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1 **Impacts of metallic trace elements on an earthworm community in an urban wasteland:**

2 **emphasis on the bioaccumulation and genetic characteristics in *Lumbricus castaneus***

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19   **Highlights**

20   Impact of soil pollution on earthworms at different levels of biological organisation.

21   Differential effects of MTEs on earthworms were investigated in an urban wasteland.

22   Community structure and *L. castaneus* physiology and genetics were studied.

23   MTEs affected earthworm community and bioaccumulation, but not population genetic.

24   *L. castaneus* is a promising model to study the molecular basis of MTE tolerance.

25   **Graphical abstract**

26

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Abstract

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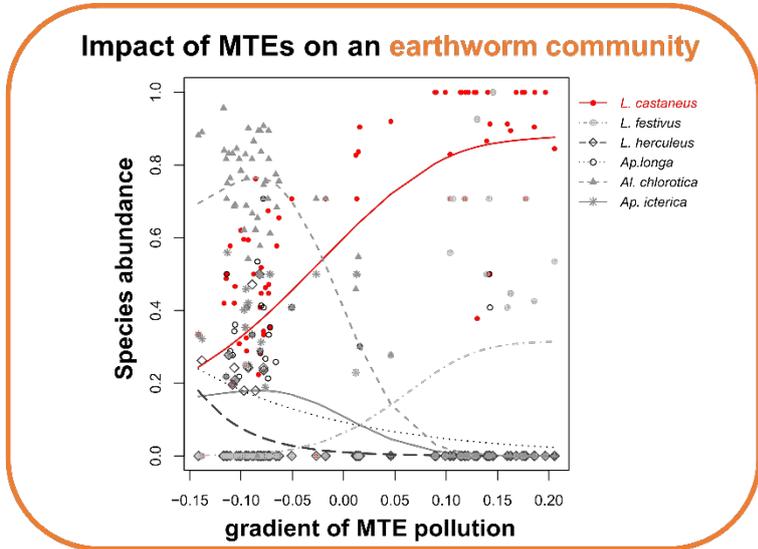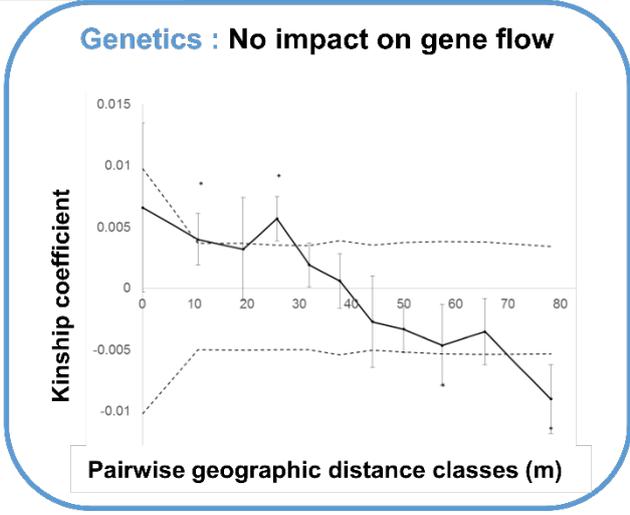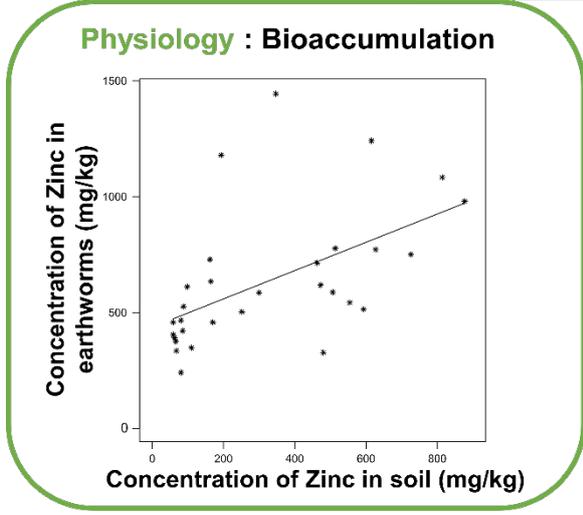
29   Metallic trace elements (MTEs) soil pollution has become a worldwide concern,

30   particularly regarding its impact on earthworms. Earthworms, which constitute the dominant

31   taxon of soil macrofauna in temperate regions and are crucial ecosystem engineers, are in direct

32   contact with MTEs. The impacts of MTE exposure on earthworms, however, vary by species,

33   with some able to cope with high levels of contamination. We combined different approaches

34   to study the effects of MTEs at different levels of biological organisation of an earthworm

35   community, in a contaminated urban wasteland. Our work is based on field collection of soil

36   and earthworm samples, with a total of 891 adult earthworms from 8 species collected, over 87

37   quadrats across the study plot. We found that MTE concentrations are highly structured at the

38   plot scale and that some elements, such as Pb, Zn, and Cu, are highly correlated. Comparing

39   species assemblage to MTE concentrations, we found that the juvenile and adult abundances,

40   and community composition, were significantly affected by pollution. Along the pollution

41   gradient, as species richness decreased, *Lumbricus castaneus* became more dominant. We thus

42   investigated the physiological response of this species to a set of specific elements (Pb, Zn, Cu,

43   and Cd) and studied the impacts of MTE concentrations at the plot scale on its population

44   genetic. These analyses revealed that *L. castaneus* is able to bioaccumulate high quantities of

45   Cd and Zn, but not of Cu and Pb. The population genetic analysis, based on the genotyping of

46   175 individuals using 8 microsatellite markers, provided no evidence of the role of the

47   heterogeneity in MTE concentrations as a barrier to gene flow. The multidisciplinary approach

48   we used enabled us to reveal the comparatively high tolerance of *L. castaneus* to MTE

49   concentrations, suggesting that this is a promising model to study the molecular bases of MTE

50   tolerance.

51 **Keywords**

52 Soil contamination, MTEs, community structure, population genetics.

1. Introduction

Metallic trace elements (MTEs) occur naturally in the earth's crust, but increasing quantities of metals are being released into the environment by human activities. Soil pollution by MTEs has become a global concern (Hou et al., 2017; Weissmannova & Pavlovsky 2017; Rodriguez-Eugenio et al., 2018), particularly in urban areas, as urban soil appears to be more contaminated than agricultural and natural soils (Ajmone-Marsan & Biasioli 2010). In urban soils, anthropogenic sources of MTEs include traffic emissions, industrial discharges and municipal wastes (McLlwaine et al., 2017; Weissmannova & Pavlovsky 2017; Jia et al., 2018). While the most common hazardous MTEs in soils (namely arsenic As, cadmium Cd, chromium Cr, copper Cu, mercury Hg, nickel Ni, lead Pb and zinc Zn) can be non-degradable, persistent, and bioaccumulate and biomagnify in food chains, the specific impact and toxicity of the different MTEs are dictated by their chemical forms (Knox et al., 2000; Weissmannova & Pavlovsky 2017). MTEs tend to accumulate in soil, and sometimes in food webs, representing, beyond certain concentrations and durations of exposure, a significant risk to the health of living organisms, including humans (Tyler et al., 1989).

Earthworms constitute a dominant taxon of soil macrofauna, and their activities of recycling organic matter and modifying soil structure are crucial to the functioning of the soil ecosystem (Blouin et al., 2013). These keystone species are affected by MTEs present in the soil as they are in direct contact with the bioavailable contaminants through the soil porewater (van Gestel et al., 2009). At the community level, fieldworks showed that earthworm biomass and species richness are inversely correlated to metal concentrations in the soils, and especially to Pb, Zn, and Cd, contents (Terhivuo et al., 1994; Spurgeon & Hopkin 1996, 1999; Nahmani et al., 2003; Leveque et al., 2015; Wang et al., 2018b). At the species level, studies have aimed at evaluating metal toxicity, either focusing on their effect on fitness or assessing MTE lethal and sub lethal concentrations (reviewed in Nahmani et al., 2007). However, there are great

differences among species in their sensitivity to MTEs. Some species of earthworms, such as *Lumbricus rubellus*, *L. castaneus*, and *L. terrestris*, were shown to persist even in highly contaminated sites (Spurgeon & Hopkin 1996) and this persistence can often be linked with differences in physiological abilities. For instance, these species have the ability to protect themselves from the toxic effects of metals by sequestering, detoxifying, and storing excess metal (Spurgeon & Hopkin 1996; Vijver et al., 2004; Iordache & Borza 2012; Grumiaux et al., 2015). This protection involves the induction of a gene coding a metal sequestering metallothionein (Sturzenbaum et al., 1998; Brulle et al., 2006), allowing species to tolerate high concentration of MTEs through bioaccumulation processes. In particular, cadmium and zinc are MTEs that have been shown to bioaccumulate in numerous earthworms (van Straalen et al., 2001; Tischer 2009), among which *L. castaneus* (Tischer 2009). *L. castaneus* also accumulated higher quantities of Pb and at higher pace than *L. rubellus*, *Aporrectodea caliginosa* and *A. rosea,* in a laboratory rearing experiment (Terhivuo et al., 1994).

