-1} protein.

LPO occurrence was evaluated by determining thiobarbituric acid-reactive substances (TBARS) formation in the midgut gland S9 fraction of caged snails as previously described by Sturba et al. (2018). A 200 µL aliquot of sample was added to 150 µL 0.1 M phosphate buffer (pH 7.4) and 250 µL of 20% TCA-0.01% BHT solution and centrifuged at 5670 × *g* at 4 °C for 10 min. 400 µL of the resulting supernatant was mixed with 80 µL 0.6 M HCl and 320 µL of 25 mM TRIS-120 mM TBA solution (pH 7.9) and incubated at 80 °C for 15 min. The supernatant was used to spectrophotometrically quantify the MDA content at 530 nm using a Victor 3 1420 Multilabel Counter spectrophotometer (Wallak). Levels of MDA were expressed as mmol MDA mg $protein^{-1}$.

Total proteins in S9 homogenates were measured according to Bradford (1976) using a Shimadzu UV-160A visible recording spectrometer and bovine serum albumin as a standard, reading at 595 nm.

The MTs protein content was quantified in hepatopancreas using a spectrophotometric method at 420 nm (Viarengo et al., 1997). Two g of tissue were homogenized (1:3 w/v) in 0.5 mM sucrose buffer mixed with 20 mM Tris, 0.5 mM PMSF, 0.006 mM leupeptin, and 0.001% β-mercaptoethanol (pH 8.6) and centrifuged at 31,986 × *g* for 20 min at 4 °C. One mL of supernatant was mixed with 1.05 mL of ice-cold ethanol and 80 µL chloroform and centrifuged at 6000 × *g* for 10 min at 4 °C. The supernatant was again mixed with 40 µL HCl 37% and 3 volumes of ice-cold 70% ethanol. After 1 h at −20 °C, the solution was centrifuged at 6000 × *g* for 10 min. The pellet was resuspended in 2 mL of a 70% ethanol chloroform/buffer solution (0.5 mM sucrose, 20 mM Tris, pH 8.6) (87/1/12 v/v/v) and centrifuged at 6000 × *g* for 10 min at 4 °C. After 10 min of nitrogen flow evaporation, the pellet was resuspended in 150 µL 25 M NaCl and 150 µL 4 mM EDTA. The solution is mixed with 4.2 mL of 0.43 mM DTNB (in 0.2 M phosphate buffer pH 8 with 2 M NaCl) and centrifuged at 3000 × *g* for 5 min. The supernatant was analysed at 420 nm on a UV–visible spectrometer.

2.4. Trace element analysis

Snails were sacrificed by freezing (−80 °C), freeze-dried for 48 h, homogenized and subsequently divided into three pools of similar

weight. Individuals of each pool were digested with a mixture of 3 mL HNO_3 and 0.5 mL H_2O_2 (ultrapure trace-grade reagents) in Teflon bombs placed in a microwave digestion system (Milestone Ethos 900). A certified reference material and a blank of the employed reagents were included in each digestion batch. Solutions were filtered (0.45 μm), diluted to 50 mL with ultrapure water and stored in PE bottles before analysis. Concentrations of heavy metals (Cd, Hg, Pb, Tl, V and Zn) in whole soft tissue of snails were determined by inductively coupled plasma-mass spectrometry (ICP-MS) using a Perkin Elmer NexION 350 spectrometer. Analytical accuracy was assessed by determination of heavy metal concentrations in the standard reference material SRM 2977-mussel tissue (Organic Contaminants and Trace Elements) of the National Institute of Standards and Technology. The analytical precision was determined by using five replicate analyses of each snail sample. Percentage of recovery of the trace elements ranged from 95% (Pb) to 110% (Tl) of the certified concentration in the standard reference material.

