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Insect Life Traits Are Key Factors in Mercury Accumulation and Transfer within the Terrestrial Food Web

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► **To cite this version:**

Loïc Yung, Coralie Bertheau, David Cazaux, Nicole Regier, Vera Slaveykova, et al.. Insect Life Traits Are Key Factors in Mercury Accumulation and Transfer within the Terrestrial Food Web. Environmental Science and Technology, 2019, 53 (19), pp.11122-11132. 10.1021/acs.est.9b04102 . hal-02355645

HAL Id: hal-02355645

<https://hal.science/hal-02355645v1>

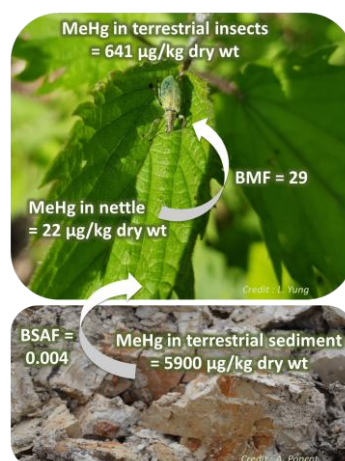
Submitted on 4 Jan 2022

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16 **ABSTRACT**

17 As plants and associated insects are at the bottom of some terrestrial food webs, they are the
18 primary contributors to mercury (Hg) fluxes in ecosystems. In addition to the trophic position
19 of these organisms, factors related to their life traits have been hypothesized to influence their
20 exposure to Hg. This study investigates the transfer of Hg in a soil-nettle-insect system and
21 the insect-related factors affecting their Hg concentrations in a revegetated chlor-alkali
22 landfill. Twenty-three insect species were identified and classified according to their life
23 traits, their relationship with nettle and their morphological characteristics. We observed low
24 total mercury (THg) concentrations in nettles, with only 1% methylmercury (MeHg) being
25 detected, while concentrations ranged from 5 to 3700 $\mu\text{g}/\text{kg}$ dry wt. in insects with a MeHg
26 percentage of up to 75%. The nettle-related insects were primarily exposed to Hg through the
27 food web with significant biomagnification, particularly at the level of secondary predators.
28 Within the nettle-unrelated group, the insect habitat was the most explanatory factor, with the
29 highest enrichment being for the insects that spent part of their cycle in direct contact with Hg
30 sources. Therefore, these insects require special attention because they are an essential vector
31 of Hg transfer for terrestrial top predators.



32
33

34 INTRODUCTION

35 Landfills in the chlor-alkali industries require stabilization of mercury (Hg) to prevent
36 air pollution by evaporated Hg¹. Revegetation of these sites offers a suitable solution to
37 confine the Hg released from the soil in plant biomass², as plants are net sinks that incorporate
38 the atmospheric Hg into leaf tissues³. These plantations, mainly from Salicaceous species (*i.e.*,
39 willow), favour the appearance of spontaneous herbaceous cover highly dominated by the
40 common stinging nettle, *Urtica dioica* L. (Urticaceae)⁴. In natural conditions, this nettle is
41 considered an invertebrate “super-host” providing food for a large diversity of insects with
42 different biology⁵ and is involved in strong trophic interactions^{6–8}. In this context, herbivorous
43 insects on revegetated landfills are potentially exposed to Hg and, more precisely, to
44 methylmercury (MeHg), the form of Hg that biomagnifies readily from soil and sediment to
45 plants⁹. The accumulation of MeHg in herbivorous insects can cause serious physiological,
46 neurological, behavioural and reproductive disorders for insects or invertebrate predators,
47 birds and mammals through their diet^{10,11}.

48 Studies and reviews focusing on Hg transfer in the terrestrial food chain and its
49 toxicity on specific groups (*i.e.*, birds, invertebrates, mammals, and microorganisms) have
50 increased since 2010^{12,13}. Some recent works, mainly targeting high trophic levels taxa,
51 concluded that elevated MeHg concentrations in high trophic wildlife are ubiquitous across
52 the world’s ecosystems and that there is a lack of accurate information on the impact of Hg in
53 terrestrial ecosystems^{14–17}. Investigations in Chinese industrial areas showed that Hg level in
54 the soil-plant-insect system increased from plants to herbivorous insects and to predators,
55 showing a biomagnification primarily conditioned by trophic factors^{15,18–21}.

56 Other factors such as the insect life stages can play a major role in the transfer of Hg
57 through food webs and particularly from aquatic to terrestrial ecosystems^{22–24}. Indeed,
58 emerging aquatic insects such as chironomids²⁵ or dragonflies²⁶ that move to terrestrial biota

59 can serve as both prey²⁷ and biovector of contaminants for local terrestrial consumers,
60 including ants or spiders^{28,29}. Consequently, the exposition of terrestrial insects is linked to the
61 vicinity to aquatic biota and the Hg bioavailability, conditioned by the physical and chemical
62 factors of these systems (*e.g.*, dissolved organic carbon, quantity of aqueous nutrients)^{25,30}.
63 For strictly terrestrial habitats, factors such as insect habitats, have been hypothesized to
64 influence Hg accumulation in terrestrial insects^{18,31}, but no studies examining biological
65 and/or ecological factors affecting Hg concentrations in these organisms exist, as previously
66 performed in lakes and wetlands^{25,32}.

67 The present study aimed to study Hg uptake and biomagnification through an original
68 and specific soil-plant-terrestrial insect system in a Hg-contaminated area in France and to
69 estimate the extent to which insect-related factors affected Hg uptake. To that end, we used a
70 chlor-alkali landfill revegetated by poplar plantations where the stinging nettle, *Urtica dioica*
71 L., colonized spontaneously the herbaceous layer, hosting a large diversity of insects. The
72 specific objectives were i) to quantify THg (total Hg) and MeHg in soil, different
73 compartments of nettles and a large number of well-identified insect taxa; ii) to characterize
74 insect taxa by their trophic guilds, feeding guilds, dietary range and habitats at both the larval
75 and adult stages, their relationship with the nettle and their morphological characteristics (*i.e.*,
76 life stage and weight); and iii) to assess the relative importance of these factors on THg and
77 MeHg concentrations in insects at the revegetated landfill.

78

79 **MATERIALS AND METHODS**

80 **Site description**

81 A phytomanagement field trial was implemented in 2011 on a chlor-alkali sediment
82 landfill located in Saint-Symphorien-sur-Saône in the Bourgogne Franche-Comté region

83 (France). The location, history and phytomanagement set-up at the sediment landfill have
84 been described in detail in previous studies^{33,22}. Five years after planting, biotic and abiotic
85 environmental conditions have promoted the emergence of a spontaneous herbaceous cover
86 strongly dominated by stinging nettle (*Urtica dioica* L.). The control site is a natural area
87 located at Courcelle-les-Montbéliard, 140 km east of the chlor-alkali plant, not directly
88 influenced by the point-source.