At the population level, MTEs are likely to induce microevolutionary processes through (i) mutations and increased allelic diversity, (ii) emphasis of the effects of genetic drift and bottlenecks and (iii) natural selection, leading to the disappearance of the most sensitive genotypes (Ribeiro & Lopes 2013). In that respect, population genetic approaches using neutral molecular markers ('neutral' refers to a locus that has no effect on fitness, Holderegger et al., 2006) are commonly used to infer microevolutionary processes such as mutation, genetic drift and gene flow (Kirk & Freeland 2011).

Since the responses of individual species and communities are highly dependent on the soil physicochemical properties and the MTE cocktail present locally, the results found in the literature are not always consistent (see the contrasted responses of earthworm communities to Pb concentration from van Gestel et al., 2009 and Leveque et al., 2015). Here, we aimed at providing a comprehensive study of the effects of MTEs on different levels of the biological

103   organisation of a community of earthworm species in a polluted site. Our approach was first

104   exploratory and aimed at studying in their entirety the links between the concentrations of

105   MTEs found on the study site and the earthworm community (abundance and richness). Second,

106   we focused on the specific effect of Pb, Zn, Cd, and Cu, on *L. castaneus.* Pb, Zn, Cd, and Cu,

107   are known to be the most widespread anthropogenic contaminant elements in urban soils

108   (Argyraki et al., 2018), with Pb being one of the major concern in many cities (Ajmone-Marsan

109   and Biasioli 2010). The species, *L. castaneus,* was selected as it was found throughout the site,

110   which makes it possible to study variations in the response of this species to a range of MTE

111   concentrations. Specifically, we hypothesized that *L. castaneus* would show increased levels of

112   bioaccumulation with increased concentrations of these MTEs. We also investigated the neutral

113   genetic variation of this species using 8 microsatellite markers in order to determine how

114   mutation, genetic drift, and gene flow, affect the genetic characteristics of earthworm

115   populations in a contaminated urban soil. To our knowledge, this is the first field study that

116   combines different approaches to assess the responses of earthworms to metallic trace elements

117   in the field.

2. Material and methods

2.1 Study area

The study was carried out in an urban wasteland of 8 ha situated east of the city of Villeneuve-Le-Roi, in the region of Paris (2°26'16.7"E 48°44'26.8"N, France). The area is located inside a loop of the Seine River and near an industrial zone. In 2013, a quantitative evaluation of sanitary risks was carried out at the request of the city, which wanted to convert the site and dedicate it to urban agriculture. The report concluded that an area of 3 ha north of the site presented high levels of 5 MTEs (Cd, Cu, Hg, Pb, and Zn); this polluted zone was then separated from the unpolluted zone by a fence (Guittard 2013). Further, the agro-pedological report by Sol Paysage (2013) defined the soil from the polluted zone as mainly composed of coarse sand with a C/N ratio of 14.5, a cation exchange capacity (CEC, 0-25 cm depth) of 18.7 meq/100g, and a pH of 8.2. The soil from the unpolluted zone is defined as loamy with a C/N ratio of 9.6, a CEC (0-25 cm depth) of 10.1 meq/100g, and a pH of 7.7 (Sol Paysage 2013).

The study plot covered a surface of 50 m x 60 m that straddles in approximately equal proportions the zones initially identified as polluted and unpolluted. Based on previous work on related species, this surface area is appropriate to study the fine-scale population genetic structure of earthworms in order to infer microevolutionary processes at the intra-population scale (Novo et al., 2010; Dupont et al., 2015).

2.2 Earthworms collection

Earthworms were collected over two years. The first sampling aimed to collect specimens for the studies of community structure and population genetics. It was done over three consecutive days in 2016 (March 29th, 30th, and April 1st). Earthworms were collected from 87 quadrats (50 cm x 50 cm), chosen following a stratified sampling protocol across the

141   study plot (Fig. 1a). 52 quadrats were sampled in the polluted zone and 35 in the unpolluted

142   zone. We sampled more densely in the polluted zone, in order to better capture the variations

143   in soil pollution, than in the unpolluted zone, where the soil is assumed to be homogeneous.

144   Note that the resolution of our sampling is likely to have captured the heterogeneity of soil

145   pollution, which is at a smaller spatial scale than the 50 cm x 50 cm quadrat of our sampling.

146   Over each quadrat, 10L of AITC (allyl isothyocyanate) and isopropanol diluted in water

147   (1:100:10000L) were poured in two stages. All earthworms were collected as they came out of

148   the soil until no more individuals came out (we waited up to 15 minutes for each quadrat). They

149   were first transferred to a solution of 10% dilution alcohol and water, and then stored in 100%

150   alcohol before taxonomic identification in the laboratory and analyses. The number of adults of

151   each species as well as the number of juveniles were counted per quadrat. Earthworms were

152   identified using the taxonomic keys of Bouché (1972) and Sims and Gerard (1999). Further,

153   the identification of a subset of 55 individuals, for which either the taxonomic identification

154   was uncertain or belonging to species rarely occurring within our sampling, was confronted

155   with the results of the barcode identification, carried out using a fragment the cytochrome c

156   oxidase subunit I (COI) mitochondrial gene (Genbank accession number MN519732 -

157   MN519786, Hebert et al., 2003; Dupont et al., 2019).

158   For the second sampling, carried out the 6th of April 2017, only individuals from *L.*

159   *castaneus* were collected in order to study, in this species, the bioaccumulation of a subset of

160   MTEs. 136 *L. castaneus* individuals were sampled from 30 out of the 87 quadrats. These

161   quadrats were selected to reflect a lead (Pb) concentration gradient (estimated on the basis of

162   2016 soil data, Fig. 1a).

2.3 Soil collection and measure of MTE pollution

163

164   Soil samples were taken simultaneously with the first earthworm sampling (in 2016) to

165   measure pH and metal pollution of soils. For each quadrat, three soil samples were collected on

166   three of the four sides of the quadrat, and pooled prior to be analyzed. In the laboratory, soil

167   samples were dried at 40°C for 24 hours, grounded, and sieved to 2 mm. Soil pH was measured

168   in 1 M KCl and in distilled H_2O according to the ISO 10390:2004 standard. The concentrations

169   of the different elements in the soils, including the MTEs, were measured by X-ray

170   Fluorescence (XRF) using the Epsilon 3XL panalytical and were analyzed with the software

171   Omnian. As soil moisture is known to significantly affect XRF-measurements (Parsons et al.,

172   2013), 10 randomly chosen soil subsamples were dried for 24 hours at 104°C in order to

173   measure their total humidity. The average soil moisture of these 10 soils was of $1.4 \pm 0.5\%$

174   (mean $\pm$ sd), which testify of the accuracy of XRF-measurements (Parsons et al., 2013).

175   However, a pilot investigation on a subset of 10 soil samples showed relatively high variance

176   of the concentrations of the different elements between 3 repeated measurements of each soil

177   sample. Therefore, for the rest of the soil samples, the measurements were performed twice per

178   sample in order to increase the reliability of measured concentrations. The average

179   concentration of each element was used in the analyses described below. Detailed data on the

180   pH and the concentrations of each element, and their variation across the soil samples, are

181   available in Table A1.

