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Interactive effects of metals and carbon nanotubes in a microcosm agrosystem

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Abstract

Agricultural soils are exposed to multiple contaminants through the use of agrochemicals or sewage sludge, introducing metals, nanomaterials and others. Among nanomaterials, carbon nanotubes (CNTs) are known for their large surface area and adsorption capabilities, possibly modifying other element behavior. However, to date, very little is known about the impacts of such interactions in agrosystems. In this study, we aimed at understanding the transfer and toxicity of contaminants (Cd, Pb, Zn and CNTs) in microcosms including native soil bacteria, earthworms and lettuce. After a 6 week exposure, no effect of the addition of CNTs to metal contaminated soils was detected on bacterial concentration or earthworm growth. However, in lettuce, an interactive effect between CNTs and metals was highlighted: in the soil containing the highest metal concentrations the addition of 0.1 mg kg⁻¹ CNTs led to a biomass loss (-22%) and a flavonoid concentration increase (+27%). In parallel, the addition of CNTs led to differential impacts on elemental uptake in lettuce leaves possibly related to the soil organic matter content. For earthworms, the addition of 10 mg kg⁻¹ CNTs

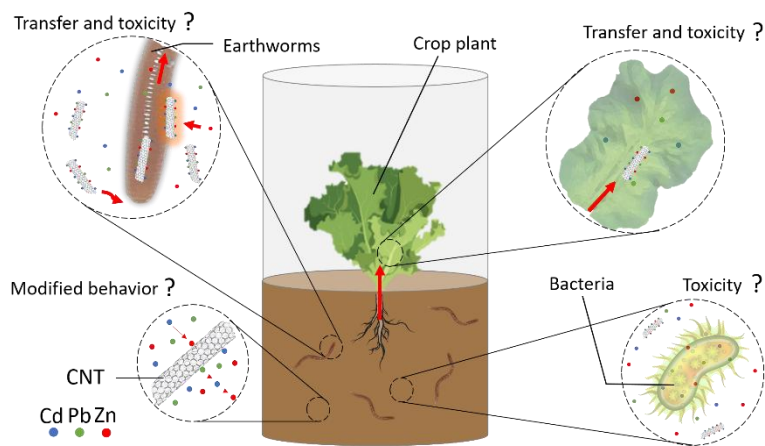
25 resulted in an increased body elemental transfer in the soil with the higher organic matter content
26 (Pb: + 34% and Zn: + 25%).

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28 **Keywords:** multiple stress, cocktail contaminants, risk assessment, toxicity, transfer

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30 **Graphical abstract**



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42 **1. Introduction**

43 For several decades, most agrosystems have faced problems related to diverse agricultural practices.
44 For instance the continuous spreading of organic and inorganic fertilizers has progressively led to the
45 accumulation of metals in soils^{1,2}. Additionally, the application of sludge from wastewater treatment
46 plants as fertilizer is reported to be one of the most important sources of metal contamination in
47 agricultural soils^{2,3}. In the long term, this also implies a risk of toxicity to organisms in these
48 ecosystems and contamination of the food chain⁴. Indeed, many studies demonstrated that soil
49 contamination with metals reduces the overall bacterial biomass, leads to compositional changes in
50 bacterial communities and microbial processes that would result from a difference in tolerance to
51 metals depending on the bacterial species^{5,6}. For earthworms, studies have shown a reduction in
52 their growth through a delay in biomass gain compared to controls⁷⁻⁹. Metals can also modify
53 earthworm reproduction through a reduction in the number of spermatozoa produced¹⁰, cocoon
54 production^{8,11,12}, cocoon hatching success^{9,13,14} and number of juveniles produced^{8,9,11}. Finally, studies
55 have observed a slowdown in plant growth parameters following exposure to metals such as
56 inhibition of seed germination, reduction and/or malformation of the root system, reduction of
57 mineral nutrition, photosynthetic activity, activity of certain enzymes, leaf area and aerial biomass
58 and ultimately fruit production^{15,16}. In response, authorities such as the European Union (Directive
59 86/278/EEC) and the United States (Title 40 of the Code of Federal Regulations, Part 503) have
60 established legislation defining the permitted limits for metals in sewage sludge used for land
61 application^{17,18}.

62 However, with society habit changes, new contaminants have emerged recently such as
63 nanomaterials. In particular, since their first synthesis in 1962¹⁹, and because of their remarkable
64 physical, electronical and chemical properties, graphene-based materials have been increasingly
65 used by industrials. The nanoscale of graphene-based materials provides a common characteristic to
66 these materials, a high specific surface area, which reveals increased energy and surface reactivity²⁰.
67 Carbon nanotubes (CNTs) are among the most widely used graphene-based materials²¹. Nowadays,