89 **Sample collection**

90 To ensure the most representative diversity in insect species, the study was carried out
91 over two consecutive years (2017 and 2018). Six poplar plots were selected at the
92 experimental site, three with the Skado cultivar (*Populus trichocarpa* x *P. maximowiczii*) and
93 three with the I-214 cultivar (*P. deltoides* x *P. nigra*). Soil and plant samples were collected in
94 June 2017 and monthly in 2018 from April to September (*i.e.*, during the vegetation period)
95 for the experimental site and in July 2018 for the control site. Three samples of soil and nettle
96 plants were composed of three mixed subsamples taken randomly within each sampling plot.
97 Soil samples consisting of bulk soil were collected from the first 20-cm layer, cleared from
98 roots and other plant materials. Soil and plant samples were placed into plastic and paper
99 bags, respectively, stored into a refrigerated container and shipped to the laboratory, where
100 they were dried at ambient temperature (24 °C) for two weeks before Hg analyses. In parallel,
101 an exhaustive sampling of the entomofauna related to the stinging nettle was carried out in the
102 six studied plots monthly from April to September (*i.e.*, period of insect activity) during the
103 two consecutive years (2017 and 2018). At each plot (chlor-alkali and control sites), insects
104 from six nettle patches of similar size were caught using a sweep net and pooled in a collector
105 tube with absolute ethanol or ethyl-acetate as usually used to assure optimal preservation and
106 identification. All samples were stored at -20°C until identification and Hg analyses.

107 **Insect identification and characterization**

108 Among the whole insect diversity collected from the entomological survey, in the
109 present study we targeted the most abundant taxa, representing 63% of the total insects
110 collected on nettles at the experimental site during the two years. Prior to the analyses, each
111 taxon was identified to the lowest possible taxonomic level using several references that
112 included some identification keys^{35,36}, on-line sources, such as insecte.org and/or specialists
113 from the Franche-Comte Natural Spaces Conservatory. Each taxon was classified according
114 to their life traits using published literature (SI Table S1). First, taxa were assigned according
115 to their habitat both at the larval and adult stages, which allowed them to be split into two
116 groups, "the nettle-related group", which included insects hosted by nettles (*i.e.*, insects living
117 on the nettle during their whole life cycle), and the "nettle-unrelated group", which refers to
118 all other insects. We further classified taxa according to their trophic levels (*i.e.*, herbivores,
119 predators I or predators II), their larval and adult feeding guild (*i.e.*, plant parts for herbivores
120 and type of prey for predators) and their dietary range (*i.e.*, insects that are host specific
121 within a family were categorized as "specialists", and those that feed on plants or insects from
122 more than one family were categorized as "generalists"). Finally, to test the influence of the
123 morphological characteristics of insects on THg content, we identified the life stage of each
124 taxon (larvae or adult) and measured the dry weight (mg).

125 **THg and MeHg analyses in soil, plants and insects**

126 Before analyses, dried nettle stems and leaves were separated and ground into a
127 homogenous powder in a MM40 Mixer Mill (Retsch, Eragny sur Oise, France) for 4 min at 30
128 Hz and 7 min at 30 Hz for leaves and stems, respectively. Soil samples were manually
129 homogenized and sieved to <4 mm. Insects euthanized with ethyl acetate were freeze-dried,
130 and those euthanized with absolute ethanol were transferred into new plastic tubes and opened
131 overnight at ambient temperature (24 °C) for the evaporation of ethanol. As the mass of some
132 dried insects was <1 mg, they were carefully weighed using a NewClassic MS analytical

133 balance (Mettler-Toledo AG, Greifensee, Switzerland) with a five decimal resolution.
134 Preliminary tests showed no significant influence of the washing and preservation methods on
135 THg concentrations measured in insects (SI, Figure S1). Moreover, in trophic interactions, the
136 whole insects are usually ingested by predators, including the potential fraction of Hg
137 adsorbed on the cuticle or internally accumulated through associated microorganisms (*e.g.*,
138 fungi and bacteria). Thus, whole insects were directly analysed without pretreatment to be
139 closer to natural conditions. For the small insects, multiple individuals of the same taxa were
140 pooled together to reach a minimal mass of 1 mg. THg concentrations were measured in the
141 soil, plant and insect samples using an AMA-254 cold vapor atomic absorption (CV-AAS) Hg
142 analyser (Altec Co., Czech Republic), as fully described in a previous study³⁷. The instrument
143 detection limit (DL) and the quantification limit (QL) using this method were 0.006 and 0.07
144 ng of THg, respectively. For samples with non-detected Hg, we calculated a THg
145 concentration using the QL value.

146 To ensure that the preservation of different taxa in ethanol did not induce cross-
147 contaminations, ethanol blank samples were regularly analysed. One hundred microliters of
148 ethanol was placed into the sample container and evaporated. No Hg was detected in the
149 ethanol fraction, which shows that Hg was not released into that fraction during storage.

150 MeHg was extracted from freeze-dried ground material using a HNO₃ leaching /
151 CH₂Cl₂ extraction method, as described by³⁸. Briefly, 0.02 to 0.5 g of insect, nettle or soil
152 material were weighed into a 50-mL centrifuge tube and homogenized into 2 mL 1 M CuSO₄,
153 5 mL 5 M HNO₃ and 10 mL CH₂Cl₂, shaken for 1 h and centrifuged for 25 min at 3000 rpm.
154 The solvent layer was transferred to a new tube and MeHg back-extracted into 10 mL H₂O
155 (MilliQ) under 50 mL/min N₂ flow in a water bath at 50°C. MeHg was analysed as described
156 elsewhere³⁹. The number of samples are provided in SI Table S2.

157 **Data analysis**

158 Statistical analyses were performed with R software v.3.5.1 (R Development Core
159 Team, 2013). All variables were checked for their homoscedasticity (Levene test) and normal
160 distribution (Shapiro–Wilk test). Non-normal data were log transformed. Data that did not fit
161 a normal distribution after transformation were analysed with nonparametric tests. All
162 statistical tests were considered to be significant at $P < 0.05$. The results are presented as the
163 means with their standard error (SE). Differences in THg concentrations in soil, nettles and
164 insects between sites (experimental *vs.* controls) were assessed using the Mann-Whitney-
165 Wilcoxon test. In the experimental site, a comparison of THg and MeHg concentrations
166 between soil, nettle, insect families and between nettle-related and nettle-unrelated insects
167 was performed using a Kruskal-Wallis test and a Mann-Whitney-Wilcoxon test, respectively.
168 A redundancy analysis (RDA) was performed on both the nettle-related and nettle-unrelated
169 groups to assess the correlation between THg concentrations and the insect life traits studied
170 using the “rda()” function available in the “vegan” package. The significance and rank of each
171 trait was then tested with the “envfits()” function (Monte Carlo test with 999 permutations).
172 Then, the effect of each significant insect life trait on THg concentrations for each group was
173 investigated with a Kruskal-Wallis test. For the morphological characteristics of insects, a
174 Mann-Whitney-Wilcoxon test was performed to assess whether THg concentrations differed
175 among insect stages. Pearson’s or Spearman’s correlation coefficients were calculated to
176 check for relationships between THg content and weight of insects grouped according to their
177 life traits.