182   The variation in soil chemical composition was first explored using a PCA on zero-

183   centred and normed data of pH and concentrations of the different elements. We only included

184   in the PCA analysis the elements that were detected in a minimum of 80 quadrats. The missing

185   values were replaced by the average concentration of the element on all the quadrats, so as not

186   to influence the centroid of the PCA. Then, we tested for each element their spatial

187   autocorrelation at the scale of the sampling with a Mantel test based on 9999 permutations

188 (Table A1). All the above-mentioned statistical analyses were done in R 3.6.1 (R core Team

189 2019) using the ade4 library (Dray & Dray & Dufour 2007).

190   In order to investigate bioaccumulation processes in Cd, Pb, Zn, and Cu, in *L. castaneus*

191 (see below), the composition of the soil samples of the 30 quadrats where *L. castaneus* were

192 collected for the bioaccumulation study was also quantified using inductively coupled plasma-

193 optical emission spectrometry (ICP-OES). Soil samples were first lyophilized and grinded with

194 a mortar and a pestle. The mineralisation consisted in the digestion of 300 mg of sample in 7

195 mL of concentrated HNO3, using a Berghof microwave digestion system (speed wave MWS-

196 2-Microwave pressure digestion). The soil samples were analyzed by ICP-OES (ICPOES IRIS

197 Interpid II XSP Thermo, Thermo Scientific, Whatman, MA, USA). We used commercial

198 mussel tissue (ERM®-CE278) as the certified reference material, here and in the

199 bioaccumulation study in *L. castaneus* described below. Measured concentrations in the

200 reference material never differed by more 10% from the certified concentrations.

2.4 Earthworm community and soil pollution

202   The link between earthworm assemblage and the changes in the set of chemical elements

203 detected in a minimum of 80 quadrats was performed using a canonical redundancy analysis

204 (RDA) with the Vegan package (Oksanen et al., 2018). We followed the recommendation of

205 Legendre and Gallagher (2001) and Hellinger–transformed the species data prior to analysis to

206 tune down dominant species. The statistical significance of the RDA, the canonical axes, and

207 the different elements were tested by the mean of permutation tests (n = 999 permutations). For

208 the species whose abundances showed to be significantly structured by the first axis of the

209 RDA, the shapes of these relationships were investigated. In order to model changes in

210 Hellinger-transformed abundances of species along RDA1, we used generalized additive

211 models with a quasi-Poisson family and specified the number of knots to 3.

212   Moreover, MTEs do not only affect adult earthworm populations. MTEs are also known

213   to interfere with species reproduction and, thereby, influence the population dynamics of these

214   species. The effects on the abundance of juveniles of the soil score on the axes of the PCA

215   (PCA1 and PCA2), while correcting for the effect of the abundance of adults present in each

216   quadrat, were tested using a generalized linear model with a Poisson distribution.

217   **2.5 Bioaccumulation of metals in whole *L. castaneus* bodies**

218   The level of MTEs in *L. castaneus* specimens were measured on individuals that were

219   starved for 48 hours to empty the intestinal content, frozen for at least 48 hours, and finally

220   lyophilized for about 60 hours. Then, the specimens were reduced to powder using liquid

221   nitrogen before mineralisation. The mineralisation is a digestion in acid medium (using HNO_3,

222   H_2SO_4 and HCl) at high temperature (for details on the method, see Bernard et al., 2010). The

223   obtained solution was analyzed by ICP-OES (Varian 720-ES, USA) and Cd, Cu, Pb, and Zn,

224   were quantified in the samples. Bioaccumulation factors (BAFs) were calculated according to

225   the following equation: $BAF_{Me} = \frac{c_{Me\ earthworm}}{c_{Me\ soil}}$, where $c_{Me\ earthworm}$ is the total metal

226   concentration in the body of the earthworm (mg.kg^{-1}) and $c_{Me\ soil}$ is the total concentration of

227   the same metal in the soil (mg.kg^{-1}). Based on preliminary visual investigations of the data, the

228   relationships between the concentration of Cd, Cu, Pb, and Zn, in the specimens and in the soil

229   were investigated using a linear model for Zn and Cd, and included a quadratic term for Pb and

230   Cu.

231   **2.6 Analysis of neutral genetic variation: microsatellite genotyping**

232   Neutral fine-scale genetic structure of *L. castaneus* populations was investigated to

233   determine if the population in the contaminated zone has undergone a strong demographic

234   bottleneck that might be linked to high mortality and to investigate if there was any limits to

235 gene flow between both plots. Total genomic DNA of 175 *L. castaneus* sampled across 63 of

236 the 87 study quadrats (Fig. 1a) was extracted using the NucleoSpin® 96 Tissue kit (Macherey-

237 Nagel). Individuals were genotyped at the eight microsatellite loci described in Dupont et al.

238 (2019). Loci were amplified by polymerase chain reaction (PCR) following the protocol

239 detailed in Dupont et al. (2019). The migration of PCR products was carried out on an ABI

240 3130 xl Genetic Analyzer using the LIZ500 size standard (Applied Biosystems); alleles were

241 scored using GeneMapper 5 software (Applied Biosystems). All PCR results were repeated and

242 individuals missing three or more loci (e.g. failed PCR, poor-quality DNA extract) were

243 excluded from our data set.

244   The genetic diversity of the *L. castaneus* population was analyzed by computing allele

245 frequencies, number of alleles (N_{all}), and expected heterozygosity (H_e) using Genetix V 4.05

246 (Belkhir et al., 2004). The null independence between loci was tested from statistical genotypic

247 disequilibrium analysis using Genepop V4.4 (Rousset 2008). Null allele frequencies were

248 estimated using the software Microchecker (Van Oosterhout et al., 2004; van Oosterhout et al.,

249 2006). Departure from Hardy-Weinberg expectation was quantified by calculating the Weir and

250 Cockerham's (1984) estimator of the fixation index, F_{is}, and conformity to Hardy-Weinberg

251 equilibrium was assessed with exact tests implemented in Genepop V4.4.

252   Moreover, we investigated the occurrence of a cryptic population structure using the

253 Bayesian model implemented in Geneland V 4.0.3 (Guillot et al., 2005) that simultaneously

254 analyses the spatial and genetic data. The analyses were conducted using both the uncorrelated

255 and correlated allele frequency models. The correlated frequency model is more powerful at

256 detecting subtle differentiation, but it is also more sensitive to departures from model

257 assumptions (e.g. presence of isolation-by-distance), and more prone to algorithm instabilities,

258 than the uncorrelated frequency model (Guillot et al., 2005). The putative presence of null

259 allele(s) was taken into account in the model (Guillot et al., 2005). The Markov Chain Monte

260   Carlo (MCMC) was run 5 times to check for convergence allowing K to vary from one to three

261   clusters and using 10^6 MCMC iterations.

262   To further examine the spatial genetic structure of *L. castaneus* at the individual scale,

263   a spatial autocorrelation analysis was conducted using Spagedi 1.2 (Hardy & Vekemans 1999;

264   Hardy & Vekemans 2002). Such an analysis provides a measure of the genetic relatedness

265   between pairs of individuals as a function of their Euclidean distances. Kinship coefficients

266   between individuals (F_{ij}) were estimated as described in Loiselle et al. (1995). We identified

267   10 classes of spatial distance in order to reach approximately 1400 pairs of individuals per

268   spatial distance class, apart from the 0 distance class with 301 pairs of individuals coming from

269   the same quadrat and having the same spatial coordinates. The average multilocus relationship

270   coefficients per distance class were estimated and their significance per class was tested with

271   10000 permutations of multilocus genotypes. To visualize the spatial genetic structure, we

272   plotted the kinship coefficient against geographical distance.

273   **3. Results**

274   **3.1 Soil pollution heterogeneity**

275   XRF-measurements allowed the relative quantification of 38 elements over all soils

276   sampled. First, it was shown that the concentrations of elements greatly varied among samples.