2.5. Statistical analysis

Multiple regression analysis represents a powerful tool to predict indicators which may depend upon different variables. These models can disentangle the effects of focal predictors from those of other confounding variables by isolating and testing their net contributions on the measured indicator (Zuur et al., 2009; Zuur & Ieno, 2016). Extensions of such models can also address hierarchical/nested sampling designs or grouping factors via random effects, e.g., to handle repeated measurements conducted within the same experimental unit (Zuur et al., 2009; Harrison et al., 2018). These models have been widely used in ecological field studies, where most variables cannot be controlled by the experimental design itself (Bolker et al., 2009; Zuur et al., 2009). However, their use in ecotoxicological research is still limited (Johnson & Omland, 2004). Through linear mixed models and generalized linear mixed models (LMMs and GLMMs; Zuur et al., 2009) we modelled the following six biological parameters (response variables): CAT, GST, MDA, MTs, MN and NRRT. The MN and NRRT, being continuous and bounded variables, were standardized within the range 0–1 (i.e., divided respectively by 1000 and 100) to be modelled through beta errors (link function: logit). Statistical analyses were conducted following the information-theoretic approach (Burnham & Anderson, 2002), by evaluating multiple competing *a priori* hypotheses for each indicator. Multi-model selection approaches have become invaluable tools whenever multiple hypotheses are plausible and are employed for assessing the combination(s) of predictors which best contribute to support empirical data (Grueber et al., 2011; Harrison et al., 2018). In particular, model selection allows for ranking of competing models and selection of those with the lowest uncertainty through various criteria, e.g., by balancing model simplicity and goodness of fit (Aho et al., 2014). Once a subset of candidate models has been generated using such criteria, selected models can be used to predict the measured indicator by estimating the effects of predictors. Previous studies identified major influencing predictors of our biological parameters (Table S2, Supporting information). We could not discard any combination of these explanatory variables, as all the relevant underlying hypotheses could be meaningful biologically. Consequently, for each biological parameter, we performed a model selection to rank all possible models, each corresponding to the relevant combination of fixed effects. In the full model, whole soft tissue concentrations (mg Kg^{-1}) of V, Hg, Zn, Tl, Cd, Pb in snails as well as distance from industrial setting (in a straight line; km) were set as fixed effects, i.e. covariates. Sample size (n = 10 observations) allowed for the number of included predictors (7), because, as a rule of thumb, at least ten observations per predictor should be used (Bolker et al., 2009; Grueber et al., 2011). Multicollinearity among covariates was assessed through the Spearman correlation coefficient (Press et al., 1992), to allow checking for both linear and nonlinear correlations. All the Spearman correlation coefficients were $< |0.6|$, indicating no multicollinearity between explanatory variables (Zuur et al., 2009).

As snails were transplanted at each site inside a box, relevant measured levels of biological response could be site-specific (confirmed by discriminant function analysis; see Results). We therefore included the sampling site as a random intercept in every model to account for repeated measures conducted at the same site.

Model selection was performed using the minimum Akaike's Information Criterion corrected for small samples (AICc: Burnham & Anderson, 2002), a metric which considers both model parsimoniousness and goodness of fit (Aho et al., 2014). For each response variable, all alternative models, each one representing a candidate *a priori* hypothesis, were ranked and weighted from the full model (number of models run per full model: $2^7 = 128$, where 7 is the number of predictors). The null model (i.e., that including the random intercept only) was also included in model selection to allow for assessment of model performance relative to a fixed baseline (Mac Nally et al., 2018). We used a conservative approach by following the 'nesting rule', to avoid retaining overly complex models (Burnham & Anderson, 2002; Richards, 2008; Richards et al., 2011; Harrison et al., 2018): models with $\Delta AICc \geq 2$ with respect to the best model (i.e., the model with the lowest AICc value), as well as models with an AICc value greater than that of any simpler alternative were not selected. Akaike model weight the probability that a given model is the best model (Burnham & Anderson, 2002: 75; Symonds & Moussalli, 2011; Galipaud et al., 2014, 2017), was thus standardized within each subset of selected models: the highest the weight, the lowest the model uncertainty. Results of model selection are reported in Table 1.

In order to estimate the effects of predictors and relevant uncertainty, inference is performed either by model averaging, or based on each of the selected models separately (Richards et al., 2011; Galipaud et al., 2017). Whenever no single best model is determined, it is recommended that ecological conclusions be drawn from a set of selected models rather than on the first-ranked model only (Burnham & Anderson, 2002; Galipaud et al., 2014, 2017). Following the more cautionary approach suggested by Richards et al. (2011), we based inference on selected models. For each response variable, coefficient of predictors, 95% confidence intervals and variance of random intercepts were estimated for each of the selected models separately. Parameters estimated from best models are shown in Table 2, while parameters estimated from other selected models are reported in Table S3. The

Table 1
Result of model selection: best models and models with $\Delta AICc < 2$, each with K, logLikelihood, AICc value, ΔAICc, and Akaike weight (standardized within the subset of selected models). Top-ranked models are shown in bold. DIS = distance to industrial settings.