178 The Biota-Soil Accumulation Factor (BSAF) was calculated for the nettles from the
179 experimental site and the control site according to the following equation:

$$\text{BSAF nettle} = \frac{[\text{THg}] \text{ nettle}}{[\text{THg}] \text{ soil}}$$

180 According to the information collected from the literature on trophic interactions (**Table S1**),
181 we calculated the Bio-Magnification Factors (BMF) for nettle-related insects from the
182 experimental site according to the following equation:

$$\text{BMF taxa} = \frac{[\text{THg}] \text{ taxa}}{[\text{THg}] \text{ preys}}$$

183 Trophic Magnification Factors (TMF) were determined for both THg and MeHg at the
184 experimental site. TMF considered all taxa along the five level trophic web (*i.e.*, plants,
185 herbivorous insects, predator I, predator II, predator III) except those from the Elateridae
186 species, which were removed from the herbivorous group as they accumulated large quantities
187 of Hg through their life cycle. Hence, TMF were determined from the slope of a regression
188 between the Hg concentrations and trophic level of insects in the food web and calculated as
189 the antilog of the regression slopes (b):

$$\text{Log [Hg]} = a + b\text{TL}$$

190 TMF refers to the average rate of change in contaminant concentrations from diet-to
191 consumer along the trophic web. In TMF, slope values above zero are indicative of
192 biomagnification (*i.e.*, $\text{TMF} > 1$)⁴⁰.

193

194 **RESULTS**

195 **Characterisation of targeted taxa**

196 The present study covered insects from six orders: Coleoptera, Diptera, Hemiptera,
197 Hymenoptera, Lepidoptera and Mecoptera, belonging to 16 common families within these
198 orders (SI Table S1). The 23 targeted insect taxa were identified up to the family level for
199 100% of them, to the genus level for 96% and to the species level for 74%. The identification
200 of 16 caterpillars could not go beyond the order level. The 23 selected taxa were then

201 classified according to their relationship or lack of relationship with nettle as a host-plant and
202 their life traits (SI Table S1). The nettle-related group included 12 different genera of insects
203 belonging to orders of Hemiptera and Coleoptera and unidentified families of Lepidopteran
204 larvae (SI Table S1). These insects belonged to three different trophic guilds: herbivores,
205 predators I and predators II. Herbivores belonged to taxa feeding on nettle sap, seeds or leaves
206 during their larval or adult stages. Predators I were mainly specialists belonging to the family
207 Coccinellidae and Miridae, which fed almost exclusively on aphids. Predators II were
208 represented by the genus *Nabis*, which included certain species known as generalist predators
209 that hunt herbivorous or other predatory species (*i.e.*, aphids, Lepidopteran or Coleopteran
210 larvae and some Heteropteran and Dipteran adults) on herbaceous layers. The nettle-unrelated
211 group included all the other abundant taxa represented by 8 families (*i.e.*, Formicidae,
212 Panorpidae, Aphrophoridae, Chrysomelidae, Curculionidae, Elateridae, Chironomidae, and
213 Sciomyzidae) and 9 genera. All taxa were sampled on nettles but were not directly related to
214 it. Most of these taxa were characterized by a high mobility and a specific life cycle that
215 occurred at different habitats, such as a larval cycle in the soil, in water or in snails as
216 parasitoids.

217

218 **Hg concentrations in the soil-plant-insect system**

219 THg concentrations of soil, plant and insects were significantly higher at the
220 experimental site than at the control site. The THg concentrations measured in soil samples
221 from the experimental site were in the range of 4382 – 7119 $\mu\text{g}/\text{kg}$ and 10-fold higher than
222 THg concentration ($570 \pm 70 \mu\text{g}/\text{kg}$) at the adjacent undisturbed forest soil ³³. Nettle stems
223 and leaves were significantly more enriched in THg at the experimental site than their
224 counterparts collected at the control site (Table 1). The BSAFs calculated for the experimental
225 site and the control site were 0.004 and 0.011, respectively, suggesting a noticeable dilution at

226 both sites. With an average THg of $31 \pm 1 \mu\text{g}/\text{kg}$, leaves were significantly ($P < 0.01$) more
227 enriched than stems ($11 \pm 1 \mu\text{g}/\text{kg}$ THg). A significant enrichment was observed in the nettle
228 leaves during the season, with THg concentrations ranging from $18 \pm 5 \mu\text{g}/\text{kg}$ in samples
229 collected in April to $31 \pm 5 \mu\text{g}/\text{kg}$ in samples collected in September ($P < 0.01$).

230 A total of 312 and 159 analyses of THg concentrations was performed on insects from
231 the experimental and control sites, respectively (Table 1). Average THg concentrations for
232 insects collected at the experimental site were significantly higher than those at the control
233 site ($P < 0.001$), and this difference was found more specifically for 12 of the 14 compared
234 families and orders (Table 1). Taxa (Cicadellidae family and Lepidoptera order), for which no
235 significant differences were observed, had THg concentrations ranging from $< \text{QL}$ to 271
236 $\mu\text{g}/\text{kg}$ at the experimental site and from $< \text{QL}$ to $109 \mu\text{g}/\text{kg}$ at the control site. No THg was
237 detected in Nabidae from the control site, whereas elevated THg concentrations (ranging from
238 107 to $1805 \mu\text{g}/\text{kg}$) were measured for the same taxa collected at the experimental site, and
239 the same phenomenon was observed for Aphididae and Aphrophoridae, although to a lesser
240 extent. For most insects collected at the control site, THg concentrations remained < 53
241 $\mu\text{g}/\text{kg}$, except for the highly mobile flying insects belonging to the Chironomidae and
242 Panorpidae families, which presented the highest background concentrations (Table 1).

243

244 **Table 1.** Total mercury (THg) concentrations measured in nettles and the 16 families and
245 order of insects from the sediment landfill and the control site. n represents the number of
246 samples analysed. All concentrations are expressed in $\mu\text{g}/\text{kg}$ dry wt \pm SE. 'NA' = taxa not
247 collected at the control site. Concentrations measured in the sediment landfill were compared
248 to those measured in the control site using the Wilcoxon rank sum test and the two-sample t-
249 test. The results from the statistical comparisons are represented with the following legend: *P*-

250 value '****' 0.001 '**' 0.01 '*' 0.05 'NS' not significant. ^a = data from an adjacent undisturbed
 251 forest soil²¹