277   Al, As, Ca, Cr, Fe, K, Mg, Mn, Pb, Rb, S, Si, Sr, Ti, V, Zn, Zr were found in each of the 87

278   soils sampled (Table A1). Ba, Eu, Ga, Hg, Ir, Mo, Nb, Ni, Os, Re, Sb, Ta, Te, Th, Yb were

279   found in less than 64 of the sampled soils (Table A1). The accuracy of the XRF-measurements

280   greatly varied between measurements and between elements. For example, the coefficient of

281   variation between replicate measurements for As, S, and Cr were of 52.5, 26.1, and 15.8%,

282   respectively. Therefore, even though these elements were also found to significantly vary

among samples, suggesting that they are likely to structure the environment, their potential effect should be interpreted with caution as the reliability of their quantification is uncertain. However, for Pb, Cu, and Zn, which are the MTEs of interest, the coefficients of variation among XRF-measures were quite low (from 6.5 to 11.2, Table A1). The XRF-measures for these elements were also highly correlated with the measurements obtained by ICP-OES (Pb: r = 95.3, n = 30, p < 0.001; Cu: r = 78.0, n = 28, p < 0.001; Zn: r = 99.4, n = 30, p < 0.001), suggesting that their quantification by XRF is reliable. Cd, which is also a MTE of interest, was always below the level of detection by XRF.

The two first axes of the PCA capture 66.6% of the variation in chemical composition found between soil samples (Fig. 1b). The first axis accounts for most of the variation, 52.9%, found among soil samples. This axis is characterized by soils mainly rich in Ti, K, Al, Rb, and Si, at one end, and in Sr, Pb, Zn, Ca, and Cu, at the other end. Soil contamination in Pb, Zn, and Cu, is highly correlated (Fig. 1b). Besides, Mantel's tests have shown that most of the elements that contribute the most to PCA1 also exhibit strong spatial autocorrelation, as is the case for Pb, Zn, and Cu (Mantel test: Pb = 0.44, p < 0.001 ; Zn = 0.40, p < 0.001 ; Cu = 0.25, p < 0.001, see Table A1). Such a spatial autocorrelation suggests that the pollution is structured at the scale of our sampling. The visual inspection of the interpolation plot for PCA1 shows that the pollution is indeed structured with a source pollution at the northwest of the site (Fig. 1a, Appendix B).

3.2 Community composition

A total of 891 adults, 21 subadults, and 1129 juveniles, were collected over the 87 samples. On average, we found 10.24 ± 0.92 adults, 12.98 ± 1.33 juveniles, and a juvenile to adult ratio of 1.54 ± 0.15 in each quadrat (mean ± se). No earthworm was collected in four out of the 87 quadrats. Eight species were found: *Al. chlorotica* (L1, n = 418)*, L. castaneus* (n =

279)*, Ap. rosea* (L4, n = 49)*, Ap. icterica* (n = 47)*, Ap. longa* (n = 38)*, L. festivus* (n = 25)*, Ap.*

giardi (n = 15)*, L. herculeus* (n = 14). We were also able to identify subadults of *Ap. longa* (n

= 7)*, L. festivus* (n = 6)*, Ap. giardi* (n = 4)*, Al. chlorotica* (L1, n = 2), and of *Ap. rosea* (L4, n =

2)*.*

The canonical RDA showed that soil chemical composition accounted for 43.1% of the

variation in species abundance and the permutation test confirmed the significance of this model

(F = 3.88, p = 0.001, Fig. 2a). Only the first axis of the RDA (RDA1) was significant (F = 81.84,

p = 0.001) and explained 35.4% of the total variance. pH H_2O, Mg, and Fe, significantly

explained the variation captured by the RDA (F(pH H_2O) = 32.75, p = 0.001; F(Mg) = 26.73,

p = 0.001; F(Fe) = 4.09, p = 0.009). Although not found to be significant, the first axis aligns

closely with an increase in the concentration of soils in Cu, Zn, and Pb (Fig. 2a). Thus, in what

follows, we interpret the changes in community composition along the first axis of the RDA as

a response to an increase of MTE pollution.

The generalized additive models built to investigate for each species changes in the

Hellinger-transformed abundance data along RDA1 were significant for 6 of the 8 recorded

species and highlighted species differences of sensitivity to soil pollution by MTEs (Fig. 2b,

Table A2). Note that while the models were not significant for *Ap. rosea* and *Ap. giardi,* these

species were not observed in soils with high scores on the RDA1. Still, in the following our

interpretations are limited to the changes in these 6 species, which significantly vary along

RDA1. For high RDA1 values, species richness is restricted to *L. festivus* and *L. castaneus*, the

latter being the dominant species. For low RDA1 values, the communities are much more

diverse, with the all 6 species being observed in soils with low scores on RDA1. The relative

dominance of *Al. chlorotica* in the community sharply decreased with increasing values along

the RDA1 axis, as for *Ap. icterica, Ap. longa,* and *L. herculeus,* but the amplitudes of their

variation were weaker (Fig. 2b). *L. festivus* was not observed for soils with a low score on

17

332 RDA1. The explained deviations of the models were high, up to 90.3% for *Al. chlorotica,*

333 suggesting that changes in species abundance and of community assemblage is to a large extent

334 determined by changes in soil chemical composition (Table A2). More specifically, these

335 changes in species abundance along RDA1 are likely to reflect, in part, the increase in

336 concentrations of MTEs in soils, and in particular of Zn, Pb, and Cu (Fig. 2a).

337 Last, the number of juveniles is positively correlated to the number of adults (estimate

338 = 0.051, z-value = 16.74, p < 0.001, Table A3). In addition, the number of juveniles is

339 negatively correlated to PCA1 (estimate = -0.090, z-value = -6.57, p<0.001) but positively

340 correlated to PCA2 (estimate = 0.13, z-value = 6.47, p<0.001).

341 **3.3 Bioaccumulation in *L. castaneus***

342 *L. castaneus* showed high BAFs for Zn [0.68 – 7.81] and Cd [5.41 – 56.97]. The models

343 testing the relationship between the concentration of MTEs in earthworms and the soils showed

344 that bioaccumulation of Zn and Cd by *L. castaneus* was linear (Fig. 3). The models explained

345 25.9 and 22.3 % of the variance in the data for Zn and Cd, respectively. In contrast, *L. castaneus*

346 showed low BAFs for Cu [0.0027 – 0.26] and Pb [0.005 – 0.23], indicating that at the site, these

347 MTEs do not bioaccumulate in *L. castaneus*. Yet, the concentrations of Cu and Pb in

348 earthworms and the soils are correlated according to a quadratic relationship. The models

349 captured 33.5% and 39.7% of the variance in the data for Cu and Pb, respectively (Fig. 3).

350 **3.4 Population genetic structure of *L. castaneus***

351 A high neutral genetic diversity was observed within the 175 analysed genotypes (N_{all}

352 = 16.25; H_e = 0.670). Two pairs of loci departed significantly from linkage equilibrium (LC18

353 – LC33 and LC10-LC36). Since Dupont et al. (2019) showed no physical linkage between these

354 loci, this result could be explained by inbreeding (Nordborg 2000). A second dataset composed

of only one genotype per quadrat (i.e. 63 genotypes) was created. All loci were unlinked in this second dataset and a lower proportion of them displayed heterozygote deficiency (i.e. significant F_{is} estimate, Table 1). Geneland Bayesian analysis requiring linkage equilibrium was carried out with this second dataset and identified only one group of individuals in the study plot, regardless of the model of allelic frequency chosen.

Spatial autocorrelation analysis revealed, however, local genetic structure at the scale of the study plot. We found a significant negative relationship between the kinship coefficient and the geographic distance between pair of individuals (b ± se = -0.211 ± 0.079, p < 0.001). In particular, positive values of kinship coefficient are measured between individuals collected in close quadrats (mean distance of 10 m and 25 m), which means that neighbouring individuals have a higher genetic relatedness than random pairs of individuals (Fig. 4). Conversely, negative kinship coefficient values, are observed between individuals collected in more distant quadrats (mean distance of 60m and 80m) and indicate isolation by distance (Fig. 4).

4. Discussion

The study plot displays a high heterogeneity in soil chemical composition. In particular, the concentrations of MTEs are highly structured and reflect the division of the plot into the polluted and unpolluted zones. The levels of pollution in Pb, Cu, Cd, and Zn, overlap with the range of variation found elsewhere in lawn and forest soils of the Paris region (Foti et al., 2017) and the median value of European urban soils and world soils for Pb, Cu, Zn, Ni, Cr, Cd, and As (Baize 1997; Adriano 2001; Desaules 2012; Luo et al., 2012, summarized in Foti et al., 2017). The concentrations of MTEs were, for part, highly correlated, which makes it difficult to disentangle their respective impact on earthworms, suggesting potential cocktail effects. Based on the existing knowledge and literature on the impact of MTEs on earthworm species, in the following we relate the variation of soil chemical composition along RDA1 to a gradient

379   of pollution, since this axis is associated with increasing concentrations of Pb, Cu, and Zn (Fig.