68 they are found in products such as kitchen utensils, tires and sport equipments²², and subsequently
69 possibly end up into the environment during their journey from production to recycling²³. In
70 particular, they tend to accumulate in sewage sludge²³. According to a review published by Holden *et*
71 *al.* (2014), the concentration of carbonaceous nanomaterials in biosolids could reach up to 10 mg kg⁻¹
72 while in natural soil it could be up to 0.01 mg kg⁻¹²⁴. In addition, CNTs concentration in agricultural
73 soils could increase in the future as their use as nano-fertilizer is considered for the production of
74 plant-based biofuels and crop food²⁵. It is thus necessary to better understand the fate of these CNTs
75 in agrosystems. Recent work has demonstrated that CNTs can decrease soil microbial activity²⁶⁻²⁹;
76 another study has observed a decrease in soil bacterial biomass when exposed to CNTs²⁸ (up to
77 1000 mg kg⁻¹). The structure of bacterial communities may also be altered by exposure to CNTs^{29,30}.
78 For earthworms, DNA damage in a dose-response relationship was demonstrated in *Eisenia fetida*
79 after exposure to CNTs over a wide range of concentrations (50, 250 and 500 mg kg⁻¹)³¹. Another
80 study showed a reduction in cocoon production of up to 60% and 78% at 495 and 1000 mg
81 MWCNT.kg⁻¹ of food, respectively³². However, a majority of studies did not observe any apparent
82 effect on earthworms following exposure to CNTs, probably due to their depuration abilities³³⁻³⁵,
83 which limit the toxicity of CNTs to these organisms^{34,36-38}. Finally, for crop plants several studies
84 showed a significant increase in seed germination³⁹⁻⁴², root length^{39,43,44}, biomass production⁴⁴⁻⁴⁶, and
85 flower and fruit productions⁴⁷ at different CNTs concentrations (40, 50, 100 and 500 mg.L⁻¹).
86 However, some studies highlighted contrasting results with slower growth of some crop plants⁴⁸⁻⁵¹.
87 Overall, it is interesting to note that most experiments were carried out with very high CNTs
88 concentrations in comparison with expected environmental concentrations.

89 The multiplicity of pollutants found in the environment and their different effects raise questions
90 about their possible interactions. Indeed, organisms are exposed to a wide variety of contaminants,
91 the cumulative effects of which are as yet uncertain. Studies have shown that there can be a "cocktail
92 effect" and that individual toxic substances present at seemingly innocuous concentrations can
93 synergistically cause harm to organisms when mixed⁵²⁻⁵⁵. To date, very few studies have been carried

94 out on co-contamination of CNTs and metal(oid)s (*e.g.* Cd^{56,57} and As⁵⁶), although recent research has
95 shown possible interactions between them^{58,59}. Indeed, CNTs are capable of adsorbing metals to their
96 surfaces in aqueous solution⁶⁰⁻⁶³.

97 Finally, in agrosystems, interactions between soil micro-organisms, earthworms and crop plants are
98 of uttermost importance for ecosystem services; however, these interactions are rarely considered in
99 ecotoxicology studies which rather focus on single organism exposure. For instance, in a review that
100 compiles data from 67 different studies on the effect of earthworms on plants, Stefan Scheu⁶⁴
101 observed in 79% of the cases a significant increase in the biomass of seedlings in the presence of
102 earthworms. He explained this phenomenon in particular by the action of the worms as bioturbbers,
103 which modified the soil structure, mineralized and remobilized nutrients and microorganisms⁶⁴.

104 The objectives of this study were to investigate the transfer and toxicity of these cocktail
105 contaminants (Cd, Pb, Zn and CNTs) at environmentally relevant concentrations on above- and
106 belowground components (microbial communities, earthworms and crop plants) in a microcosm
107 agrosystem experiment. Our aims were to: (1) assess the joint toxicity of CNTs and metals on soil
108 microbial communities (total abundance), earthworms (mortality, biomass and sexual maturity) and
109 crop plants - here lettuce - (height, leaf number, aerial biomass, photosynthetic activity), (2)
110 determine whether the transfer of metals was modified in the presence of CNTs in exposed
111 organisms (earthworms and lettuce plants), and (3) discuss the processes and the factors involved in
112 the interactions.

113

114 **2. Material and methods**

115 **2.1. Soil characterization**

116 To avoid adding “fresh” metals to the soils right before the beginning of the experiment which can
117 bias their behavior⁶⁵, we used “historically” contaminated soils (see supporting information for more

118 details)⁶⁶. As a result, two clay-loam soils with different metal concentrations were collected: Lower
119 Contamination (LC, Cd = 13.2, Pb = 523.9, Zn = 689.3 mg kg⁻¹) and Higher Contamination (HC, Cd =
120 27.7, Pb = 1471.4, Zn = 1825.1 mg kg⁻¹) in metals (see section 3.1 in results for more information).
121 These soils were chosen to be as similar as possible in term of physico-chemical properties (texture
122 and pH).

123 The main physico-chemical characteristics (texture, pH, C/N) of soil (sieved to 2 mm) were
124 determined for each microcosm at the beginning of the experiment (see supporting information for
125 more details). Total Cd, Pb and Zn concentrations and others elements (B, Ba, Ca, Cu, K, Mg, Mn, Na,
126 Ni, P, S, Sr) were determined by inductively-coupled plasma atomic emission spectrophotometry
127 (ICP-AES, see section 2.7).

128

129 **2.2. Characterization and suspension of CNTs**

130 CNTs were synthesized at the Interuniversity Center of Materials Research and Engineering (CIRIMAT,
131 Toulouse University) and observed by transmission electron microscope. They were multi walled
132 CNTs with length varying from 1 to 20 μm, an average diameter of 16.8 ± 6.7 nm and a specific
133 surface area of 293 m².g⁻¹. CNTs were suspended in ultrapure water at 100 mg.L⁻¹ and homogenized
134 for 15 min in an ultrasonic bath before use.

135

136 **2.3. Experimental set-up**

137 HC and LC bulk dry soils were contaminated by addition of the required volume of CNTs suspension
138 to reach a final concentration of 0.1 or 10 mg kg⁻¹ dry soil; this resulted in a full-factorial design
139 crossing each metal contamination level with each CNTs concentration (LC0, LC0.1, LC10, HC0, HC0.1
140 and HC10). This resulted in 36 microcosms: 6 treatments x 6 replicates. The soils were mixed

141 manually for 10 min after contamination. This last step was repeated every day for a week so that
142 the CNTs were homogeneously distributed in the soil.