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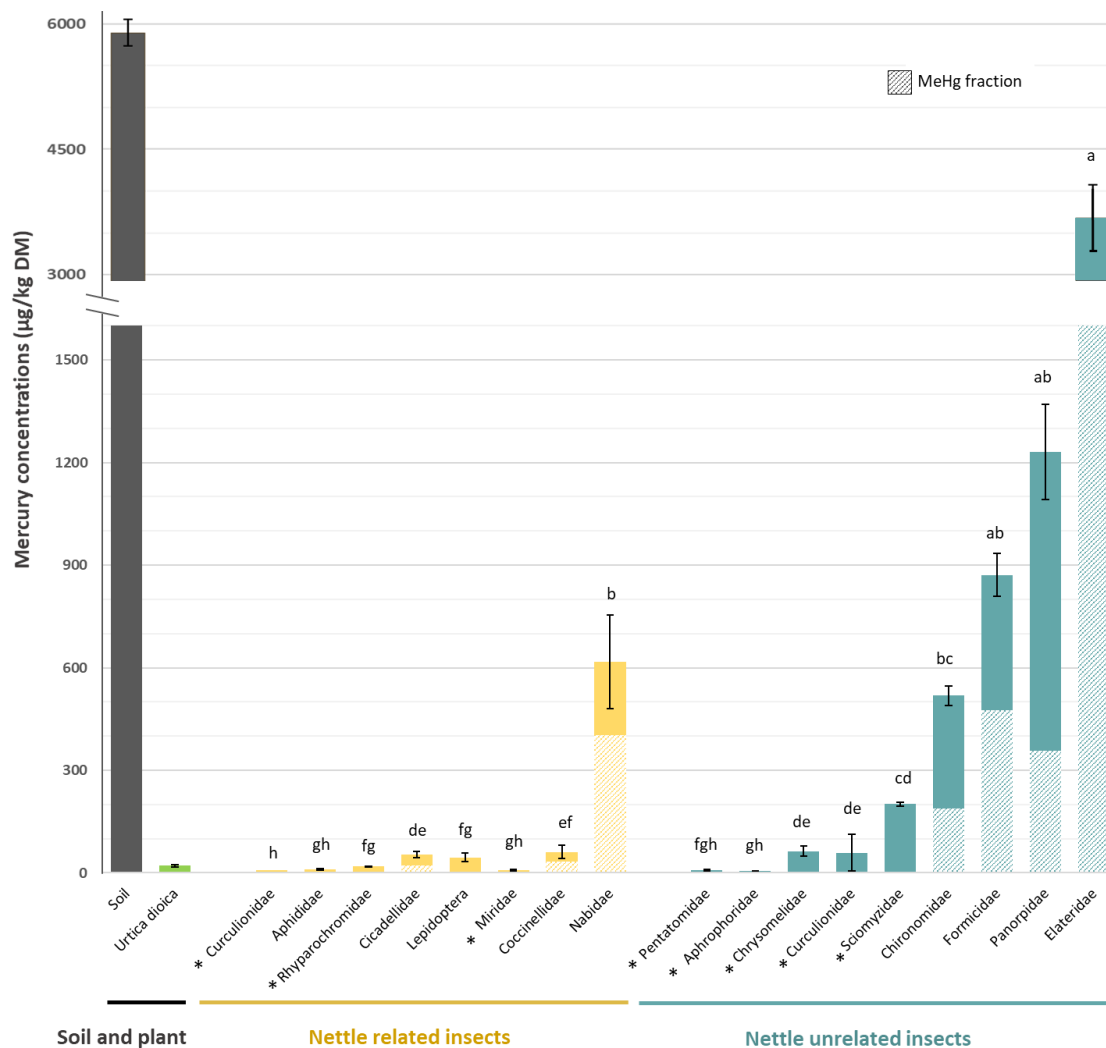
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255

Organism		Experimental site		Control		Significance test: comparison of the means
		Experimental site THg (µg/kg)		Control sites THg (µg/kg)		
		n	Mean ± S.E	n	Mean ± S.E	
Soil		37	5894 ± 111	7^a	570 ± 70^a	****^a
Nettles		187	21.4 ± 1.0	11	6.3 ± 0.9	***
	Stems	95	11.6 ± 0.7	5	3.8 ± 1.1	***
	Leaves	92	31.5 ± 3.4	6	8.4 ± 0.7	***
Insects	Taxa	312	388.5 ± 38.5	159	24.0 ± 3.7	***
	Elateridae	8	3679.8 ± 372.7	19	18.1 ± 4.2	***
	Panorpidae	6	1231.3 ± 261.0	3	178.6 ± 96.2	**
	Formicidae	21	871.6 ± 46.9	16	11.8 ± 3.4	***
	Nabidae	67	617.3 ± 47.2	19	<DL	***
	Chironomidae	32	518.1 ± 29.3	11	131.0 ± 12.7	***
	Sciomyzidae	5	200.8 ± 38.9	NA	NA	NA
	Chrysomelidae	16	63.5 ± 9.4	12	18.0 ± 4.7	***
	Coccinellidae	13	59.8 ± 20.4	17	4.7 ± 1.5	**
	Curculionidae	39	59.3 ± 15.0	15	6.1 ± 2.9	***
	Cicadellidae	21	53.2 ± 9.6	9	52.8 ± 10.4	NS
	Lepidoptera	18	45.2 ± 18.1	8	38.7 ± 10.5	NS
	Rhyparochromidae	17	19.3 ± 4.1	NA	NA	NA
	Aphididae	22	10.1 ± 1.2	7	<DL	***
	Miridae	17	8.9 ± 2.4	13	4.2 ± 3.3	*
	Pentatomidae	3	7.1 ± 2.7	6	0.5 ± 0.3	*
	Aphrophoridae	7	5.7 ± 1.8	4	<DL	**

256

257 The 16 studied families from the experimental site were plotted according to their THg
 258 concentration (Figure 1).



259

260 **Figure 1.** Mean THg concentrations (full bar, µg/kg dry wt.), including the MeHg fraction
 261 (hatched bar, %), plotted for soil and nettles (*Urtica dioica*) and for all the investigated insect
 262 taxa from the nettle-unrelated and nettle-related groups. * = The MeHg concentrations were
 263 not analysed. Bars with the same letter did not differ significantly from each other using a
 264 pairwise Kruskal–Wallis test ($P < 0.05$).

265 The highest THg concentrations were measured in Elateridae, Panorpidae and
 266 Formicidae followed by Chironimidae and Nabidae, with concentrations higher than 500
 267 µg/kg dry wt., which are significantly different from Elateridae (3679.8 ± 372.7 µg/kg). The
 268 measured THg concentrations dropped to 200 µg/kg for Sciomyzidae and below 100 µg/kg for
 269 each other family. Within the Curculionidae family, THg concentrations were variable,

270 ranging from < QL to 321 $\mu\text{g}/\text{kg}$, with concentrations over 150 $\mu\text{g}/\text{kg}$ for *Phyllobius sp.* and
271 up to 56 $\mu\text{g}/\text{kg}$ for the two other considered species (SI Table S1). All other less enriched
272 families had THg concentrations in the same order of magnitude as those measured for
273 nettles. Overall, nettle-unrelated insects showed mean THg concentrations ($981 \pm 96 \mu\text{g}/\text{kg}$)
274 six times higher than nettle-related insects ($124 \pm 14 \mu\text{g}/\text{kg}$) (Figure 1). Within the nettle-
275 related group, mean THg concentrations ranged from 8.9 ± 2.4 for Miridae to 617.3 ± 47.2
276 $\mu\text{g}/\text{kg}$ for Nabidae. Concerning the nettle-unrelated group, the lowest mean THg
277 concentrations were obtained for Aphrophoridae and Pentatomidae, whereas the highest
278 concentrations were found in Elateridae. However, the THg enrichment seems not to be only
279 related to the relationship with nettle. Indeed, the nettle-related taxon Nabidae had THg
280 concentrations not significantly different from the nettle-unrelated taxa Panorpidae,
281 Formicidae and Chironimidae (Figure 1).

282 Additionally, no significant difference ($P > 0.05$) was found between the lowest mean
283 THg concentrations in the nettle-unrelated group and those in the nettle-related group. Despite
284 high THg concentrations, the percentage of MeHg in the soil was very low ($0.03 \pm 0.002\%$),
285 slightly increased in nettles ($1.2 \pm 0.1\%$) and considerably increased in insects (ranging from
286 5.6% to 74.2%). Insect MeHg (average 29.2%, a) being significantly different ($P < 0.05$)
287 from nettle MeHg (8.0%, b), and soil MeHg (average 4.5%, b).