Biomarker	Models selected	k	logLik	AICc	ΔAICc	Weight
CAT	**DIS + Pb + (Site)**	5	−505.83	1022.2	0	0.281
	DIS + V + (Site)	5	−505.87	1022.3	0.09	0.268
	DIS + (Site)	4	−507.34	1023.1	0.83	0.186
	Cd + (Site)	4	−507.62	1023.6	1.39	0.140
	Hg + (Site)	4	−507.75	1023.9	1.55	0.124
GST	**DIS + Cd + Tl + V + (Site)**	7	−847.00	1709.1	0	0.297
	DIS + Tl + V + (Site)	6	−848.25	1709.3	0.22	0.268
	Tl + (Site)	4	−850.57	1709.5	0.43	0.241
	V + (Site)	4	−850.79	1710.0	0.86	0.193
MDA	**Pb + (Site)**	4	−781.35	1571.1	0	0.587
	Cd + (Site)	4	−781.70	1571.8	0.70	0.413
MTs	**DIS + Pb + (Site)**	5	−298.54	607.7	0	0.541
	DIS + Cd + (Site)	5	−299.33	609.2	1.59	0.244
	DIS + (Site)	4	−300.56	609.5	1.85	0.214
MN	**DIS + Zn + (Site)**	5	229.99	−449.4	0	0.630
	DIS + Cd + (Site)	5	229.46	−448.3	1.06	0.370
NRRT	**DIS + (Site)**	4	171.05	−337.7	0	1

Table 2
Parameters estimated (B: coefficient, CI: 95% confidence interval, variance of random intercepts) from the best models of biomarkers. Predictors whose confidence intervals do not include 0 are marked with an asterisk. DIS = distance to industrial settings.

Biological response	Predictor	B	95% CI
CAT (Site) variance = 513	intercept	327.3250	304.0270; 350.6228 *
	DIS (km)	−1.8880	−3.3164; −0.4595*
	Pb (mg Kg^{-1})	26.440	−3.1296; 56.0093
GST (Site) variance = 96933	Intercept	3217.1	2218.849; 4215.289 *
	DIS (km)	−30.3	−56.602; −3.995*
	Cd (mg Kg^{-1})	−297.6	−660.148; 64.969
	Tl (mg Kg^{-1})	1191.5	278.311; 2104.759 *
	V (mg Kg^{-1})	4727.9	546.457; 8909.292 *
MDA (Site) variance = 164598	Intercept	4500	4178.144; 4821.03 *
	Pb (mg Kg^{-1})	425	72.503; 776.66 *
MT (Site) variance = 8.36	Intercept	17.0495	13.8525; 20.2466*
	DIS (km)	0.8112	0.6225; 0.9999 *
	Pb (mg Kg^{-1})	4.6219	0.1676; 9.0761 *
MN (Site) variance = 0.108	Intercept	−0.7217	−1.0975; −0.3459 *
	DIS (km)	−0.0267	−0.0460; −0.0074 *
	Zn (mg Kg^{-1})	−0.0025	−0.0044; −0.0006 *
NRRT (Site) variance = 1.05	Intercept	2.3473	1.5130; 3.1816 *
	DIS (km)	−0.0733	−0.1326; −0.0140 *

effect of each predictor was assessed by checking whether its 95% confidence interval included 0, which would imply uncertainty in the predictor effect on the biological response (Bolker et al., 2009; Grueber et al., 2011; Zuur & Ieno, 2016; Leroux, 2019). Validation of the best models was made by visual inspection of residual patterns (Zuur et al., 2009). Model selection, LMMs and GLMMs were performed through the R packages *MuMIn* (Bartoń, 2012), *lme4* (Bates et al., 2015) and *glmmTMB* (Brooks et al., 2017), respectively.

In order to validate the risk levels selection, we assessed differences in biological responses measured in snails and trace elements levels in whole soft tissue, across the three risk levels and across sites within the same risk levels using discriminant function analysis (DFA; Rencher, 1995). DFA reduced our multivariate dataset to two discriminant functions (graphically represented by discriminant scores) which are a linear combination of the original variables providing the greatest separation of groups and explaining > 90% of their variability. We tested overall differences between groups through PERMANOVA tests based on Euclidean distances between discriminant scores (significance was computed by permutation of group membership, with 99,999 replicates). Analyses were performed in Past (Hammer et al., 2001).

3. Results

After 30 days of exposure caged snails show significant relationships among biological responses investigated and the distance from the main industrial setting (low, medium and high-risk levels). At the end of the exposure period mortality averaged 30% among sites.

Data for CAT, GST, MN and NRRT were negatively influenced by distance from the industrial setting: the lower the distance, the higher the biological responses both at cellular (NRRT and MN) and tissue levels (CAT and GST) (Table 2; Fig. 2). Conversely, MTs content increased with distance to industrial settings (Table 2; Fig. 2). The effect of this predictor was further confirmed for most of the other selected models (Table S3, Supporting information), supporting its marked contribution in affecting biological responses.

Significant relationships among biological response and concentration of heavy metals in whole soft tissue were observed in snails from all 10 sites. MDA content and MTs protein content were predicted to increase with increasing concentration of Pb (Table 2; Fig. 2). The second-best model of MDA content also provides support to an increase of this biomarker with increased concentration of Cd (Table S3, Supporting information). GST activities were positively related to increasing concentrations of V and Tl (Table 2; Fig. 2), whereas MN frequency was negatively influenced by increased concentration of Zn (Table 2; Fig. 2).