143 Three hundred grams of soil were distributed in each experimental unit (L = 45 cm, D = 10 cm); a
144 lettuce seedling (*Lactuca sativa*, var. Laitue romaine provided by the company Germinance) and 4
145 earthworms (*E. fetida*, provided by the Laboratory of Civil Engineering and Geo-Environment) were
146 added (Figure S1). Each earthworm was selected at the sub-adult stage (puberculum without
147 clitellum) and in a restricted body mass range (1.37 ± 0.22 g). They were depurated for 24h before
148 being introduced into the microcosms. To avoid additional trophic stress to the earthworms, they
149 were fed every two days with 4.5 ± 0.2 mg of crushed (<2 mm) moistened horse dung added in small
150 clumps on top soil. The experiment lasted 6 weeks (for complete development of organisms) in an
151 environmental chamber with controlled parameters (10 h/14 h day/night cycle, 24°/22 °C and a
152 hygrometry rate of 85%).

153

154 **2.4. Bacterial abundance**

155 After exposure to contaminants, 3 mg of previously homogenized dried soil were placed in a tube
156 with 10 ml of ultrapure water for each microcosm. After 2 hours of stirring, the samples were
157 centrifuged. The supernatant, containing bacteria, was filtered at 10 μm to remove suspended
158 matter coarse particles. Then, bacterial samples (1 mL) were stained with SYBR Green I (0.1x final
159 concentration) and incubated in the dark for 15 minutes. Finally, the samples were run for flow
160 cytometry (Guava, Merck, United States) at a medium speed of $2 \mu\text{L s}^{-1}$ and a count rate not
161 exceeding $1000 \text{ events s}^{-1}$.

162

163 **2.5. Toxicity endpoints for *Eisenia fetida***

164 After exposure, the soil was manually disaggregated to recover the worms. The survival rate and
165 juvenile density were determined. After 24 hours of depuration, earthworms were weighed. Finally,
166 they were frozen, then freeze-dried and ground to determine their elemental concentration by ICP-
167 AES (see section 2.7.).

168

169 **2.6. Phytotoxicity endpoints**

170 After exposure, several parameters were recorded: length of the longest leaf, number of leaves,
171 fresh and dry biomasses, leaf surface area and effective quantum yield. Part of the leaf biomass was
172 dried in an oven (60°C) to determine elemental concentrations by ICP-AES (see section 2.7.). The
173 remaining biomass was used fresh for methanol-based extraction. Photosynthetic pigments
174 (chlorophyll a and b) were then quantified⁶⁷ and secondary metabolites *i.e.* total phenolic compound,
175 flavonoid and tannin concentrations were assessed thank to the Folin-Ciocalteu assay⁶⁸, aluminum
176 chloride method⁶⁹ and vanillin method in acid medium⁷⁰, respectively as described in Liné *et al.*
177 (2021)⁷¹. More details for phytotoxicity endpoints can be found in supporting information.

178

179 **2.7. Sample preparation and chemical analysis by ICP-AES**

180 Acid digestion was performed in a DigiPrep heating system, using HNO₃, NH₄F, and HClO₄, as well as
181 H₂O₂, with a temperature of 100 °C and several evaporation steps for plant and worm samples⁷². For
182 soils, a two-step digestion was performed in the same system with respectively 4 ml of HCl and 2 ml
183 HNO₃ during 90 minutes at 95°C, then addition of 1.5 mL of H₂O₂ and heating at 100°C until near
184 complete evaporation. For soil, a filtration step (0.45 µm) was added. Digested samples were then
185 diluted in 5% HNO₃ before analysis on an ICP-AES (IRIS Intrepid II XDL, Thermo Scientific, United
186 States). along with control samples (blanks with only chemicals and standard reference materials:

187 NIST SRM 1515: apple leaves, irmm BCR 679: white cabbage, NRCC PRON-1: river prawn). Percentage
188 of recovery for reference materials are given in Table S1.

189

190 **2.8. Calculations and statistical analyses**

191 Transfer factor was determined as the ratio between metal concentration in a given organism (plant
192 or earthworm) divided by the total metal concentration in the soil.

193 For statistical analyses, first, 2-way ANOVAs were performed on the toxicity endpoints to test the
194 effects of metal concentration (p_{metals} -value), of CNTs addition (p_{CNTs} -value) or the interaction
195 between both ($p_{CNTs/metals}$ -value). The normality, independence and homoscedasticity of the residues
196 were checked using Shapiro, Durbin-Watson and Breusch-Pagan tests, respectively. If one of the
197 three conditions of validity was not met, the data were log-transformed or square root transformed.
198 In the case of failed transformations, a non-parametric Kruskal-Wallis test was performed. All the
199 details about statistical analyses can be found in Table S2. Principal component analyses (PCA) were
200 performed on the elemental concentrations in earthworms and plants. All statistical analyses were
201 performed on RStudio software (version 3.4.1).

202

203

204 **3. Results**

205

206 **3.1. Soil characterization**

207 Cd, Pb and Zn concentrations were significantly different between LC and HC soils (Table S3, p -value
208 < 0.01 for each metal, t-test). HC soils had 2.1 times more Cd than LC soil (28 vs. 13 mg kg⁻¹), 2.9
209 times more Pb (1471 vs. 524 mg kg⁻¹) and 2.6 times more Zn (1825 vs. 689 mg kg⁻¹). More information
210 about other elemental concentrations (Ag, Al, As, Ba, B, Ca, Ce, Co, Cr, Cu, K, Li, Mg, Mn, Na, Ni, P, Pb,

211 S, Sr) in soils is available in Table S4. HC soil was composed of 1.7% sand, 88.4% silt and 9.8% clay
 212 while LC soil had 5.9% sand, 82.2% silt and 11.8% clay (Table S5). LC and HC soils had pH of 7.2 and
 213 7.0, respectively. Finally, carbon and nitrogen contents were 1.7 and 3 times more important in HC
 214 soil than in LC one (p -value < 0.01).