288 The percentages of MeHg were not significantly different between the nettle-related
289 group and the nettle-unrelated group ($P > 0.05$) with mean values of $43 \pm 7\%$ and $49 \pm 5\%$,
290 respectively. Within the nettle-related group, for the 5 studied families, the percentage of
291 MeHg was higher than 25% and reached 65% for Nabidae, except for Lepidoptera, for which
292 MeHg only represented 6% of THg. Elateridae from the nettle-unrelated group exhibited 74%
293 MeHg, representing over 2700 $\mu\text{g}/\text{kg}$. The Panorpidae family presented the most variable
294 MeHg percentages, ranging from 4 to 70%.

295 **Overall factors influencing THg concentrations in insects**

296 The present study clearly demonstrated that insect life traits, such as habitat, trophic
 297 guild, feeding guild, and dietary range, at both the larval and adult stages significantly
 298 influenced the mean THg concentrations of insects living at the landfill ($P < 0.01$). However,
 299 the relative importance of each of these life traits on THg concentrations of insects in the two
 300 groups (nettle-related vs. nettle-unrelated insects) was different (Table 2).

301

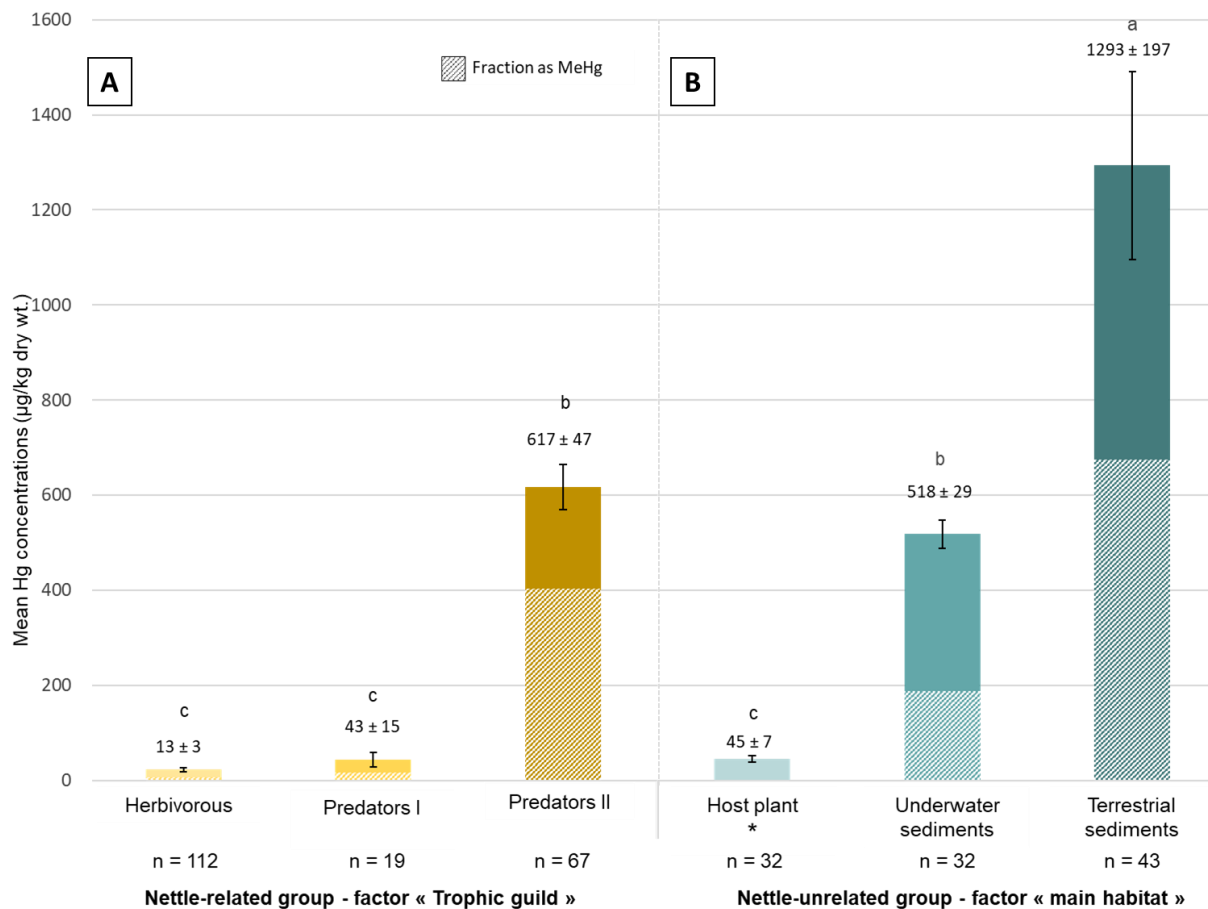
302 **Table 2.** Hierarchical analysis of factors affecting THg concentrations within the nettle-
 303 unrelated and nettle-related groups. P -value '****' 0.001 '**' 0.01 '*' 0.05 'NS' not significant.

	Factors	r2	Pr(>r)	Significativity
Nettle-related group				
	Trophic guild	0.39	0.001	***
	Feeding guild (adult)	0.39	0.001	***
	Feeding guild (larvae)	0.39	0.001	***
	Dietary range	0.37	0.001	***
	Habitat (larvae)	0.07	0.017	*
	Habitat (adult)	0	1	NS
Nettle-unrelated group				
	Habitat (adult)	0.22	0.001	***
	Feeding guild (larvae)	0.21	0.001	***
	Habitat (larvae)	0.16	0.002	**
	Feeding guild (adult)	0.06	0.115	NS
	Dietary range	0.05	0.037	*
	Trophic guild	0.02	0.475	NS

304

305 According to the RDA, THg concentrations in nettle-unrelated insects were mainly
 306 influenced by the adult habitat ($P < 0.001$), then by the dietary range at larval stage ($P <$
 307 0.001) and by larval habitat ($P < 0.002$), while the trophic guild and adult feeding guild
 308 factors were not critical. Considering adult habitat, THg concentrations followed the

309 following order: insects living on host plants < insects having their larval cycle in underwater
 310 sediments < insects living a part of their life cycle in terrestrial sediments (**Figure 2A**).



311
 312 **Figure 2.** Mean THg concentrations (full bars, µg/kg dry wt.) ± SE and the MeHg fraction
 313 (hatched bars, %), measured in classes related to the main factors influencing the Hg
 314 enrichment for both the nettle-related group (factor = trophic guild, A) and external group
 315 (factor = main habitat, B). *: The MeHg concentrations were not analysed. Bars with the same
 316 letter did not differ significantly from each other using a pairwise Kruskal–Wallis test ($P <$
 317 0.05).