For specific values of CAT and GST activities, MDA levels and MTs content in snail hepatopancreas and MN frequency and NRRT in snail haemocytes, the reader is referred to Table 3. Levels of heavy metals in whole soft tissue are reported in Table 4, those of control snails are reported in Table S1 (Supporting information).

Concerning differences in biological responses measured in snails and levels of heavy metals across the three risk levels and across sites within the same risk levels, we found that the three risk levels were significantly distinguished by discriminant scores (Fig. 3), i.e. both kinds of variables differed across risk levels. Moreover, within all risk levels, sampling sites were significantly distinguished by discriminant scores (Fig. 4), meaning that sites within all risk levels differed to each other.

4. Discussion

In order to investigate the land snail *C. aspersum* as bioindicator of air pollution in an industrialized setting, specimens were caged for 30 days and biological responses analysed at cellular and biochemical levels. Levels of heavy metals in whole soft tissue as well as distance from the industrial setting were used as predictors. Mortality was higher for all investigated sites compared with what reported in the literature (Regoli et al., 2006; Gomot-de Vaufleury, & Phian 2000). This result underscores the importance of the application of a battery of biomarkers at different biological levels in biomonitoring studies. The reported results indicate a more marked response of the antioxidant defence system in snails located in proximity of the industrial setting, which tends to decrease with distance from the contamination sources. The present study is the first attempt to predict biological response of caged snails with distance from a contamination source (industrial setting) with the aim of further validating the use of translocation to monitor air pollution in the field. Moreover, a time-integrated assessment of environmental quality will not be affected by fluctuation of airborne heavy metal concentrations.

The results from our models confirm that levels of heavy metals in snails soft tissues are related to biological responses and that the most impacted sites are closest to the industrial setting. Results of discriminant function analysis further support that variability within sampling sites should be accounted in our models, as well as that the

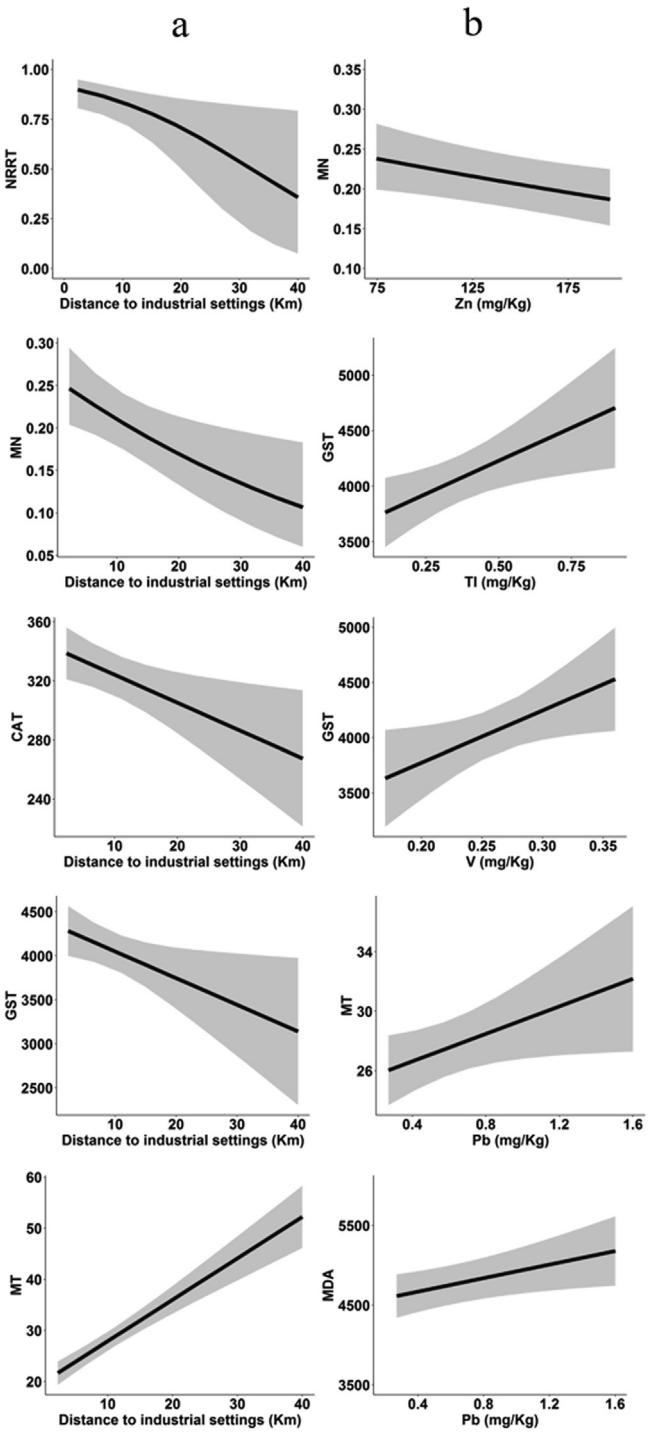

Fig. 2. a) Relationship between distance to industrial settings and predicted levels of biomarkers in snails. Lines: predicted values; bands: 95% confidence intervals. b) Relationship between concentration of relevant heavy metals and predicted levels of biomarkers in snails. Lines: predicted values; bands: 95% confidence intervals.

three risk levels should be controlled by considering the distance from industrial settings.

