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The downside of copper pesticides: An earthworm's perspective

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Abstract

The widespread use of copper-based pesticides, while effective in controlling plant diseases, has been identified as a major source of copper contamination in soils. This raises concerns about potential adverse effects on earthworms, key players in soil health and ecosystem function. To inform sustainable pesticide practices, this study aimed to establish copper toxicity thresholds for earthworm avoidance in agricultural soils impacted by copper-based pesticides. We collected 40 topsoil samples (0-5 cm) from orchards and vineyards in the O'Higgins Region of central Chile, and 10 additional soils under native vegetation as background references. A standardized avoidance bioassay using *Eisenia fetida* assessed the impact of copper-based pesticides on the soils. Total copper concentrations ranged between 23 and 566 mg kg⁻¹, with observed toxic effects on earthworms in certain soils. The effective

26 concentration at 50% (EC₅₀) for total soil copper, determined by *Eisenia fetida*'s avoidance response,
27 was 240 mg kg⁻¹, with a 95% confidence interval of 193-341 mg kg⁻¹. We further compared our EC₅₀
28 values with existing data from agricultural soils impacted by mining activities. Interestingly, the results
29 revealed a remarkable similarity between the thresholds for earthworm avoidance, regardless of the
30 source of copper contamination. This observation underscores the universality of copper toxicity in
31 agricultural ecosystems and its potential impact on soil biota. This study provides novel insights into
32 copper toxicity thresholds for earthworms in real-world, pesticide-contaminated soils.

33

34 **Keywords:** Lumbricidae; bioavailability; ecotoxicity; heavy metals; orchards; field-contaminated soil.

35

36 **Introduction**

37

38 Copper-based pesticides have been identified as a significant contributor to soil contamination by
39 copper, as documented in several studies (Komárek et al. 2010; Brunetto et al. 2016; Tóth et al. 2016;
40 Schoffer et al. 2020). These pesticides have been utilized in agriculture for over a century to control
41 bacterial and fungal diseases in crops, since Pierre Millardet's serendipitous discovery of the Bordeaux
42 mixture, which is composed of CuSO₄+Ca(OH)₂ (Johnson 1935), in 1880. Since then, a plethora of
43 other copper-based pesticides have been introduced and widely adopted in agriculture.

44

45 Copper-based pesticides are frequently administered via foliar spraying, yet a substantial fraction of
46 these compounds may become weakly bound to plant surfaces (Pérez-Rodríguez et al. 2016),
47 ultimately leading to their migration into the soil. Our investigations have demonstrated that leaf litter
48 can serve as a safeguard against the leaching of copper-containing pesticides into the mineral soil
49 horizon (Schoffer et al. 2021). Notwithstanding this finding, the issue of high copper levels in vineyard
50 and orchard soils remains prevalent on a global scale (Schoffer et al. 2020).

51

52 The majority of ecotoxicological studies for metal toxicity analysis use artificially contaminated
53 (metal-spiked) soils (review of Nahmani et al. 2007a), while investigations utilizing real-world
54 contaminated soils remain limited (review of Santa-Cruz et al. 2021b). However, multiple
55 environmental toxicology experts have emphasized that the toxicity of metals is dependent on the
56 length of time they remain in the soil, known as “aging”, and is not accurately reflected in metal-spiked
57 soils (Spurgeon and Hopkin 1995; Nahmani et al. 2007b; McBride and Cai 2016; Zeng et al. 2017).
58 Moreover, studies have consistently shown higher metal toxicity to soil biota in artificially
59 contaminated soils compared to actual soils exposed to pollution a few decades ago (Santa-Cruz et al.
60 2021a). Therefore, the traditional approach to analyzing metal toxicity in soils, based on metal
61 enrichment, has become outdated.

62

63 The rarity of monometallic contaminated sites (Dovletyarova et al. 2023) further complicates the
64 identification of metal-specific toxicity effects in real-world contaminated soils (Yáñez et al. 2022).
65 Therefore, the identification of soil environments contaminated predominantly with a single metal,
66 such as agricultural soils impacted by copper-based pesticides, represents an opportunity to investigate
67 the ecotoxicological impacts of that specific metal on soil biota.

68

69 It is worth noting that invertebrates are more vulnerable to copper contamination than plants. A review
70 by Santa-Cruz et al. (2021a) found that the effective concentrations at 50% (EC₅₀) of total copper in
71 soil for invertebrates in real-world contaminated soils were significantly lower than those for plants,
72 with values of $303 \pm 108 \text{ mg kg}^{-1}$ and $987 \pm 491 \text{ mg kg}^{-1}$, respectively.