318 The THg concentrations in *Aphrophora alni* (Aphrophoridae), which perform their
 319 complete life cycle on plants (especially Salicaceous species), did not exceed 12 µg/kg dry wt.
 320 In contrast, taxa with part of their life cycle in soil, such as *Agriotes sp.*, *Phyllobius sp.*,
 321 *Chironomus sp.*, *Trypetoptera punctulata*, *Myrmica rubra* and *Panorpa sp.* (SI, Table S1),

322 had average THg contents higher than 200 $\mu\text{g}/\text{kg}$ with a mean percentage of MeHg over 50%.
323 Insects belonging to the genus *Crepidodera*, which also feed on Salicaceae, had the lowest
324 THg content for insects with a part of their life cycle in the soil but still significantly higher
325 than those measured for *Aphrophora alni* ($P < 0.01$) (Figure 1).

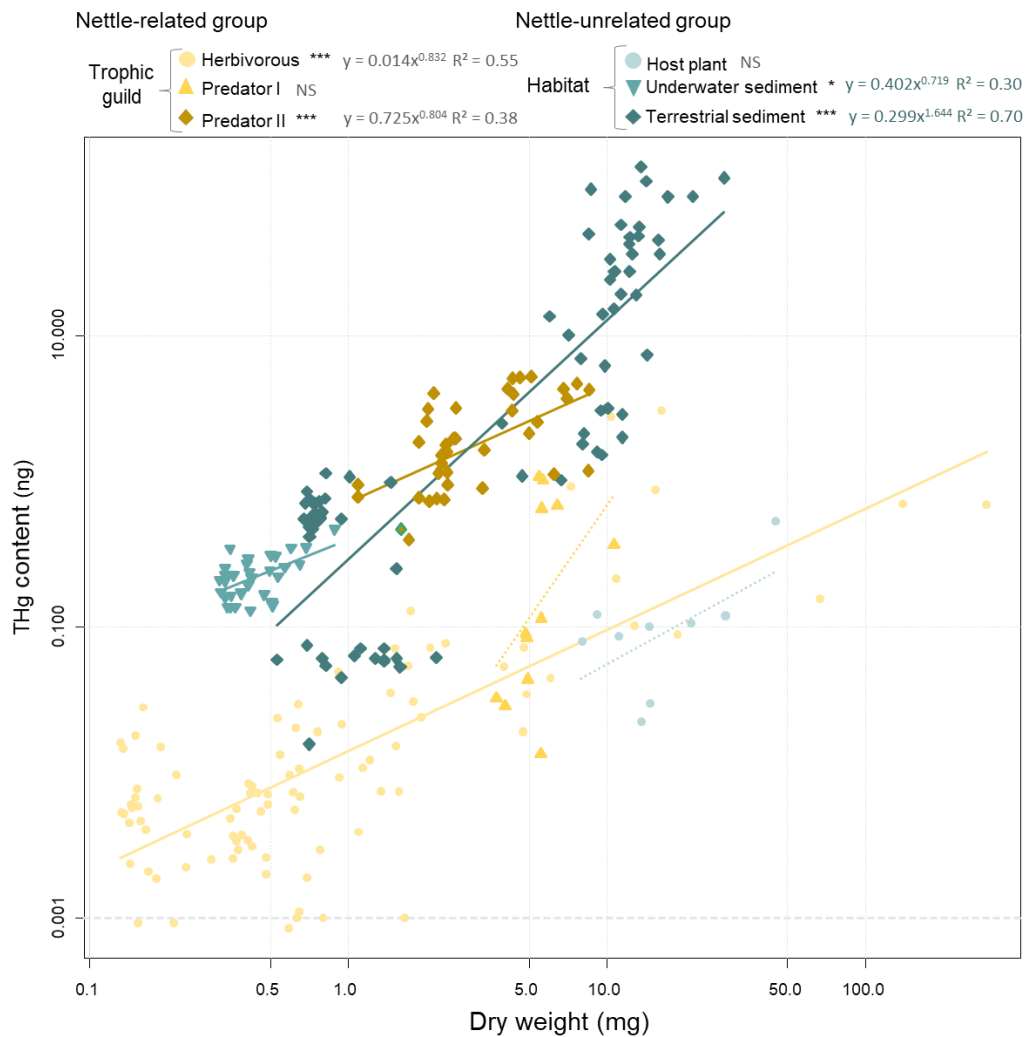
326 For nettle-related insects, feeding guild at larval and adult stages ($P < 0.001$), trophic
327 guild ($P < 0.001$) and then dietary range ($P < 0.001$), accounting individually for more than
328 37% of the total variability, were the most significant life traits (Table 2). Thus, in the nettle-
329 related food web, the THg contents were in the following order: herbivores < soft-body insect
330 predators < generalist insect predators (Figure 2B), with a percentage of MeHg following the
331 same trend: 24% < 37% < 65%. Herbivorous insects presented no significant THg enrichment
332 compared with nettles, but the mean MeHg concentrations increased from 0.3 $\mu\text{g}/\text{kg}$ in nettles
333 to 9.1 $\mu\text{g}/\text{kg}$ in herbivorous insects. The biomagnification within the nettle-related group was
334 also observed while considering all the studied taxa (including those from the nettle-unrelated
335 group), with TMF of 2.8 for THg and 6.3 for MeHg.

336 Each family of insects feeding on nettle sap or nettle seeds had a BMF lower than 1.0,
337 except for leafhoppers (Cicadellidae), which had the highest BMF (4.6) among all
338 herbivorous insects. THg concentrations ($\mu\text{g}/\text{kg}$) for herbivorous insects increased as follows:
339 seed-feeder ($13 \pm 3 \mu\text{g}/\text{kg}$) < sap-sucking ($22 \pm 5 \mu\text{g}/\text{kg}$) < leaf-feeder ($37 \pm 10 \mu\text{g}/\text{kg}$) with
340 no significant differences between these feeding guilds. With regard to defoliating insects,
341 Lepidoptera larvae, which included unidentified taxa with potential generalist feeding
342 specificity, had a BMF of 1.4, whereas that of a specialist species of weevils (Curculionidae)
343 hosted by nettles was 0.6. No significant THg enrichment was obtained for predators I, which
344 included ladybirds (Coccinellidae) and a predatory bug (Miridae), compared to herbivorous
345 insects. Damsel bugs (Nabidae), which were the only studied generalist predators II of the

346 nettle-related food web, had average concentrations 15 times (THg) and more than 30 times
347 (MeHg) higher than those measured in the lower trophic guild, resulting in a BMF of 4.8.

348 The influence of the development stage on THg concentrations was studied on two
349 predatory taxa: the ladybirds, *Harmonia axiridis* (Coccinellidae) and the damsel bugs, *Nabis*
350 *sp.* (Nabidae). THg concentrations measured in the imago ladybirds (n = 6) were 10-fold
351 higher ($P < 0.001$) than those measured in larvae (n = 7). In contrast, no significant difference
352 was obtained for Nabidae species between the imago (from 148 to 1786 $\mu\text{g}/\text{kg}$; n = 43) and
353 the larvae (from 108 to 1805 $\mu\text{g}/\text{kg}$; n = 24). A significant positive correlation between the
354 weight of insects and their THg content was observed when all taxa were pooled ($r_s = 0.62$, P
355 < 0.001). The THg content in insects increased proportionally with their weight but
356 differently according to the group studied. We also considered the main life traits, that is, the
357 habitat of nettle-unrelated taxa and the trophic guild for nettle-related taxa. In doing so, the
358 correlation between THg and weight was confirmed for herbivores ($r_s = 0.60$, $P < 0.001$) and
359 predators ($r_s = 0.62$, $P < 0.001$) trophic guilds as well as for underwater sediments ($r_s = 0.44$,
360 $P < 0.01$) and terrestrial sediments ($r_s = 0.81$, $P < 0.001$) habitats (Figure 3).