380   2a).

381   **4.1 Impact of MTEs on earthworm community**

382   Along the gradient of pollution, we observed marked changes in community

383   composition and abundances. *Al. chlorotica* and *L. castaneus* were the dominant species in our

384   sampling. *Al. chlorotica* dominates low-polluted quadrats and is absent from the most polluted

385   part of the plot, while the relative abundance of *L. castaneus* increases along the pollution

386   gradient. Previous studies have already shown that epigeic species, such as *L. castaneus*, are

387   more resistant than endogeic species, such as *Al. chlorotica*, to the effects of MTE pollution

388   (e.g. Spurgeon & Hopkin 1999; Mirmonsef et al., 2017). An explanation for this difference is

389   that endogeic species that live and feed in the mineral soil layers are probably more exposed to

390   the bioavailable fraction of metals, per comparison with epigeic species which are active in the

391   superficial soil and litter layers (Mirmonsef et al., 2017).

392   Overall, species richness decreased along the pollution gradient, as did the abundance

393   of juveniles compared to that of adults. The latter result is consistent with the literature as Cd,

394   Pb, Cu, and Zn, are known to reduce reproduction rates in several species. For instance,

395   Spurgeon et al. (1994) found a significant negative effect of high concentrations of these four

396   MTEs, particularly of Cd and Cu, on the cocoon production of the epigeic earthworm *Eisenia*

397   *fetida* in an artificial soil. Significant decreases in cocoon production was also observed for *L.*

398   *rubellus* in Cu-amended sandy soil and sandy loam (Ma 1984). Conversely, Reinecke et al.

399   (2001) showed that in three different species, *Eudrilus eugeniae*, *Perionyx excavates*, and *E.*

400   *fetida*, cocoon viability, but not production, was detrimentally affected by Pb concentrations.

401   Although MTEs are known to have an impact on earthworms, we cannot exclude the

402   hypothesis that the observed changes of community composition across the plot may be

403   explained by differences in soil properties between zones. The unpolluted zone is characterized

404   by a sandy soil while a loamy soil was found in the polluted zone, and these differences also

405   have certainly an impact on the earthworm community composition. Indeed, spatial variations

406   in the abundance of earthworms are commonly observed and can be partly explained by

407   variations in soil properties (e.g. Nuutinen et al., 1998). It is however noteworthy that the most

408   abundant earthworm species in the polluted zone is *L. castaneus*, an epigeic species known to

409   resist to and bioaccumulate MTEs (present study, Terhivuo et al., 1994; Spurgeon & Hopkin

410   1996; Tischer 2009).

411   **4.2 Impact of MTEs on the bioaccumulation of *L. castaneus***

412   We showed that *L. castaneus* bioaccumulate Zn and Cd, and that the bioaccumulation

413   of these two MTEs was highly correlated ($R^2 = 0.89$, t = 10.5, p < 0.001).

414   Zn is an essential element necessary for earthworm growth, maturation, and

415   reproduction, and might therefore be required to a number of metabolic processes (Nannoni et

416   al., 2014; Wang et al., 2018a). High concentrations of Zn in earthworm tissues have been

417   recorded elsewhere (Wang et al., 2018a) but it has also been shown that some earthworm

418   species, e.g. *Eisenia fetida*, are able to regulate their uptake of Zn and, thus, do not accumulate

419   this metal (Bernard et al., 2010; Brulle et al., 2011). In our sampling, *L. castaneus* was found

420   to accumulate Zn up to 7.8 fold the concentration found in the soil. The relationship between

421   the concentration of Zn in *L. castaneus* and in soil is linear, suggesting that the bioaccumulation

422   of Zn has not reached a threshold.

423   Although the concentration of Cd was relatively low in the soil, ranging from 0.15 to

424   1.06 mg/kg, this metal accumulated in *L. castaneus* up to 18.06 mg/kg, a result highlighting its

425   strong bioavailability. *L. castaneus* wasshown to bioaccumulate up to 56.9 fold the

426   concentration of Cd found in the surrounding soil. This strong ability to accumulate Cd has

been reported for other epigeic and endogeic earthworm species (Bernard et al., 2010; Latif et al., 2013).

Conversely, the bioaccumulation factors of Pb and Cu in *L. castaneus* were low. Other studies have shown that Cu and Pb are bioaccumulated by earthworms when their concentrations in soils are particularly high ([Pb] > 900 mg/kg Bernard et al., 2010). The soil concentrations of Cu and Pb at our study site are, comparatively, lower, which might explain why these metals were not found to bioaccumulate in *L. castaneus.* Alternatively, Mirmonsef et al. (2017) proposed that the bioaccumulation of Cu or other heavy metals in earthworm populations can occur in populations that have been exposed for many generations to these metals, as natural selection and genetic adaptation in these populations would have resulted in an increase in their efficiency to sequester and detoxify these MTEs. The low bioaccumulation of Cu and Pb by the population of *L. castaneus* at our study site could then reflect the yet limited duration of their exposure to pollution.

4.3 Impact of MTEs on the genetic characteristics of *L. castaneus*

The genetic variability of a population exposed to MTEs may be altered in different ways. Genetic changes in the population may either result from genotoxic exposure (i.e. direct effect) or from microevolutionary processes (i.e. indirect effect). Direct effects are related to DNA or chromosome alterations which, when they are exerted on gametes and are passed on to the next generation, can significantly impact exposed populations (Medina et al., 2007). In this work, we focused on indirect effects of MTEs, which are population-mediated processes. We assumed that soil pollution by MTEs may alter the diversity of neutral genetic markers, such as microsatellites, through random genetic drift associated to drastic reduction in population size. However, the indices of genetic diversity computed in the population were high, similar to those of other *L. castaneus* populations genotyped with the same markers

22

451 (Dupont et al., 2019), providing no support for the hypothesis that this population would have

452 undergone a significant reduction of genetic diversity through genetic drift. Alternatively, the

453 genetic structure estimated from neutral markers could be shaped by natural selection of

454 resistant phenotypes. In such instance, we would expect to observe at least two differentiated

455 genetic clusters, corresponding to the polluted and unpolluted zones. Yet, no genetic clustering

456 was revealed at the scale of the study plot. Last, the spatial autocorrelation analysis revealed a

457 pattern of isolation by distance that does not support the role of the heterogeneity of soil

458 pollution by MTEs as a barrier to gene flow in this species. This result, added to the fact that

459 the abundance of *L. castaneus* was found to be high in the polluted quadrats, suggest that direct

460 genotoxic effects might be negligible for this species.

461 **5. Conclusion**

462   It is particularly difficult to study the consequences of MTE pollution on soil

463 biodiversity in the field, mainly because of confounding and cocktail effects (e.g. Ye et al.,

464 2017). Although laboratory experiments are valuable in testing theory and in providing

465 quantitative estimates of survival and reproduction rates of species under controlled conditions

466 (e.g. level of contamination), they can be difficult to implement when the pollution is

467 multifactorial and heterogeneous, as generally observed in urban areas. Moreover, these

468 experiments frequently use the laboratory earthworm models, *E. fetida* and *E. andrei,* while

469 they are often rare in the field (e.g. Coelho et al., 2018).

470   Here, we used a multidisciplinary approach to study in the field the response to MTE

471 pollution of an earthworm community in an urban area and to further our understanding of the

472 bioaccumulation capacities, population genetic structure and gene expression of a MTE tolerant

473 species in response to pollution. *L. castaneus* was identified as the most tolerant species to

474 MTEs of the study site. In sites contaminated by MTEs, the maintenance of earthworm

475   populations and their associated functions in the ecosystem (Pauwels et al., 2013) rely on the

476   evolution of molecular mechanisms of metal tolerance, which, however, remain poorly

477   understood. As mentioned elsewhere (Stapley et al., 2010; Vandegehuchte & Janssen 2014;

478   Evans 2015), the study of gene expression profile in populations under different selection

479   pressure should provide new insights into the molecular mechanisms of metal tolerance in

480   earthworms, and help identify candidate functional genes that may be under selection. Although

481   still expensive, the Next-generation sequencing (NGS), now permits direct transcriptome

482   sequencing, and can provide such qualitative and quantitative information on the expression of

483   genes.