73

74 It is also noteworthy that the scientific literature on anthropogenic soil contamination, particularly with
75 regards to copper, is sparse and lacks robust studies that have established threshold values using

76 earthworms as bioindicators. We are aware of merely six studies that have estimated copper toxicity
77 thresholds for earthworms using real-world contaminated soils (Table 1). Due to the limited number of
78 scenarios examined in prior studies, it is challenging to draw generalizable conclusions regarding the
79 effect of copper contamination on soil biota (Neaman and Yáñez 2023). Therefore, further research on
80 this topic is both novel and relevant.

81

82 Our previous study (Ginocchio et al. 2009) demonstrated that copper solubility differed significantly in
83 soils polluted by different sources, including smelter dust, tailings, smelter slag, copper concentrate,
84 and copper ore. However, a review of the six studies that estimated copper toxicity thresholds for
85 earthworms in real-world contaminated soils (Table 1), reveals a glaring gap: only one study focused
86 specifically on agricultural soils contaminated by copper-based pesticides (Van Zwieten et al. 2004).
87 The other studies focused on soils contaminated by industrial activities, such as mining and timber
88 treatment. This raises a crucial question: are copper toxicity thresholds for earthworms similar across
89 different pollution sources, namely copper-based pesticides and industrial activities? To address this
90 knowledge gap, this investigation had two objectives: (1) to establish copper toxicity thresholds for
91 earthworms inhabiting agricultural soils impacted by copper-based pesticides, and (2) to compare these
92 thresholds with those established in agricultural soils impacted by industrial activities.

93

94 **Materials and Methods**

95

96 *Selection of earthworm species and bioassay type*

97 The earthworm species *Eisenia fetida* is a well-established bioindicator organism for assessing toxicity
98 levels of various metals (Nahmani et al. 2007b). A recent review of studies estimating metal toxicity
99 thresholds for earthworms in real-world contaminated soils (Santa-Cruz et al. 2021a) revealed its
100 widespread use. While several studies employed other species (*Eisenia andrei* (Posthuma and

101 Notenboom 1996; Luo et al. 2014), *Lumbricus rubellus* (Spurgeon et al. 2005), *Aporrectodea*
102 *caliginosa* (Nahmani and Lavelle 2002), and *Aporrectodea tuberculata* (Mirmonsef et al. 2017)), the
103 majority relied on *Eisenia fetida* (Spurgeon and Hopkin 1995, 1996; Scott-Fordsmand et al. 2000; Van
104 Zwieten et al. 2004; Nahmani et al. 2007b; Mertens and Smolders 2013; Bustos et al. 2015; Delgadillo
105 et al. 2017).

106

107 Duque et al. (2023) demonstrated that *Eisenia fetida* is less sensitive to copper than several other
108 species (*Lumbricus rubellus*, *Aporrectodea longa*, *Lumbricus terrestris*, and *Allolobophora chlorotica*,
109 while two other species, *Aporrectodea caliginosa* and *Aporrectodea*, are less sensitive to copper than
110 *Eisenia fetida*). However, its ease of culture under laboratory conditions (OECD 2016), rapid sexual
111 maturity, and high reproductive rate make it a valuable and convenient choice for copper toxicity
112 testing, despite the differences in sensitivity compared to some other species.

113

114 Earthworm avoidance behavior provides a sensitive, non-lethal, and easily quantifiable endpoint for
115 assessing metal toxicity. This endpoint is standardized in the ISO-17527-1 protocol (ISO-17527-1
116 2008) and has been shown to be effective in numerous studies (e.g., Da Luz et al. 2004; Lukkari et al.
117 2005; Loureiro et al. 2005). A recent review by Alcívar et al. (2021) further confirms the high
118 sensitivity of the avoidance test and its comparability with more traditional endpoints such as growth,
119 reproduction, physiological changes, feeding, mortality and others for the assessment of copper
120 toxicity.

121

122 *Soil sampling and characterization*

123 It was deemed necessary to limit the extent of our research to a specific area in order to effectively
124 address our research objectives. The O'Higgins Region, located in central Chile (Fig. 1), was selected
125 due to its significance in fruit production in Chile (ODEPA-CIREN 2021). It is important to note that

126 the Cachapoal River basin in the O'Higgins Region is impacted by copper mining activities,
127 specifically, the Caletones copper smelter and the El Teniente copper mining complex (Fig. 1). The
128 presence of these mining operations has been shown to result in an increase in the concentrations of
129 several metals and metalloids in the agricultural soils of the Cachapoal River basin (Ginocchio et al.
130 2002). Since the present study attempted to search for soils predominantly contaminated with copper,
131 we deliberately chose an area that was devoid of any such mining activities (Fig. 1).

132

133 Forty topsoil samples (0-5 cm) were collected from orchards and vineyards (Supplementary Fig. S1),
134 and an additional 10 soil samples from areas covered by native vegetation. The geographical locations
135 of the soil samples studied are listed in Supplementary Table 1. All soils studied were classified as
136 Entisols (Soil Survey Staff 2022).