215

216 3.2 Toxicity of the co-contamination on belowground components

217 Overall, bacterial density was 93% higher in LC soils compared to HC soils whatever the CNT
 218 treatment (p -value < 0.001, Figure 1A).

219 After exposure, no effect was observed on the worm mortality rate among treatments (p -value =
 220 0.157, Figure S2), which was on average of 13%. No effect of metal or CNT treatments was observed
 221 and no interaction CNTs/metals was detected on worm biomass (p_{metals} -value = 0.129, p_{CNTs} -value =
 222 0.819, $p_{CNTs/metals}$ -value = 0.304) with an average biomass of 0.94 g for the 4 worms per microcosm
 223 (Figure 1B). The same was concluded for the number of juvenile (p_{metals} -value = 0.953, p_{CNTs} -value =
 224 0.97, $p_{CNTs/metals}$ -value = 0.71, Figure S3) with on average 1 juvenile per microcosm.

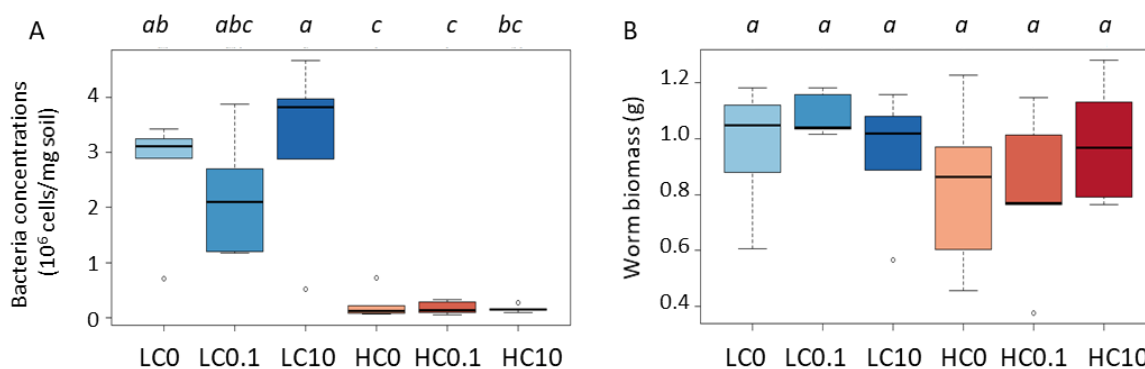


Figure 1: Soil bacterial concentration (A) and earthworm biomass (B) for the six exposure conditions (2 metal modalities: LC = Lower Contamination and HC = Higher Contamination with Cd, Pb and Zn; 3 CNT modalities: 0 = 0 mg kg⁻¹, 0.1 = 0.1 mg kg⁻¹, 10 = 10 mg kg⁻¹) after a 6 week-exposure. Same lowercase letters indicate treatments that do not differ significantly (p -value > 0.05; $n=6$).

225

226 3.3 Toxicity of the co-contamination on the aboveground component

227 Overall, lettuce grown on HC soil had a better growth than plants grown in LC soil with a significantly
 228 higher biomass (+27%, $p_{metals}\text{-value} < 0.001$, Figure 2A), longer leaves (+ 3 cm longer, $p_{metals}\text{-value} <$
 229 0.001, Figure S4). Dry weight/fresh weight ratio of lettuce was the same among all treatments ($p =$
 230 0.082, Figure S5). No significant effect of CNT addition was observed on any of the assessed
 231 parameters (Table S2, Figures S4 to S7). However, an interaction between CNT exposure and soil
 232 metals was detected for lettuce biomass ($p_{CNTs/metals}\text{-value} = 0.036$) indicating that lettuces exposed to
 233 HC 0.1 showed significantly lower biomass than HC 0 (-22%, Figure 2A). No interaction was found for
 234 other development endpoints (Table S2).