Acknowledgements

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Figure legends

697 **Figure 1** (a) Interpolation maps of soil score on PCA1. We interpolated the data using ordinary

698 kriging and validated our interpolation plot by cross validation of the residuals (see Appendix

699 B for further explanation on the interpolation plot). The pie charts indicate the location of the

700 quadrats and the purpose which the collected earthworms were used for. (b) Biplot showing the

701 contribution to the two PCA axes of pH and chemical elements, as well as the position of soil

702 samples (quadrats) in this two-dimensional space.

703

704 **Figure 2** (a) RDA triplots of the Hellinger-transformed earthworm abundance data constrained

705 by the elements measured in a minimum of 80 quadrats. (b) Changes in Hellinger-transformed

706 abundance data along RDA1 for *L. castaneus, L. festivus, L. herculeus, Ap.longa, Al. chlorotica,*

707 and *Ap. icterica*. The fitted GAM is depicted by a line for each species and the dots correspond

708 to the raw data.

709

710 **Figure 3** Relationship between the concentrations of MTEs measured by ICP in earthworms

711 and soils for Cu and Pb (left panel), Zn (central panel), and Cd (right panel).

712

713 **Figure 4** Average kinship coefficients, F_{ij}, between pairs of *L. castaneus* individuals plotted

714 against the geographical distance. Dashed lines represent 95% confidence intervals for F_{ij} under

715 the null hypothesis that genotypes are randomly distributed. Significant values: $*P < 0.05$.