137

138 In accordance with the ISO-19258 (ISO-19258 2018) protocol, the term “background concentration”
139 refers to the “concentration of an element or a substance characteristic of a soil type in an area or
140 region arising from both natural sources and anthropogenic diffuse sources such as atmospheric
141 deposition”. Given that the soil samples collected from areas covered by native vegetation were not
142 impacted by any point source of anthropogenic activities, they will be referred to as “background soils”
143 in the following discussion, in accordance with the definition provided in the ISO-19258 (ISO-19258
144 2018) protocol.

145

146 Soil samples were dried in an oven at 40°C. Subsequently, the dried samples were ground and passed
147 through a 2 mm mesh to obtain a homogenized mixture. The general physical and chemical
148 characteristics of the soil were determined using standard analytical methods (Table 2, Supplementary
149 Table 2). The soil texture was assessed using the simplified hydrometer method (Sheldrick and Wang
150 1993). The organic carbon content was quantified by wet combustion with Na-dichromate and sulfuric

151 acid, without the application of heat, while the organic matter content was estimated using the
152 conversion factor of 1.724 (Sadzawka et al. 2006). Finally, the soil pH and electrical conductivity were
153 measured in an aqueous suspension at a soil-to-water ratio of 1:5 (Sadzawka et al. 2006).

154

155 To assess potential metal and metalloid contamination from agricultural practices, the research team
156 analyzed soil samples for several trace elements. To determine total element concentrations, samples
157 were digested for 12 h in boiling nitric acid, followed by perchloric acid addition. To prevent arsenic
158 volatilization during the digestion process, a Teflon stopper with a 30 cm glass reflux tube was used
159 (Sadzawka et al. 2015). The digested samples were analyzed for trace elements using atomic absorption
160 spectroscopy. In the case of arsenic, the spectrophotometer was coupled with a hydride vapor
161 generator. The Wageningen University (ISE 859) standard reference material was used throughout,
162 yielding recovery values (i.e., certified value \times 100% / obtained value) of 101%, 97%, 87%, 99%, and
163 106% for arsenic, cadmium, copper, lead, and zinc, respectively. To further ensure accuracy, duplicate
164 measurements were performed on every tenth sample, and blank measurements were also performed,
165 which consistently yielded results below the detection limit.

166

167 In this study, we did not measure soluble metal concentrations because our previous studies (Delgadillo
168 et al. 2017; Bustos et al., 2015) showed that soluble metal concentrations are not a good proxy for
169 metal bioavailability and toxicity to *Eisenia fetida* in soils of central Chile.

170

171 *Earthworm avoidance bioassay*

172 The earthworms utilized in the study were procured from a reputable earthworm farm
173 (www.espacioelpuente.cl), where they were nourished with the residuals of fruit and vegetables. The
174 species *Eisenia fetida* was subjected to a conditioning phase of approximately one month within an
175 environmental chamber maintained at a temperature of 20 ± 2 °C and a constant light intensity ranging

176 from 400–800 lx, with a photoperiod of 12 h of daylight and 12 h of darkness. The breeding medium
177 utilized during this phase consisted of an equal proportion of horse manure and peat.

178

179 The earthworm avoidance bioassay was executed in accordance with the standardized protocol outlined
180 in ISO-17527-1 (ISO-17527-1 2008), with a modification. It is important to note that prior
181 reconnaissance experiments had revealed that earthworm avoidance was influenced not only by the
182 presence of toxic substances but also by the soil organic matter content and electrical conductivity
183 (Delgadillo et al. 2017). Thus, the organic matter content and electrical conductivity of the artificial
184 substrate were adjusted to match the properties of the field-collected soil by incorporating peat and
185 NaCl solution, respectively. The field-collected soils utilized in the study remained unaltered.

186

187 By adjusting the physicochemical properties of the artificial substrate, potential interferences from
188 factors such as organic matter and electrical conductivity were mitigated, thus enhancing the accuracy
189 of the toxicity threshold determination. Despite the relatively narrow range of pH values observed in
190 the studied soils (6.4 ± 0.71) and a similar pH value in the artificial substrate (6.4), pH was not deemed
191 a confounding variable in this study. Nevertheless, further investigations are required to assess the
192 efficacy of controlling the pH of the artificial substrate when testing soils with a wider pH range.

193

194 Specifically, for the earthworm avoidance bioassay, one-liter plastic conic pots wrapped in aluminum
195 foil (11 cm lower diameter \times 12 cm in height \times 15 cm upper diameter) were utilized. The pots were
196 partitioned by a vertical separator, equally dividing the container into two sections. One half of the pot
197 was filled with the soil under study, while the other half was filled with an artificial substrate consisting
198 of peat, kaolinite, and quartz sand (ISO-17527-1 2008). Both the field soils and the artificial substrate
199 were moistened with deionized water to 60% of their water holding capacity one day prior to the start

200 of the bioassay. Subsequently, the separator was removed, and 10 earthworms with a well-developed
201 clitellum and a fresh weight in the range of 0.3-0.5 g were placed on the dividing line of each test pot.