361



362

363 **Figure 3.** Relationships between the dry weight (mg) and the THg content (ng) of insects
 364 measured in 6 classes, 3 related to the “trophic guild” or 3 related to the “habitat” factors. The
 365 results from Pearson’s and Spearman’s correlation tests are presented with the following
 366 legend: *P*-value '****' 0.001 '***' 0.01 '*' 0.05 'NS' not significant. The regression equations
 367 were calculated, as well as the regression coefficient (R^2) for each class, for which a
 368 correlation was obtained.

369 The first cluster, characterized by a high equation coefficient ($a > 0.3$), comprised
 370 most taxa from the nettle-unrelated insects that spent part of their life cycle in direct contact
 371 with Hg sources, *i.e.* Elateridae, Curculionidae, Chironomidae, Formicidae and Panorpidae
 372 species. This cluster also included nettle-related predators II (*i.e.*, Nabidae species), predators

373 I (Coccinellidae) and herbivores (Lepidoptera), which accumulated only Hg via
374 biomagnification from the diet. The second cluster, characterized by a lower coefficient in the
375 equation (calculated when significant, $a < 0.1$), mainly included nettle-related herbivorous
376 insects and predators I, as well as insects strictly living on Salicaceous species (*i.e.*,
377 Cerpopidae, Pentatomidae). This cluster also included a specific beetle (*i.e.*, Chrysomelidae),
378 which spent a short period of its life cycle in terrestrial sediments.

379

380 **DISCUSSION**

381 Our data provided evidence on the terrestrial biomagnification of Hg at the soil-plant-
382 insect food web at a chlor-alkali site. These results reinforce recently published work in which
383 Hg biomagnification not only concerns wetlands but also a wider range of biota, including
384 terrestrial biota⁴¹. As a result of the chlor-alkali process, Hg enrichment at the sediment
385 landfill were similar to those measured at a mining area in Turkey⁴² and the Liaoning
386 Province in China^{19,21} but 10-fold lower than the Wanshan Mercury Mining District in
387 China¹⁴. Mean THg concentration from our soil samples (5.9 mg/kg) was also 20-fold higher
388 than the average value for global agrosystem soils (0.3 mg/kg)⁴³ and 60-fold higher than the
389 mean concentration in natural surface soil (0.1 mg/kg)⁴⁴. Overall, soils in the vicinities of
390 chlor-alkali plants have Hg concentrations ranging from 0.06 to 100 mg/kg⁴⁵. The methylated
391 form of Hg only represented 0.03% of the THg, which is very low compared to the common
392 rate of 1% to 3% for terrestrial sediments⁴⁴. However, similar rates of MeHg in the previously
393 mentioned Wanshan District (from 0.02 to 0.11%) to the rates of our samples were
394 observed⁴⁶.

395 Low Hg concentrations were measured for the various tissues of nettles spontaneously
396 growing under the studied poplar plantation, with average THg concentrations not exceeding
397 100 ng/kg for stems and leaves. These concentrations were in the same order of magnitude

398 than those measured for the Skado poplar cultivar (*i.e.*, 42.5 µg/kg dry wt.)³⁴. The obtained
399 concentrations in nettles were lower than the average THg concentration (206 µg/kg)
400 measured in the shoots of 12 native herbaceous plant species collected in the previously
401 mentioned mining area in Turkey⁴². Hg concentrations in mature leaf tissues above 1-8 mg/kg
402 have been reported to be excessive or toxic to plants⁴⁵ with a potential 10% yield loss in
403 agronomic species⁴⁷. These thresholds reported in the literature are 10 to 80 times higher than
404 those measured in nettle leaves in our study. A recent study conducted on the same site
405 concluded that Hg entry into poplar leaves was exclusively through an atmospheric pathway².
406 If this property also applies to nettle, it may explain the higher Hg concentrations measured in
407 the nettle leaves compared to the stems.

408 Insects from the revegetated sediment landfill had Hg concentrations ranging from
409 background values to 5500 µg/kg, which is consistent with studies conducted in industrialized
410 areas with comparable Hg concentrations in terrestrial sediments. Lepidopteran larvae,
411 Formicidae, and Chrysomelidae, had THg concentrations comparable to those measured in
412 previous studies^{18,19}. Given the TMF for THg (2.8) and MeHg (6.3) within the 5 trophic
413 levels, our data suggest that Hg biomagnified at the soil-nettle-insect food web (Figure 4).
414 Our results are in agreement with studies that considered a wider range of taxonomic groups,
415 including insects, birds and mammals¹⁴⁻¹⁷. Our study did not include the latter two higher
416 trophic levels but rather focused on a much higher number of insect taxa and a more accurate
417 level of trophic interactions.

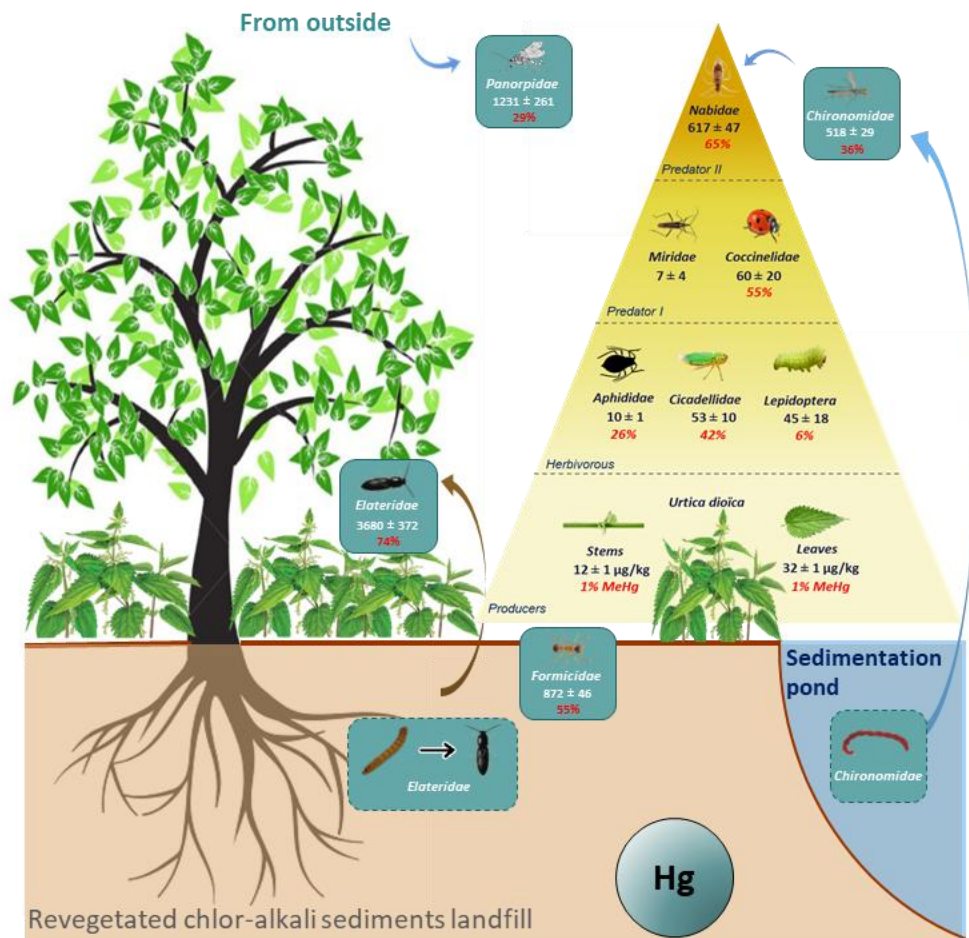
418 The present study covers a wide taxonomic range of insects for which the accuracy of
419 identification enabled the characterization of several life traits. This functional diversity
420 provided us with a decent overview of the functioning ecosystem and enabled us to study
421 these life traits as factors influencing Hg enrichment in insects. Thus, taxa from the
422 experimental site were enriched in Hg to varying degrees, depending on several factors,