716   **Figure 1**

717

718

719   **Figure 2**

Figure 3

723   **Figure 4**

724
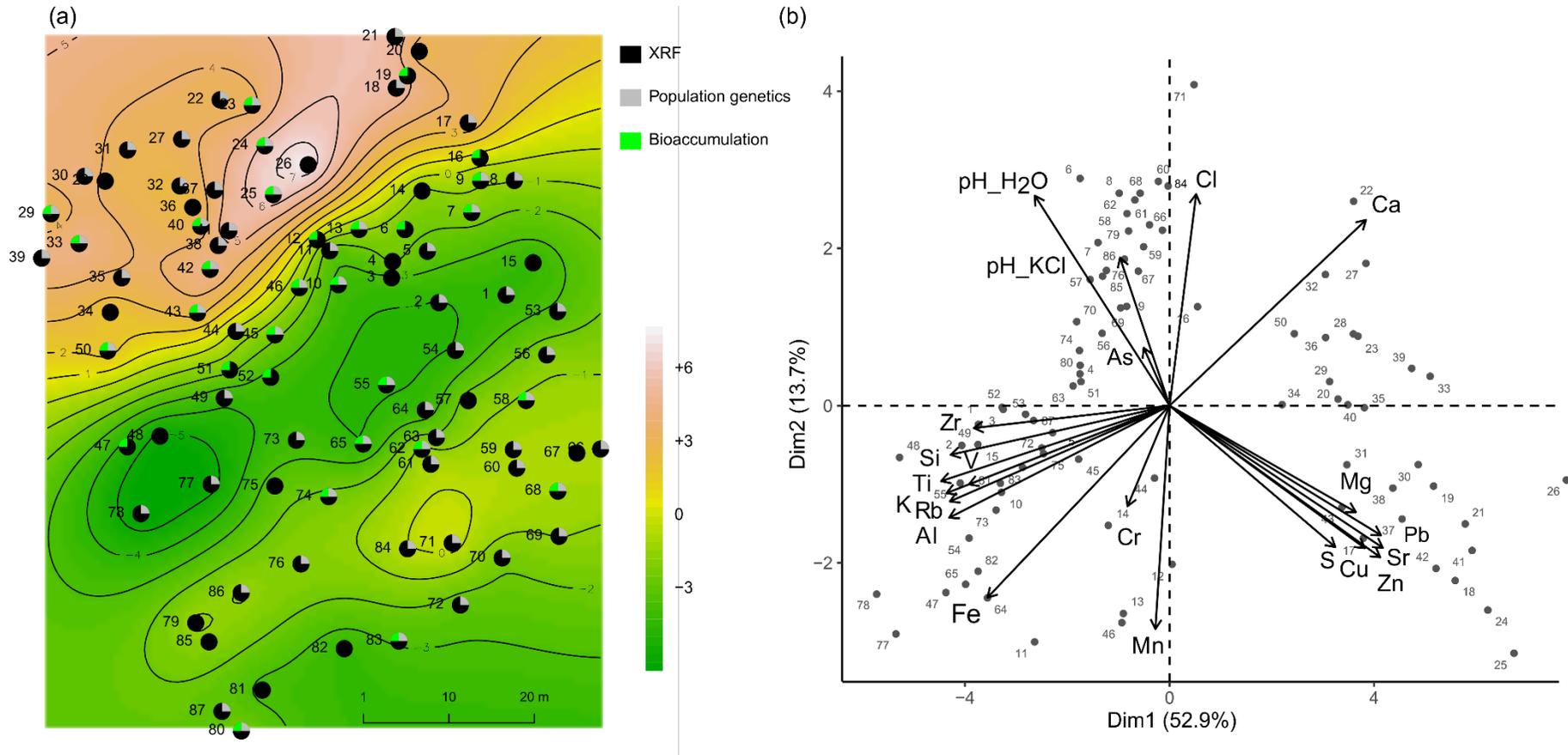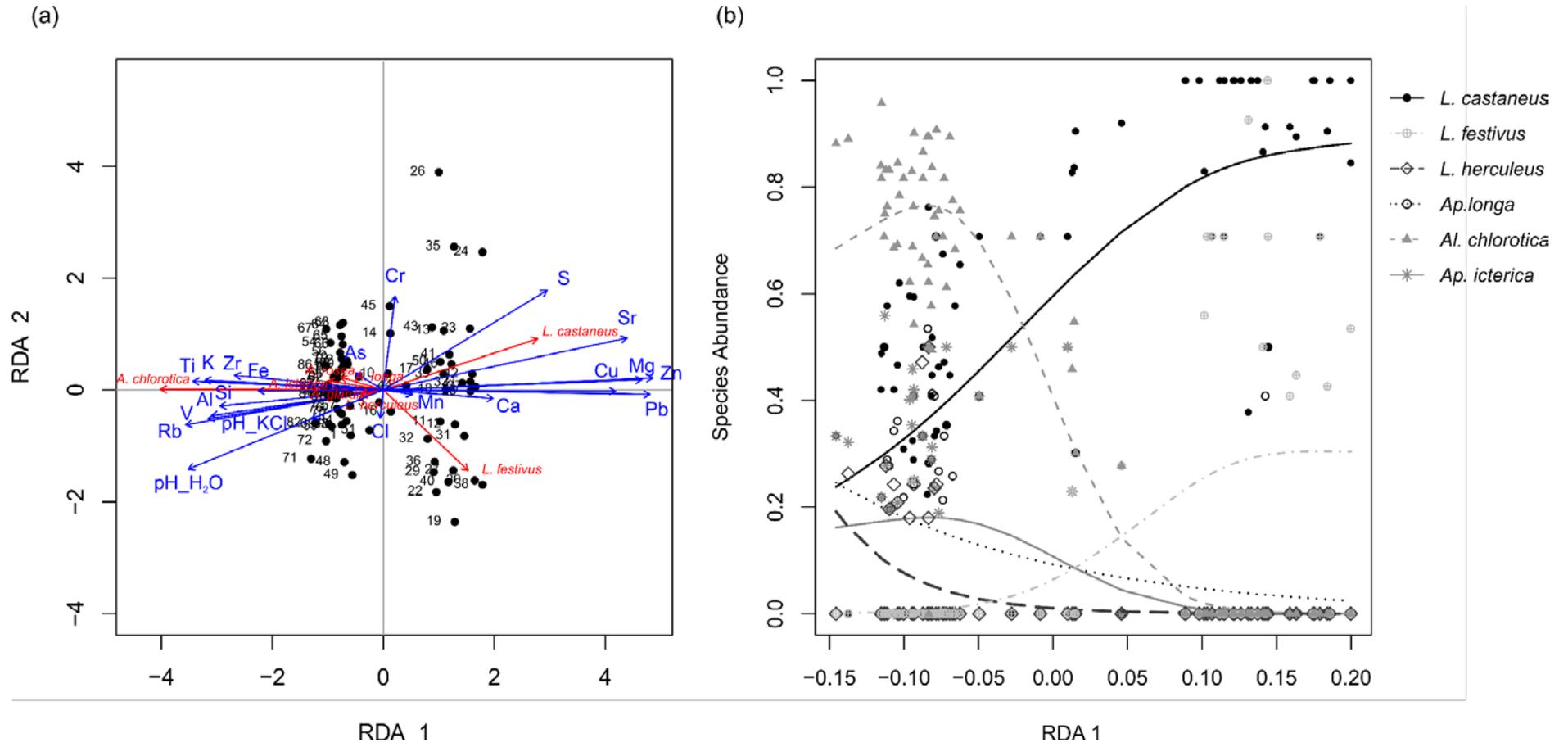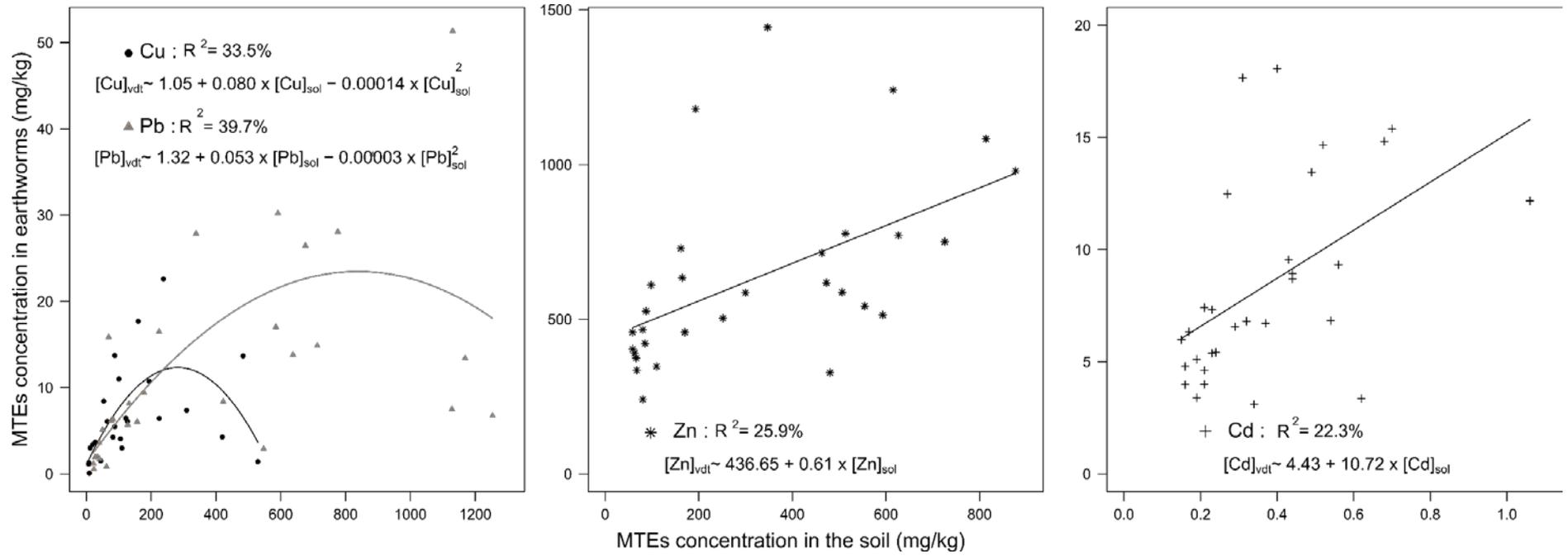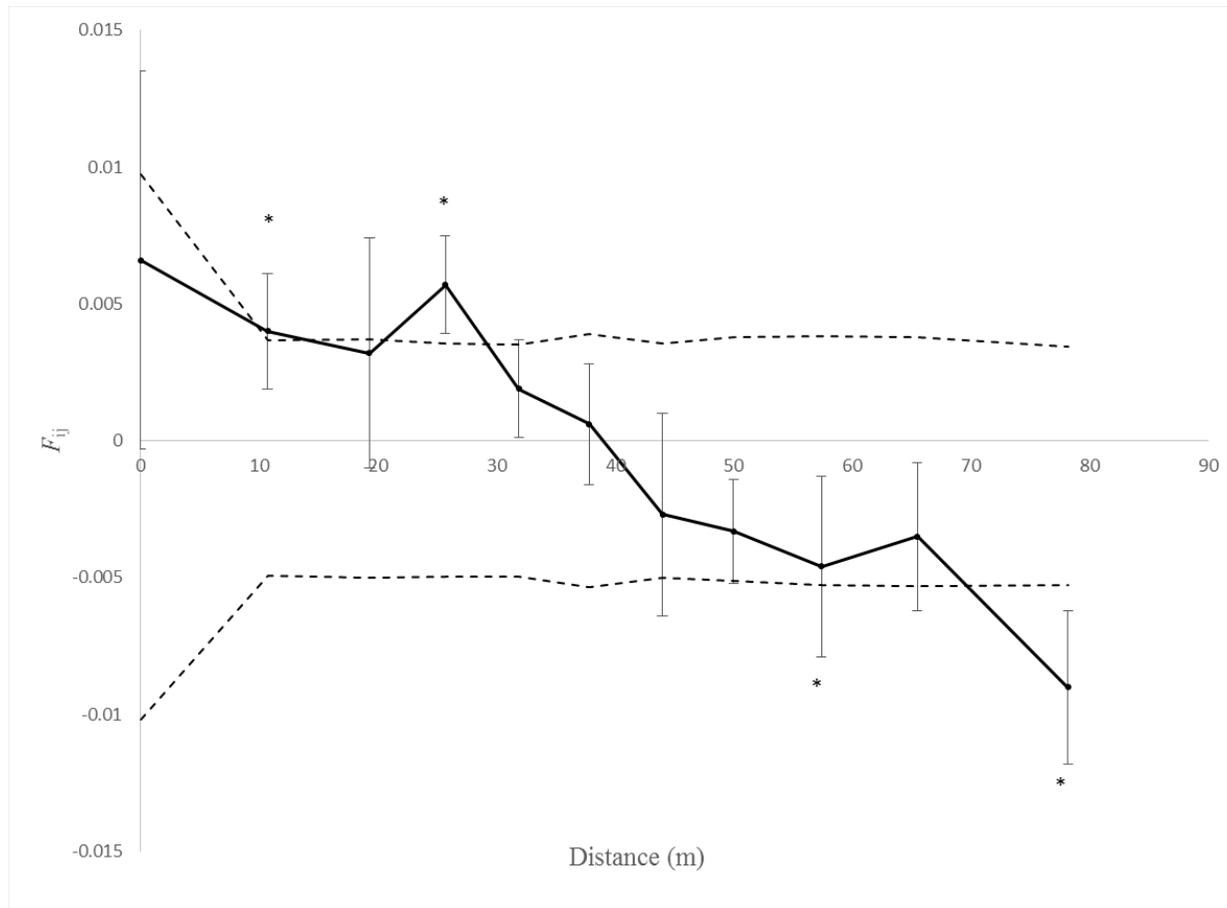
Table 1 Characteristics of microsatellite data in the global dataset (175 genotypes) and in a subsample of one genotype per quadrat (63 genotypes). Are indicated: the number of alleles for each locus (Na), the estimator of the fixation index (F_{is}) with significant value in bold, and the null-allele frequency (Null).