We further supported the experimental design with three levels of risk (low, medium and high) based on increasing distance from the industrial area (Fig. 1). Biological responses both at cellular and biochemical levels were strongly correlated to levels of heavy metals in snails and in particular genotoxicity (MN frequency), oxidative stress responses (CAT and GST) and MTs content.

Based on biological responses measured in caged land snails, a general alteration of the redox status can be hypothesized. In biological systems, heavy metals toxicity is strongly associated with production of ROS (reactive oxygen species) which affects numerous cellular processes, mostly the functioning of the membrane system (Pinto et al., 2003; Valko et al., 2005). At a subcellular level, lysosome membrane permeability increases as a result of contaminant retention. The NRRT test reflects a normal physiological process that has become compromised following membrane damage (Lowe et al., 1995) and that can serve as an early warning system due to its sensitivity to low environmental exposure concentrations (Svendsen & Weeks, 1995).

In all eleven investigated sites, NRRT in snail haemocytes were lower than 30 min with the highest percentage of lysosomal destabilization occurring within 15 min. Similar findings have been reported in land snails exposed to toxic compounds both at laboratory and field scale (Regoli et al., 2006; Itziou & Dimitriadis, 2011; Itziou & Dimitriadis, 2012; Leomannia et al., 2015; Sturba et al., 2018).

Genotoxicity expressed as MN frequencies further confirms distance as the main driver of biological response with values between 325.7 ± 37.3‰ in the site closest to the industrial setting (site 1) and significantly lower values, as 107.7 ± 8.1‰ in the most distant location (site 11). Such negative correlation also emphasises the effective performance of applied statistical models to predict biological responses with distance from contamination source. In agreement with our study, Filippi et al. (2018) recently reported higher MN frequencies in haemocytes of snails (*Helix aspersa*) closest to a coal plant both in caged (13 days of translocation) and wild individuals.

The negative correlation determined between MN frequencies and Zn levels could be explained by the fact that co-exposure to a suite of chemicals might affect DNA damage even at low contamination levels of a single element (e.g. zinc). Moreover, weakly mutagenic compounds within complex mixtures may affect biotransformation pathways or inhibit DNA repair mechanisms (Martin, 2007).

Oxidative stress occurring in caged snails is also identified by a decrease in CAT activities with distance from the industrial setting. CAT activity is considered a suitable and early biomarker of toxic chemical exposure in gastropods (Radwan et al., 2010b). Its increase prevents accumulation of reactive oxygen species (ROS). Studies have reported increased CAT activity in snails translocated to polluted urban areas (Regoli et al., 2006; El-Shenawy et al., 2012) and in laboratory conditions upon exposure to vaporized cadmium chloride (Sturba et al., 2018). The enhancement of oxygen free radical production causes an increase in oxidative stress and an activation of the antioxidant defence system that protects cells from damage (Regoli et al., 2004).

Similar findings are applicable to GST activities, which play a central role in maintaining cellular redox status and protecting cells from oxidative injury (Dickinson and Forman, 2002), operating both in antioxidant defence and in detoxifying actions (Elia et al., 2007). Moreover, positive correlations determined with levels of Tl and V in snail soft tissues further support their protective role against heavy metals exposure. Mleiki et al. (2015) reported additive effects of co-exposure to Pb and Cd in land snails, with a 10-fold increase of GST activities. Also, El-Shenawy et al. (2012) reported positive correlation between GST activity and Pb and Cu levels in local land snails *Eobania vermiculata* from a polluted site. Accumulation of ROS can initiate lipid peroxidation (LPO) and cause an intracellular excess of malondialdehyde (MDA) (Peluso et al., 2010), the main reactive aldehyde resulting from peroxidation of membrane polyunsaturated fatty acids (Ohkawa et al., 1979). In our study, intracellular excess of MDA does not correlate with distance from the industrial setting; however, a significant correlation with whole body Pb levels was observed, which is in agreement with studies on wild specimens collected from mining and urban contaminated areas (Radwan et al., 2010a; El-Shenawy et al., 2012). The measurement of MDA in caged snails can thus be considered a suitable tool for assessing lipid peroxidation induced by heavy metals.