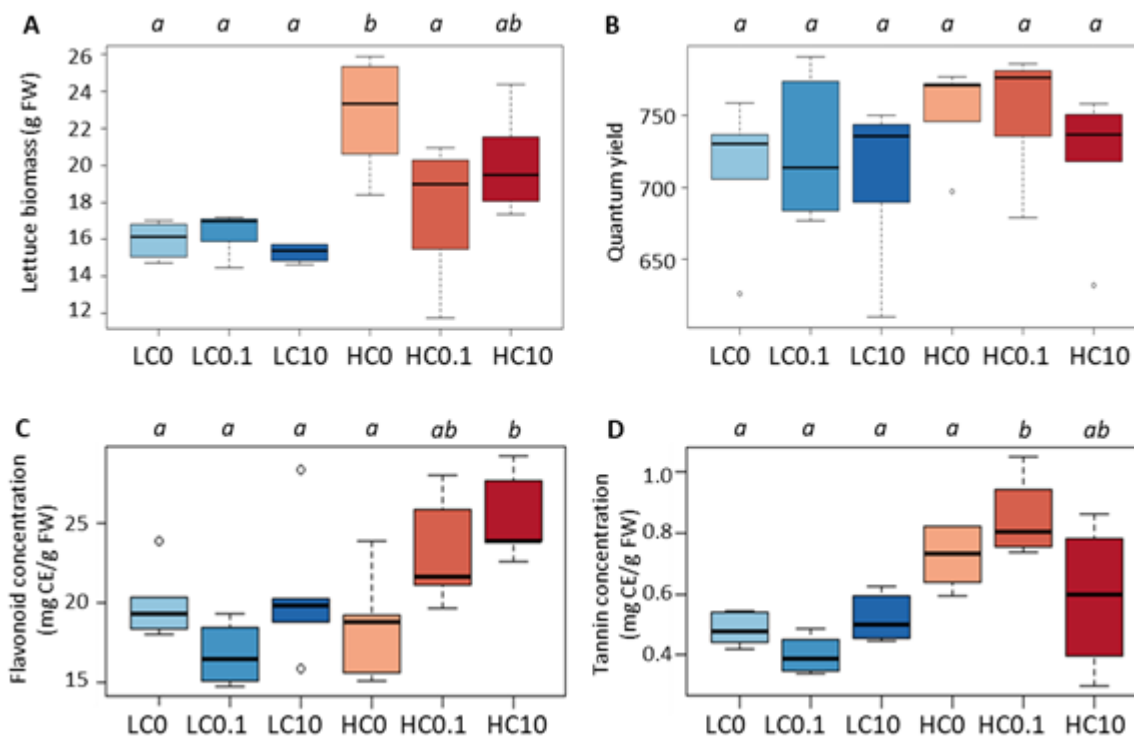


Figure 2: Lettuce aboveground biomass (A), quantum yield (B), flavonoid (C) and tannin (D) concentrations in leaves for the six exposure modalities (2 metal modalities: LC = Lower Contamination and HC = Higher Contamination with Cd, Pb and Zn; 3 CNT modalities: 0 = 0 mg kg⁻¹, 0.1 = 0.1 mg kg⁻¹, 10 = 10 mg kg⁻¹) after a 6 week-exposure. Same lowercase letters indicate treatments that do not differ significantly ($p\text{-value} > 0.05$; $n=6$). CE: catechine equivalent, FW: fresh weight.

235
 236 At the physiological level, the quantum yield of lettuce leaves showed no significant difference
 237 regardless of the treatment with in average a value of 0.730 ($p = 0.302$, Figure 2B). However, lettuces
 238 exposed in HC soils showed significantly higher chlorophyll a and b (+50% and +57%, respectively;

239 Chlo a: $p_{metals}\text{-value} < 0.001$; Chlo b: $p_{metals}\text{-value} < 0.001$, Figure S8, S9) but no impact of CNT addition
240 or interaction between these two contaminants could be highlighted. For secondary metabolites,
241 flavonoids (+17%, $p_{metals}\text{-value} = 0.014$, Figure 2C) and tannins (+53%, $p_{metals}\text{-value} = 0.001$, Figure 2D)
242 were more concentrated in the leaves of plants grown in HC soils than in those grown in LC soils; this
243 effect was not visible for total phenolic compounds (Figure S10). The addition of CNTs in the medium
244 led to an increase in flavonoids in comparison with control plants (+20%, $p_{CNTs}\text{-value} = 0.038$) which
245 was not the case for total phenolic compounds or tannins (Table S2). Interactions CNTs/metals were
246 detected for flavonoid and tannin concentrations (flavonoids: $p_{CNTs/metals}\text{-value} = 0.027$; tannins:
247 $p_{CNTs/metals}\text{-value} = 0.016$): flavonoids significantly increased with CNT concentration only in HC soils
248 and tannin concentration was higher after exposure to 0.1 mg kg⁻¹ CNTs in comparison with the
249 control in HC soils.

250

251 **3.3. Transfer and bioaccumulation of a cocktail of contaminants in below and above ground** 252 **components**

253

254 **3.3.1. Earthworms**

255 Concentrations of Pb and Zn were significantly higher in earthworm tissues exposed in HC soils with
256 in average 141.8 and 213.1 mg kg⁻¹, respectively, than in earthworm tissues exposed in LC soils with
257 45.4 mg kg⁻¹ of Pb and 157.5 mg kg⁻¹ of Zn (Pb: $p_{metals}\text{-value} < 0.001$; Zn: $p_{metals}\text{-value} < 0.001$, Figure
258 3A). This was not the case for Cd ($p_{metals}\text{-value} = 0.559$), with an average concentration of 31 mg kg⁻¹
259 whatever the condition. In addition, there was no effect of CNTs and no interaction CNTs/metals on
260 the transfer of Cd ($p_{CNTs}\text{-value} = 0.953$, $p_{CNTs/metals}\text{-value} = 0.683$). There was no effect of CNTs either
261 on the transfer of Pb and Zn in LC soils (Pb: $p_{CNTs}\text{-value} = 0.390$; Zn: $p_{CNTs}\text{-value} = 0.620$). However,
262 CNT/metal interactions were found on the transfer of Pb and Zn (Pb: $p_{CNTs/metals}\text{-value} < 0.001$; Zn:
263 $p_{CNTs/metals}\text{-value} < 0.001$). Earthworms exposed to 10 mg kg⁻¹ of CNTs in HC soils contained 34% more

264 Pb and 25% more Zn in their tissues than at other CNTs concentrations (0 and 0.1 mg kg⁻¹) which was
265 not the case for earthworms exposed in LC soils. Taking into account the initial metal concentrations
266 in soil, the transfer factors of Cd, Pb and Zn highlighted the same trend than concentration results
267 (Table S6).

268 The PCA individualized earthworms exposed to HC 10 treatment which had higher amounts of
269 certain elements (B, Mn, Ni, Ba, Na) in their tissues at the end of exposure than earthworms exposed
270 to the other treatments (Figure 3B, 3C). More information about nominal elemental concentrations
271 in earthworms (B, Ba, Ca, Cu, K, Mg, Mn, Na, Ni, P, S, Sr) are available in Table S7.