423 including the trophic level, linked to the feeding guild and the feeding specificity. However,
424 the fact that within the nettle-unrelated group, an herbivorous species (Elateridae) had THg
425 concentrations exceeding those of secondary predators, such as ants or Panorpidae, led us to
426 consider other factors, including those conditioning a direct contact with contaminant sources.

427 Among all of the taxa considered, Elateridae were the most enriched in both Hg and
428 MeHg, with concentrations in the same order of magnitude as those measured in soil. As
429 herbivorous taxa feeding mainly on the poplar cultivars with a comparable enrichment than
430 nettles³⁴, Elateridae should not have been exposed to high amounts of Hg from their diet.
431 These elevated concentrations may be related to direct cuticle contact with the dredged
432 sediments during the larval cycle. As already mentioned for ants¹⁸, Hg in soil can indeed
433 penetrate their cuticle by uptake or passive diffusion when prolonged dermal contact has
434 occurred. Elateridae larvae, also known as wireworms, have spent most of their life in the soil,
435 including 3-4 months for eggs, 2-5 years as larvae, and can overwinter as adults⁴⁸. The other
436 taxa investigated that may be exposed to Hg through prolonged dermal contact during their
437 larval cycle are Panorpidae that have spent 1-2 years in the soil⁴⁹ and Chironomidae spending
438 one to several years in underwater sediments⁵⁰. The landfill we investigated in the present
439 study was indeed constructed in the vicinity of a mitigation pond³⁷. The lower THg
440 concentrations measured in adult Chironomidae compared to Elateridae may be related to a
441 greater loss of Hg during metamorphosis, as proven for some metals, including Hg⁵¹, or to an
442 active detoxification mechanism⁵². Although the THg concentrations in the soil and in
443 Elateridae were in the same order of magnitude, the concentration of MeHg was
444 approximately 2000-fold higher in Elateridae, suggesting the entry of the methylated form of
445 Hg or the potential methylation of inorganic Hg once taken up. The diet of wireworms mainly
446 consists in decomposed plant matter, and its associated fungal and microbial biomass, which
447 are known to be involved in the methylation of Hg in forest floor⁵³. A recent study already

448 hypothesized that decomposition of the soil organic matter pool introduces additional MeHg
449 to the diets of detritivore invertebrates⁵⁴. Consequently, the high concentration of Hg
450 measured in Elateriade seemed to be related to both their feeding guild and habitat during
451 their larval stage. Further studies on insect traits related to their development intrinsic, such as
452 the duration of the larval cycle, duration and location during overwinter or diapause, are
453 needed to improve our knowledge regarding Hg accumulation in insects.

454 Within the nettle-related food web, the THg concentrations increased as follows:
455 nettles < herbivores < predator specialists < predator generalists, with a gap between predator
456 specialists (including Coccinellidae) and predator generalists, suggesting a likely entry of Hg
457 from another source at this level. Nabidae, which were the only predator generalist in the
458 nettle-related insects, only hunt on the herbaceous layer⁵⁵ with rare contact with the soil
459 surface. Adult Chironomidae, which most likely grew as larvae in the adjacent contaminated
460 pond³⁷ where they became contaminated by Hg, were abundant on nettles. In this context,
461 Chironomidae were potential prey for Nabidae and, consequently, biovectors from aquatic to
462 terrestrial environments. A previous study showed that Chironomidae, among emerging
463 insects, were the main contributors to the transfer of biomass and polyunsaturated fatty acids
464 from aquatic to terrestrial ecosystems, representing a superior food quality for terrestrial
465 consumers²². With comparable THg concentrations (Figure 1) and similar relationships
466 between THg and biomasses (Figure 3), Chironomidae probably played the role of the
467 biovector of Hg from aquatic sediments to Nabidae (Figure 4). Thus, we demonstrated that
468 the feeding specificity of insects might also be considered an explanatory factor, as generalist
469 insects are exposed to high amounts of Hg while feeding on emerging insects. These results
470 were consistent with those for predatory spiders^{24,29,56}, which are also generalist species and
471 fed on Hg-enriched Chironomidae.



472

473 **Figure 4:** THg concentrations in the nettle-related food web (yellow pyramid) and the
 474 implication of nettle-unrelated taxa in the flow of THg (blue boxes). THg concentrations
 475 (µg/kg dry wt.) are specified under each name of taxa (mean ± SE), as well as the percentage
 476 of THg as MeHg in bold red.

477 Our findings suggest that insects (*e.g.*, Elateridae and Chironomidae) that had direct
 478 contact with terrestrial or underwater sediments constitute the main entry source of Hg within
 479 the terrestrial nettle-insect food web (Figure 4). These insects may constitute direct food for
 480 higher trophic levels (birds, mammals) or food for generalist predator insects. These latter
 481 (*i.e.*, Nabidae) are likely to play a role in the export of significant amounts of Hg at higher
 482 trophic levels and become another major vector in the transfer of Hg, as recently
 483 demonstrated in temperate forest environment⁵⁴. Other insect features of interest, such as

484 dispersal capacity, vulnerability to predation, and nutritional value, would, however, need to
485 be considered to fully assess the role of insects within Hg dissemination in Hg-contaminated
486 areas.

487 **ASSOCIATED CONTENT**

488 **Supporting information.**

489 **Figure S1.** Results from preliminary tests consisting of evaluating the influence of insect
490 preservation methods on the THg concentrations measured in *Nabis sp.* (A) and *Myrmica*
491 *rubra* (B) preserved in ethanol or directly ice-dried after collection.

492 **Table S1.** Ecological characteristics of each targeted taxa, including their link with nettle,
493 habitat, trophic guild in the nettle-based food web, feeding guild and dietary range according
494 to the literature

495 **Table S2.** Details of the MeHg analyses

496

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501 Notes

502 The authors declare no competing financial interest.

503

504 **ACKNOWLEDGMENTS**

505 The authors gratefully acknowledge the funding provided by ADEME, France, under grant
506 No. 1772C0018, PHYTOFIBER Project, and the Fondation de France and ADEME for their
507 financial support to LY under a PhD grant.

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