Concerning detoxification pathways, MTs protein content of caged

Table 3
Mean ± SD of the biological response investigated in the land snail *C. aspersum* translocated for 30 days in 11 different sites of the study area. Ten replicate analyses of each site were performed.

Risk level	Site	CAT (μmoli min^{-1} mg $proteine^{-1}$)	GST (μmol min^{-1} mg $prot^{-1}$)	MDA (m molMDA mg $prot^{-1}$)	MT (ng/g)	MN (‰)	NRRT (%15 min)
High risk	Site 1	388.2 ± 37.2	2.7 ± 0.4	4.6 ± 0.4	17.6 ± 1.1	325.7 ± 37.3	79.4 ± 3.7
	Site 2	331.6 ± 22.7	2.4 ± 0.2	7.8 ± 0.4	24.2 ± 1.2	348.4 ± 21.3	67.9 ± 2.9
	Site 5	314.1 ± 21.5	2.6 ± 0.2	5.0 ± 0.4	21.9 ± 1.3	261.2 ± 31.4	85.3 ± 4.1
Medium Risk	Site 3	322.7 ± 23.5	2.6 ± 0.4	5.4 ± 0.5	24.9 ± 1.1	201.9 ± 4.1	94.8 ± 10
	Site 6	328.7 ± 48.9	2.3 ± 0.3	3.9 ± 0.4	24.1 ± 2.7	160.8 ± 2.5	95.0 ± 1.6
	Site 7	304.0 ± 33.4	2.7 ± 0.3	4.9 ± 0.5	29.9 ± 2.2	118.7 ± 15.1	89.7 ± 1.7
	Site 10	322.2 ± 53.1	2.6 ± 0.3	4.4 ± 0.4	26.0 ± 1.0	172.5 ± 23.8	70.5 ± 4.2
Low risk	Site 4	343.7 ± 38.3	2.3 ± 0.4	4.8 ± 4.9	30.0 ± 2.8	187.3 ± 6.6	88.1 ± 1.0
	Site 8	306.4 ± 44.0	2.3 ± 0.4	5.3 ± 0.5	28.2 ± 1.7	169.3 ± 4.1	94.0 ± 0.9
	Site 9	344.2 ± 48.5	2.7 ± 0.4	4.6 ± 3.9	24.1 ± 2.3	315.4 ± 5.1	58.1 ± 3.3
	Site 11	275.8 ± 23.1	2.5 ± 3.4	4.5 ± 0.6	56.1 ± 7.7	107.7 ± 8.1	33.9 ± 1.5

Table 4
Mean ± SD of trace elements concentrations (expressed in mg kg^{-1} dry weight) in in the land snail *C. aspersum* translocated for 30 days in 11 different sites of the study area. Ten replicates for each sample were performed.

Risk level	Site	V	Zn	Cd	Hg	Tl	Pb
High Risk	Site 1	0.310 ± 0.004	132.83 ± 19.09	1.88 ± 0.31	0.030 ± 0.003	0.290 ± 0.003	0.83 ± 0.13
	Site 2	0.26 ± 0.02	144.83 ± 14.74	2.54 ± 0.37	0.04 ± 0.01	0.79 ± 0.10	0.58 ± 0.09
	Site 5	0.26 ± 0.01	134.04 ± 13.37	1.73 ± 0.22	0.020 ± 0.003	0.26 ± 0.03	0.41 ± 0.08
Medium risk	Site 3	0.25 ± 0.01	114.86 ± 12.96	1.80 ± 0.38	0.03 ± 0.01	0.18 ± 0.03	0.38 ± 0.03
	Site 6	0.24 ± 0.01	155.88 ± 30. 02	1.93 ± 0.31	0.02 ± 0.01	0.27 ± 0.01	0.47 ± 0.03
	Site 7	0.22 ± 0.02	101.13 ± 3.09	2.15 ± 0.12	0.02 ± 0.01	0.13 ± 0.01	0.61 ± 0.20
	Site 10	0.27 ± 0.04	150.10 ± 50.02	1.18 ± 0.69	0.013 ± 0.006	0.16 ± 0.04	0.53 ± 0.20
Low risk	Site 4	0.28 ± 0.02	12.42 ± 15. 43	1.98 ± 0.26	0.030 ± 0.002	0.58 ± 0.08	0.48 ± 0.07
	Site 8	0.24 ± 0.04	114.29 ± 18.18	1.52 ± 0.31	0.03 ± 0.01	0.22 ± 0.03	0.68 ± 0.17
	Site 9	0.21 ± 0.01	86.72 ± 11.02	0.87 ± 0.20	< 0.01	0.34 ± 0.04	0.36 ± 0.08
	Site 11	0.33 ± 0.02	125.56 ± 17.21	1.24 ± 0.38	< 0.01	0.74 ± 0.33	1.13 ± 0.29