272

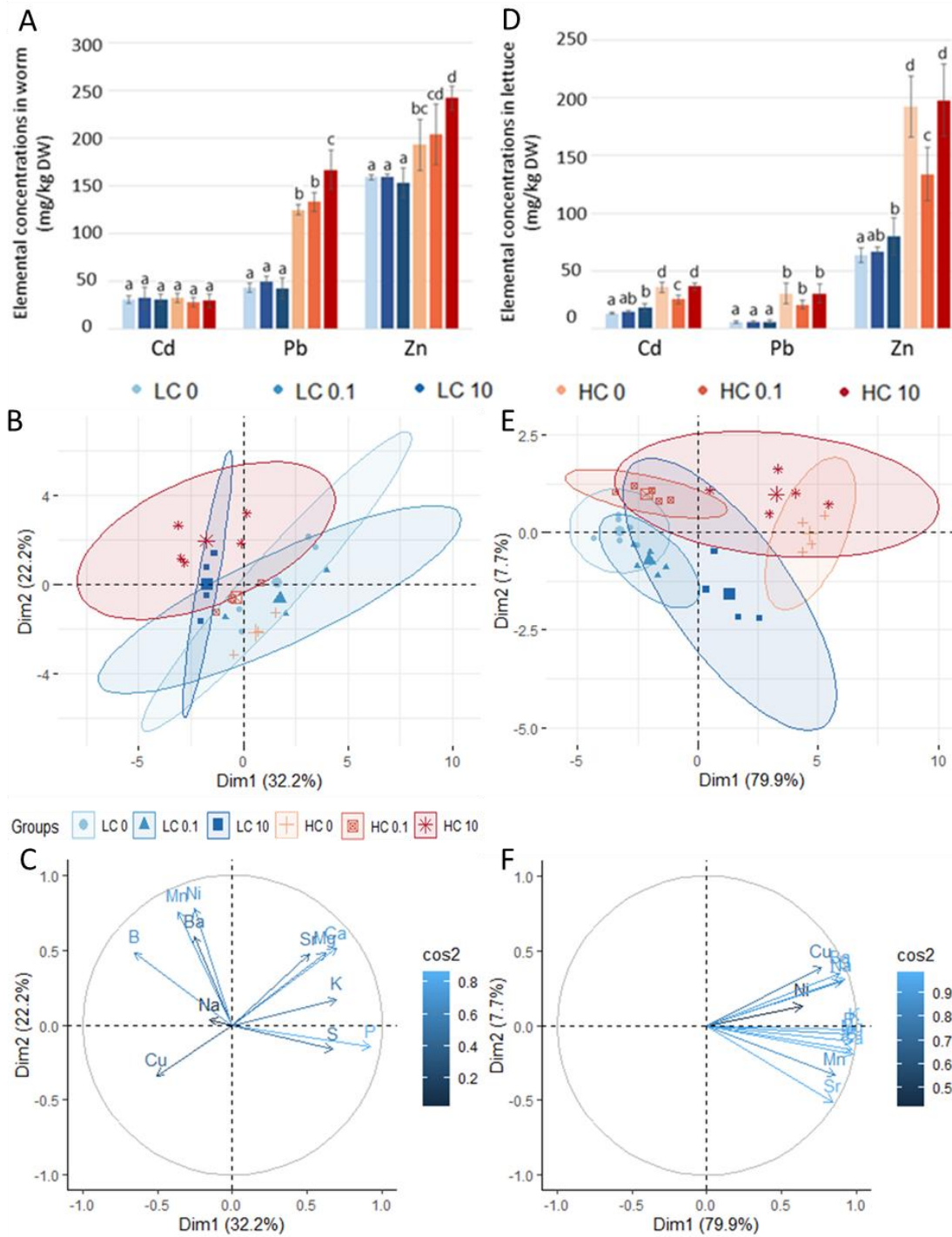


Figure 3: Concentrations of metals (Cd, Pb, Zn) in earthworm tissues (A) and lettuce leaves (D) for the six exposure modalities (2 metal modalities: LC = Low Contamination and HC = High Contamination with Cd, Pb and Zn; 3 CNT modalities: 0 = 0 mg kg⁻¹, 0.1 = 0.1 mg kg⁻¹, 10 = 10 mg kg⁻¹) at the end of exposure (6 weeks). Same lowercase letters indicate treatments that do not differ significantly (*p*-value > 0.05; *n*=6). DW: dry weight. PCA on the elemental content (B, Ba, Ca, Cu, K, Mg, Mn, Na, Ni, P, S, Sr) represented by individuals and correlation circle graphs for earthworm tissues (B and C) and lettuce leaves (E and F) for the 6 treatments. Cos2 corresponds to the level of variable contributions (elements) to dimensions 1 and 2.

274 3.3.2. Lettuces

275 Concentrations of Cd, Pb and Zn were significantly higher in leaves of lettuces exposed in HC soils
276 with in average 32.8, 27.3 and 174.6 mg kg⁻¹, respectively than in lettuce leaves grown in LC soils with
277 15.5, 5.8, 70.2 mg kg⁻¹ (Cd: $p_{metals}\text{-value} < 0.001$; Pb: $p_{metals}\text{-value} < 0.001$; Zn: $p_{metals}\text{-value} < 0.001$,
278 Figure 3D). There was no effect of CNTs on the transfer of Pb in lettuce leaves ($p_{CNTs}\text{-value} = 0.090$)
279 but it was significant for Cd and Zn (Cd: $p_{CNTs}\text{-value} < 0.001$; Zn: $p_{CNTs}\text{-value} < 0.001$). Furthermore,
280 interactions were detected for Cd and Zn with CNTs (Cd: $p_{CNTs/metals}\text{-value} < 0.010$; Zn: $p_{CNTs/metals}\text{-value}$
281 = 0.002) with a significant increase of 37% and 21%, respectively in lettuce exposed to 10 mg kg⁻¹ CNT
282 in LC soils compared to control. However, a significant decrease of 29% in Cd and 30% in Zn
283 accumulation was noticeable in plants exposed to 0.1 mg kg⁻¹ CNTs in HC soil in comparison to the
284 control. A similar pattern was observed for Pb (-32%) but was not significant ($p_{CNTs/metals}\text{-value} =$
285 0.108). Transfer factors followed the same trend than Cd, Pb and Zn absolute concentration in
286 lettuces (Table S8).