Locus		LC02	LC05	LC10	LC16	LC18	LC27	LC33	LC36
175	N_a	5	10	18	36	22	10	10	19
genotypes	F_{is}	**0.184**	-0.081	**0.117**	**0.331**	**0.175**	-0.010	**0.239**	**0.334**
	Null	**0.096**	0.000	**0.057**	**0.162**	**0.082**	0.000	**0.118**	**0.164**
63	N_a	4	7	14	28	17	9	9	17
genotypes	F_{is}	0.118	-0.077	**0.166**	**0.362**	0.093	-0.098	**0.316**	**0.301**
	Null	0.000	0.000	**0.076**	**0.177**	0.000	0.000	**0.159**	**0.155**

36

729 **Appendix A**

730 **Table A1 Summary measures of the elemental composition and pH of the soil sampled. N corresponds to the number of quadrats in which**

731 **the element were found and quantified by XRF. The variance between repeated XRF-measures for a soil sample is given by the minimum**

732 **and maximum standard error ("min se" or "max se") for all measured soils. It corresponds to range of standard error found when at least**

733 **two replicated XRF measures report the element in a sample. Mean CV corresponds to the average coefficient of variation found across**

734 **measurements. Spatial autocorrelation across the study site was measure for each element using the Mantel test. Note that Mantel tests**

735 **for Ir, Nb, Th, Mo, Sb, Te, Os, Ta were not performed as the sample size for those element was too small (< 8).**

element	pH H$_2$O	pH KCl	S	Pb	K	Zn	Cr	As	Mg	Sr	V	Al	Zr	Ti	Rb	Ca	Si	Fe	Mn
min value (in pH unit and mass concentration in mg/kg)	6.41	6.42	57.0	14.0	1741.0	25.5	13.0	3.0	372.0	91.0	8.5	2826.5	46.0	581.5	21.0	43682.0	16030.0	7962.0	178.0
max (in pH unit and mass concentration in mg/kg)	7.87	7.45	7262.5	940.0	3534.0	638.5	85.0	18.5	2205.0	441.0	33.0	8192.0	129.0	1463.0	45.5	62140.0	31495.5	13324.0	246.7
mean (in pH unit and mass concentration in mg/kg)	7.28	7.24	651.3	214.5	2629.4	182.4	26.3	9.0	821.9	162.8	19.6	5202.2	81.4	1076.8	31.2	54107.9	24380.6	9932.1	206.5
sd (in pH unit and mass concentration in mg/kg)	0.27	0.18	1143.4	243.7	486.0	177.9	8.1	2.7	466.8	87.8	5.4	1103.5	16.5	209.4	5.6	4826.3	3403.6	1231.3	16.1
N	87	87	87	87	87	87	87	87	87	87	87	87	87	87	87	87	87	87	87
max/min ratio	1.23	1.16	127.4	67.1	2.0	25.0	6.5	6.2	5.9	4.8	3.9	2.9	2.8	2.5	2.2	1.4	2.0	1.7	1.4
min se	-	-	0.9	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	10.5	0.5	0.0	0.0	5.5	6.0	0.5	0.0
max se	-	-	307.5	76.5	352.7	116.5	30.0	8.5	134.0	98.0	9.0	886.0	15.0	530.5	4.0	1559.3	1847.0	854.0	40.0
mean CV (%)	-	-	26.1	6.5	6.3	7.9	15.8	52.5	3.2	5.7	16.4	11.3	8.9	6.7	5.3	0.9	3.7	2.5	7.6
Mantel test, p-value	0.03, p=0.23	0.005, p=0.43	0.06, p=0.15	0.44, p<0.001	0.19, p<0.001	0.40, p<0.001	0.015, p=0.37	0.04, p=0.14	0.44, p<0.001	0.26, p<0.001	0.18, p<0.001	0.16, p<0.001	0.14, p<0.001	0.25, p<0.001	0.27, p<0.001	0.04, p=0.12	0.16, p<0.001	0.11, p=0.004	0.06, p=0.052

37

element	Cl	Cu	Sn	P	Br	Y	Eu	Re	Hg	Ni	Ba	Yb	Ga	Ir	Nb	Th	Mo	Sb	Te	Os	Ta
min value (in pH unit and mass concentration in mg/kg)	1.5	9.0	27.0	6.0	2.0	5.0	1.0	2.0	1.0	6.0	56.0	0.0	0.5	0.5	4.0	1.0	1.0	27.0	27.0	8	3
max (in pH unit and mass concentration in mg/kg)	44.0	608.0	98.0	256.7	5.5	13.5	130.0	10.0	5.0	19.0	203.3	101.0	4.0	3.0	6.0	4.0	1.0	36.0	27.0	8	3
mean (in pH unit and mass concentration in mg/kg)	21.3	70.6	39.4	94.3	3.9	9.2	8.5	4.8	2.7	10.7	127.1	7.2	2.1	1.8	4.7	3.0	1.0	31.5	-	-	-
sd (in pH unit and mass concentration in mg/kg)	8.7	86.0	13.1	73.9	0.8	1.6	15.7	1.5	1.1	3.5	34.7	16.5	1.0	0.9	0.9	1.4	0.0	4.5	-	-	-
N	84	79	75	71	68	66	62	50	44	43	43	34	31	8	3	3	2	2	1	1	1
max/min ratio	29.3	67.6	3.6	42.8	2.8	2.7	130.0	5.0	5.0	3.2	3.6	-	8.0	6.0	1.5	4.0	1.0	1.3	-	-	-
min se	0.5	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5	0.0	0.0	0.0	-	-	-	-	-	-	-
max se	23.0	437.0	26.5	29.5	1.0	1.0	126.0	3.5	1.5	6.5	21.0	99.0	1.0	0.6	-	-	-	-	-	-	-
mean CV (%)	42.6	11.2	9.6	22.9	8.6	5.1	27.3	39.3	38.1	31.9	6.8	46.1	37.5	63.8	-	-	-	-	-	-	-
Mantel test, p-value	0.06, p=0.054	0.25, p<0.001	0.26, p<0.001	0.40, p<0.001	0.15, p=0.007	0.18, p=0.001	-0.01, p=0.41	0.007, p=0.41	0.26, p<0.001	0.14, p=0.01	0.14, p=0.019	-0.035, p=0.47	0.31, p=0.001	-	-	-	-	-	-	-	-

737

738   **Table A2 Summary table of the GAM univariate models built for each species that showed a significant relationship between Hellinger-**

739   **transformed abundance data and RDA1.**

Model	Edf	Ref.df	F	p-value	Deviance explained (%)	Adj.R2
L. cataneus	1.75	1.94	35.01	<0.001	36.7	48.1
Ap. icterica	1.84	1.97	3.66	0.036	34.8	19.1
L. festivus	1.87	1.98	7.32	<0.001	45.5	28.2
L. herculeus	1.00	1.00	4.74	0.030	29.2	9.9
A. chlorotica	1.98	2.00	52.75	<0.001	90.3	86.0
A. longa	1.00	1.00	8.67	0.004	16.0	11.0

740

741 **Table A3. Analysis of deviance table (type II LR Chi-square tests) showing the effect of the soils score on PCA1 and PCA2 and of the**

742 **number of adults on juvenile abundances.**

743

# juveniles	df	Chisq	p
# adults	1	254.97	<0.001
PCA1	1	46.64	<0.001
PCA2	1	42.23	<0.001

744

Appendix B

746 The values of each quadrat on the first axis of the PCA (PCA1) were interpolated into a

747 pollution map with a mesh size of 50cm (similar to the sampling size). To create this map of

748 pollution we used the kriging interpolation method to account for spatial autocorrelation among

749 points and used a Matern distribution to model variogram. The reliability of the interpolation

750 map was checked through cross-validation of the residuals using 60 randomly selected points

751 for the modelling set and 27 for the validation set. Note that we did not build such a map for

752 the individual elements of particular interest in this study as they are all very much correlated

753 to each other and to PCA1. Pearson correlation tests between Pb, Cu, and Zn, followed by

754 Bonferroni correction for multiple testing showed that Pb and Cu were significantly correlated

755 to Zn at 0.94 and 0.92, respectively. Cd was excluded as it was found in none of the soils

756 analysed by XRF. All the above mentioned statistical analyses were done in R 3.6.1 (R core

757 Team 2019) using gstat (Pebesma 2004, Gräler et al. 2016) and raster (Hijmans 2019) libraries.

758

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