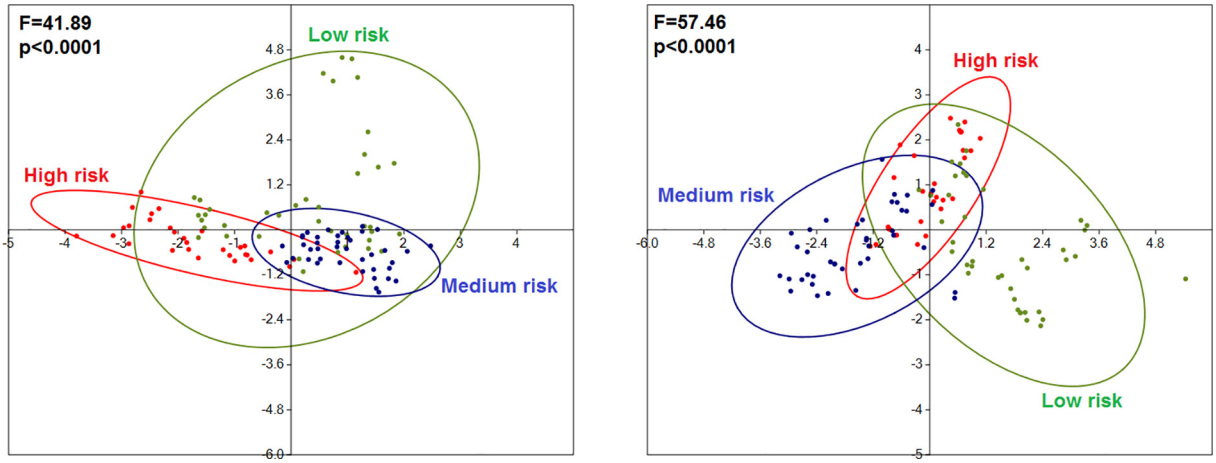

Fig. 3. Differences in biological responses (left) and body burden concentrations of heavy metals (right) between risk levels assessed through DFA. The ellipses containing 95% observations within each risk level are also shown. The x and y axes represent the scores of first and second discriminant functions, respectively, which mainly contributed (> 90%, see text) to separation between risk levels.

snails resulted in a positive correlation with distance from the industrial setting and Pb content in snails soft tissues. El-Shenawy et al. (2012) described a positive correlation between Pb content in the hepatopancreas and MTs levels in land snails, suggesting the ability of the organism to accumulate metals in a biologically active form. MTs are low molecular weight, cysteine-rich metalloproteins, responsible both for the homeostatic regulation of Zn and Cu and for protection of cells from exposure to toxic metals such as Cd or/and Hg (Brouwer and Brouwer Hoexum, 1992; Dallinger 1996; Klaassen et al., 1999; Dallinger et al., 2000). The positive correlation with the distance from the industrial setting is not in accordance with other biological responses analysed. It has been proposed that different heavy metals are able to induce specific MTs isomers. In land snails two MT pools may be envisaged: the first is mainly involved in homeostasis of copper (Cu-MT), and the second is induced by metals and involved in metal detoxification (Cd-MT).

Unfortunately, the method used for quantifying total MTs protein does not allow for distinguishing between the MTs isoforms and for quantifying the total amount including those not induced by heavy metalsexposure. Moreover, in field studies, contradictory results have often been described, with increase, decrease and transitory changes in MTs responses according to the intensity and duration of exposure. The