287 The PCA on elemental concentrations (B, Ba, Ca, Cu, K, Mg, Mn, Na, Ni, P, S, Sr) in lettuce leaves after
288 exposure (Figure 3E, 3F) highlighted the two soil modalities. Lettuces which were exposed to HC soils
289 contained higher elemental concentrations than those exposed to LC soils. In LC soils, the
290 concentration gradient of CNTs can be seen along PC 2, explaining 7.7% of the variance. This pattern
291 was not visible for HC soils. Lettuces treated with HC 0.1 had less nutrients in their leaves at the end
292 of exposure than the other HC treatments. More information about elemental concentrations in
293 lettuce leaves can be found in Table S9.

294

295

296 4. Discussion

297 4.1. Toxicological impacts of a contaminant cocktail

298 Our results demonstrated that the toxicological impacts of CNT/metal co-contamination within an
299 experimental agrosystem differed from one organism to another.

300 Bacterial populations were less abundant (-93%) in the soil containing the highest concentrations of
301 metals. This is in agreement with the results of the literature which demonstrate that microbial
302 concentration depends on the metal contamination of the environment^{5,6}. HC soils had 7, 2 and 16
303 times higher Cd, Pb and Zn concentrations than published ED₅₀ (Effective Doses) (based on the
304 inhibition of the dehydrogenase activity) obtained in a comparable soil (clay = 15%, silt = 75%, sand =
305 10 %): ED₅₀ of 90, 652 and 115 mg kg⁻¹ for Cd, Pb and Zn, respectively⁷³. The addition of CNTs in the
306 two soils, at low (0.1 mg kg⁻¹) and high (10 mg kg⁻¹) concentrations, did not result in additional
307 impacts on bacterial populations. It is interesting to note as it differs from what was expected since
308 the state of the art suggested that the addition of CNTs (in non-contaminated soil) usually leads to
309 bacterial toxicity²⁶⁻²⁹. However, these studies were mostly performed on sandy-silty soils²⁶⁻²⁹, poor in
310 clay, which could influence the mobility of CNTs and their toxicity^{72,74}. A study with a soil comparable
311 to the one used in the present study (clay = 11%, silt = 76%, sand = 13%) also did not observe a
312 significant effect of CNTs on the bacterial biomass when exposed to 0.2 and 20 mg kg⁻¹⁷⁵. No
313 interactive effects between CNTs and metals was highlighted.

314 The toxicity results obtained on earthworms also demonstrated no impact of the addition of CNTs on
315 the tested parameters (mortality, biomass and developmental stage). Nevertheless, the stress
316 imposed by metals delayed the development of the earthworms. Here, on average 35% of the
317 earthworms were still in the juvenile stage. Considering the 6 week-exposure, they should have all
318 reached the adult stage since *E. fetida* are adult in around 40-60 days. Overall, in the literature,
319 ecotoxicity studies relative to exposure to metals did not show acute effects on earthworms. Indeed,
320 *E. fetida* is able to tolerate high concentrations of metals. Spurgeon *et al.* (1994), estimated LC₅₀
321 values of 3670 mg kg⁻¹ and 745 mg kg⁻¹ for Pb and Zn, respectively during a 56-day experiment⁸. Here,
322 the concentrations were [Pb] = 524, 1471, [Zn] = 689, 1825 mg kg⁻¹ in LC and HC soils, respectively. Zn

323 concentrations, especially in HC soil, could thus have been detrimental for earthworms. However,
324 the soils used in this current study were richer in silt and clay than Spurgeon's soil (present study: 1
325 to 6% sand and 94 to 99% silt+clay, Spurgeon soil = 70% sand, 20% kaolin clay), making all the
326 elements less bioavailable because fixed in clay-humus complexes⁷⁶. In addition, it has been
327 recognized that the type of clay influences element adsorption and consequently their
328 bioavailability⁷⁷. Furthermore, the exposure soils used here came from an historically contaminated
329 area; metals have aged, which could further reduced their bioavailability⁶⁵. As for CNT
330 contamination, the literature mostly reports no apparent effects on earthworms³³⁻³⁵. Consequently,
331 our results suggest no synergic nor antagonist effects of the co-exposure of CNTs and metals on
332 earthworm fitness.

333 Opposite to the initial hypothesis, lettuces developed better in soils with higher concentrations of
334 metals (HC vs. LC). Indeed, many studies reported a slowdown in plant growth parameters when
335 exposed to metal contamination¹⁶. In particular, *L. sativa* appears to be poorly tolerant to this type of
336 contamination (LC_{50} : Cd = 2.59 mg L⁻¹, Zn = 33.88 mg L⁻¹)⁷⁸. In this experiment, the increased
337 development of lettuces in HC soils can be partly explained by the higher amounts of N in
338 comparison with LC soils (N average: LC = 0.14%, HC = 0.25%). The addition of CNTs had no overall
339 toxicological effects on the development of the crop plants except in the condition HC0.1 with a 22%
340 biomass decrease. In the literature, CNT exposure (in single stressor condition) led to controversial
341 results for *L. sativa* since some studies observed a significant reduction in root and shoot size^{51,79}
342 while another observed no effect on other developmental markers (germination and root length)⁸⁰.
343 However, for metabolism markers, it appears that the higher the concentration of CNTs, the higher
344 the concentration of flavonoids in lettuce exposed in HC soil. This is in agreement with a study which
345 observed a 160% increase in flavonoid production in *Satureja khuzestanica* following a 15-day
346 exposure to 100 mg.L⁻¹ of MWCNTs. On the other hand, exposure to lower CNT concentrations in
347 date palm shoots (0.05 and 0.1 mg L⁻¹) did not show significant differences in flavonoid production
348 compared to the control⁸¹. As reported in a review paper⁸², several studies have highlighted an

349 induction of flavonoid synthesis in plants in response to abiotic stresses such as high concentrations
350 of metals in the growing medium⁸². For instance, it has been observed that flavonoid levels increased
351 after exposure to copper sulphate and cadmium chloride in callus cultures of the legume *Ononis*
352 *arvensis*⁸³. Flavonoids are able to chelate metals, thereby reducing their toxicity to plants⁸⁴⁻⁸⁶. To sum
353 up, in the case of the crop plant, a synergic effect between CNTs and metals was detected and led to
354 a moderate toxicity.

355

356 **4.2. Controlling factors of contaminant transfer to organisms**

357 For HC soils, the accumulation of Pb and Zn in earthworm tissues was a function of CNT
358 concentrations in the medium. More generally, this observation was also valid for all other elements
359 measured in their tissues. Given their properties, CNTs may have adsorbed metals and other
360 elements on their surface and acted as a vector by facilitating their entry into earthworm tissues *via*
361 their two routes of exposure: integument and soil ingestion (probably mainly by integument for *E.*
362 *fetida*). It could be hypothesised that CNTs can damage the integument and/or the digestive tract
363 cells after ingestion by physical effects leading to an enhanced uptake of the adsorbed metals and
364 nutrients inside earthworm body like it has been demonstrated for other nanoparticles^{87,88}. However,
365 this effect of CNTs on earthworm elemental content was not observed in LC soils. An explanation to
366 these results could be related with the low organic matter content of the LC soil which is less
367 favourable for earthworm feeding and bioturbation activities and thus metal transfer⁸⁹. Finally, Cd
368 transfer did not respond in the same way as Pb and Zn; it is likely less bioavailable than the other
369 elements⁹⁰. Indeed, it has been proven that Zn presence can limit Cd absorption for *E. fetida*⁹¹, but
370 also Cd compete with Pb in *Aporrectodea caliginosa*⁹² and other organisms like collembolan⁹³.
371 Furthermore, Cd has a higher affinity than other elements for metallothioneine which could lead to a
372 more efficient earthworm detoxification^{91,94}.

373 For lettuces, CNT addition in LC soil led to increasing nutrient and metal (Cd and Zn, only)
374 concentrations in leaves at the highest CNT concentration. The low organic matter content is rather a
375 favorable factor for nanomaterial transfer to plants⁷². CNTs would adsorb elements at their surface
376 and be taken up by plant roots leading to a higher accumulation of elements in lettuce leaves⁹⁵.
377 Alternatively, CNTs could also damage root epidermis and led to enhanced elemental entry⁹⁶. On the
378 other hand, a very different effect was seen for HC soil. Indeed, in this soil, richer in organic matter
379 (on average 1.7 richer), no elemental concentration increase was detected in leaves, this is probably
380 due to the fact that complex interactions between CNTs and organic matter took place in this soil
381 making CNTs much less bioavailable for plants⁷². Furthermore, a significant decrease in metal
382 concentrations was seen in plants exposed to HC 0.1. These results might be explained by the
383 adsorption of metals on the surface of the CNTs according to the CNT concentration in the medium.
384 Indeed, at low concentrations in the medium (0.1 mg kg⁻¹), CNTs tend to be better dispersed in the
385 soil than at higher concentration (10 mg kg⁻¹)^{97,98}. Thus, at 0.1 mg kg⁻¹, exchange surface of CNTs was
386 more important because less agglomerated and therefore increased the adsorption of other
387 compounds such as metals making them less bioavailable for the plants. Likewise, in their study,
388 Zhang *et al.* (2014) observed that the agglomeration of CNTs reduced the adsorption of some organic
389 compounds⁹⁷. The reduction in elemental bioavailability induced by CNTs could, in the long term, be
390 detrimental to the proper development of crop plant or reduce their nutritional values. Indeed, here,
391 this decrease in elemental concentrations in leaves was correlated with a 22% decrease in lettuce
392 biomass.

393

394 The results obtained in this study carried out in microcosms, over a relevant duration representing
395 the life cycle of earthworms and the production of consumable biomass in plants (lettuce leaves),
396 showed that the co-contamination of CNTs and metals did not lead to acute toxicity. However, stress
397 symptoms were observed (delayed development in earthworms, increase in stress marker molecules

398 and slowdown in biomass production in lettuce). Furthermore, this study revealed that the addition
399 of CNTs to a previously contaminated soil can modify the soil-to-organism transfer of elements
400 present in the soil matrix. The results also demonstrated both synergistic and antagonist effects for
401 earthworms and lettuce. It is important to mention that it relies on several parameters: soil physico-
402 chemical parameters (in particular organic matter content), the element considered (and its affinity
403 for both soil and CNTs) as well as the organisms (including at the species level⁷¹) and their exposure
404 pathways (oral ingestion, contact, root uptake) making the prediction of their transfer and long-term
405 toxicity difficult. Finally, the interactions between these organisms are tight; consequently, the
406 impact on a single one of these organisms can lead to cascade reactions and have consequences on
407 the functioning of the entire agrosystem and the ecological services it provides. Given that CNT
408 introduction in the environment is likely to increase in the future, further studies are urgently
409 needed to understand the mechanisms of interactions between CNTs and other contaminants and
410 their impacts on organisms and the associated risks for food safety in order to guarantee their
411 rational use. In the meanwhile, their intentional introduction in both crop systems and recreational
412 gardens should be avoided as it could increase the risk of trace element uptake by humans through
413 consumption of edible parts of crops, possibly leading to health impact if consumed in large amount.

414

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