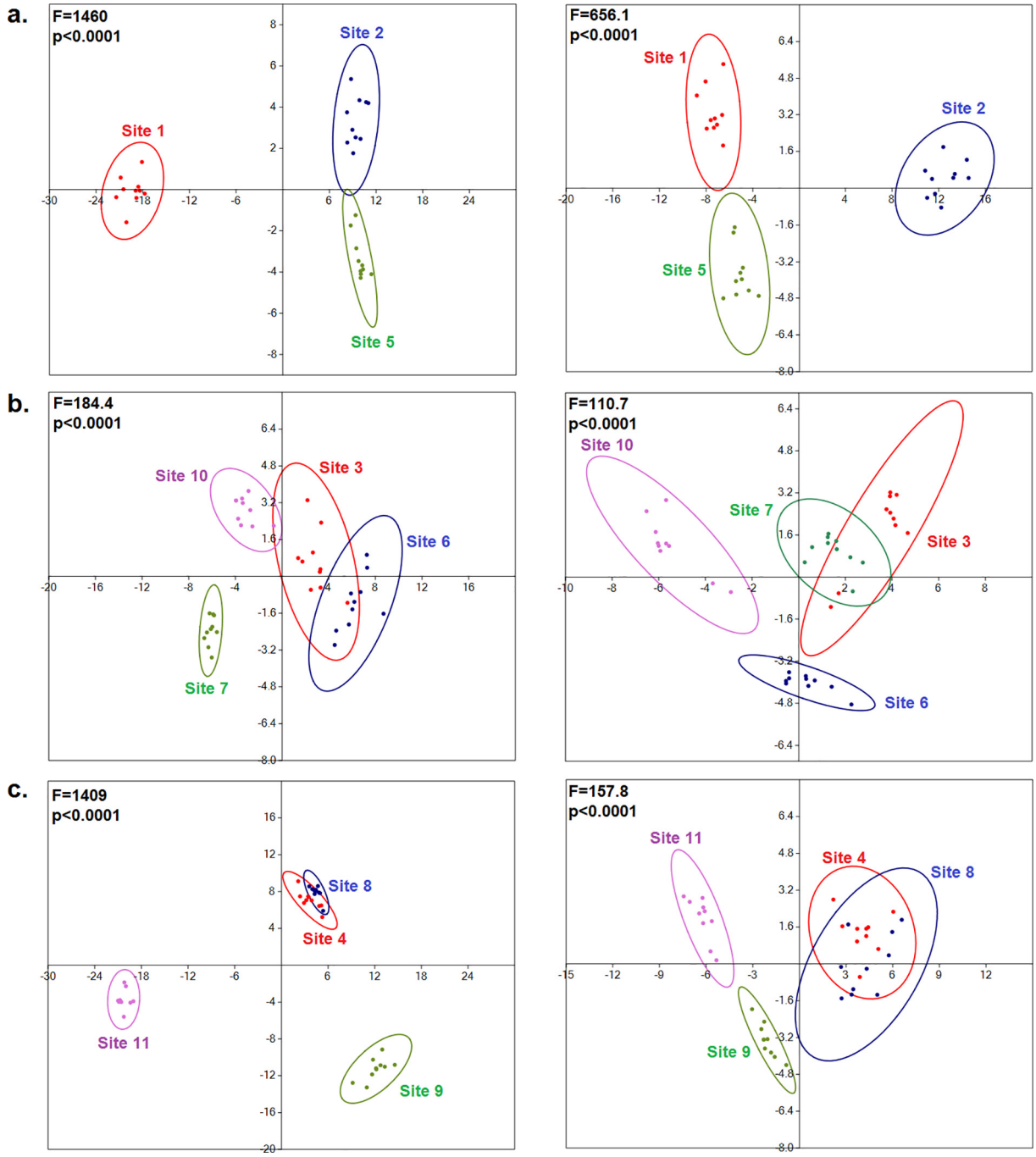

Fig. 4. Differences in biological responses (left panel) and whole soft tissues heavy metals levels (right panel) between sites within the same risk level (a: high risk; b: medium risk; c: low risk) assessed through DFA. The ellipses containing 95% observations within each site are also shown. The x and y axes represent the scores of first and second discriminant functions, respectively, which mainly contributed (> 90%, see text) to separation between sites within the same risk level.

effects on individual biomarkers is useful as warning signal of perturbation but also reflects the complex interactions occurring in cellular processes that are not easy to predict. For these reasons the application of a battery of biomarkers is recommended to better understand the imbalance of physiological processes including defence system, metabolism and REDOX status, caused by pollution.

5. Conclusion

The species *C. aspersum* was found useful for identification of air pollution source, in agreement with previous studies conducted with well-established bioindicator plant and lichen species (Abirel et al., 2014, Massini et al., 2019). Translocation was successfully proven to be an efficient and low-cost tool for air pollution monitoring and effects assessment on a geographical scale. Multi-model inference was a useful statistical method to disentangle the relative contributions of different influencing predictors such as levels of elements/pollutants and distance to contamination source and could be used to predict biological responses of indicator species.

Our models represent a starting point in the development of predictive tools for environmental risk assessment. To this end, future work may test the applicability of these models to different bioindicator species or pollutants, as well as calibrating them to handle nonlinear effects in concentration-response curves (e.g. Smetanová et al., 2015).

CRediT authorship contribution statement

L. Sturba: Data curation, Methodology, Investigation, Formal analysis, Writing - original draft. **N. Fattorini:** Methodology, Formal analysis, Writing - original draft. **G. Liberatori:** Methodology, Investigation. **M.L. Vannuccini:** Methodology, Investigation. **F. Nannoni:** Investigation, Writing - original draft. **G. Protano:** Investigation, Writing - original draft. **A. Tursi:** Project administration. **I. Corsi:** Conceptualization, Visualization, Resources, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.106688.

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