202

203 The incubation period was set to 48 h, during which earthworms were allowed to migrate between the
204 soil under study and the artificial control substrate. This test facilitated the evaluation of each soil's
205 suitability as a potential habitat for earthworms. This procedure was performed in 4 replicates for each
206 of the 40 agricultural and 10 background soils.

207

208 *Statistical analysis*

209 The effective concentrations of the metals at 25% and 50% (EC₂₅ and EC₅₀) were calculated using the
210 RegTox macro for Microsoft ExcelTM (version 7.0.7, © Eric Vindimian, 2001). The EC₁₀ value was not
211 taken into consideration as it often reflects negligible to minimal effects and typically falls within the
212 range of the control response variability (Checkai et al. 2014). To compute the effective concentration
213 values, a control response of 100% was established as the mean number of earthworms present in the
214 background soils. The earthworm response was evaluated as the number of earthworms present in the
215 tested soils, expressed as a percentage of the mean number of earthworms present in the background
216 soils (Supplementary Table 2).

217

218 **Results and Discussion**

219 As expected, the use of copper-based pesticide resulted in significantly higher copper levels in
220 agricultural soils compared to background soil. Increased copper content in soils may cause an increase
221 in soil organic matter content due to inhibition of organic matter decomposition (Sauvé 2006).
222 However, in our set of soils, there was no statistically significant relationship between copper content
223 and soil organic matter content ($p>0.05$). Therefore, interactions between copper and soil organic
224 matter were not considered in this study.

225

226 Interestingly, statistically significant ($p < 0.05$) increases were also observed for zinc, arsenic, and lead
227 in agricultural soils compared to background soil, suggesting potential co-contamination. Cadmium
228 levels, however, remained statistically similar ($p > 0.05$) between agricultural and background soils
229 (Table 2). Our previous review (Santa-Cruz et al. 2021a) summarized earthworm EC_{50} values for
230 copper, zinc, arsenic, and lead in real-world contaminated soils. These EC_{50} values are presented in
231 Table 2, along with metal concentrations in the studied soils. While total zinc and lead concentrations
232 remained below their respective thresholds (Table 2), total arsenic concentrations ranged from 3.6 to 23
233 $mg\ kg^{-1}$, slightly exceeding the EC_{50} value of $22\ mg\ kg^{-1}$. However, the absence of a clear toxicity
234 pattern in Supplementary Fig. S2 suggests that zinc, arsenic, and lead may not pose a significant threat
235 to earthworms in these soils. In stark contrast, the wide range of total copper concentrations (23-566
236 $mg\ kg^{-1}$) significantly exceeded the established EC_{50} value of $282\ mg\ kg^{-1}$ (Santa-Cruz et al. 2021a).
237 This indicates a high probability of copper-induced toxicity impacting earthworm populations in the
238 studied soils.

239

240 Indeed, Fig. 2 reveals a clear pattern of copper toxicity. The EC_{25} value for the avoidance response of
241 *E. fetida* to the total soil copper was $115\ mg\ kg^{-1}$, with a 95% confidence interval of $95-149\ mg\ kg^{-1}$,
242 while the EC_{50} value for the avoidance response of *E. fetida* to the total soil copper was $240\ mg\ kg^{-1}$,
243 with a 95% confidence interval of $193-341\ mg\ kg^{-1}$.

244

245 The EC_{50} value obtained in this study is remarkably consistent with those reported by other researchers
246 using the earthworm avoidance bioassay (Table 3). This supports the validity of our findings.
247 Specifically, Delgadillo et al. (2017) observed an EC_{50} of $213\ mg\ kg^{-1}$ (with a 95% confidence interval
248 of $172-253\ mg\ kg^{-1}$) in soils contaminated by copper mining activities (Table 3), while Van Zwieten et
249 al. (2004) found an EC_{50} of $236\ mg\ kg^{-1}$ (without reporting confidence intervals) in soils impacted by

250 copper-based pesticides. These results suggest that copper toxicity thresholds for earthworm avoidance
251 are consistent across different pollution sources, including both mining and agricultural practices.

252

253 Furthermore, a review by Santa-Cruz et al. (2021a) indicated that the EC₅₀ of total copper content in
254 anthropogenically contaminated soils was $282 \pm 113 \text{ mg kg}^{-1}$ for various earthworm response variables
255 (reproduction, neutral-red retention time, and avoidance), which is similar to the value obtained in the
256 present study.

257

258 Our results are corroborated by similar values obtained in a Danish field study (Holmstrup and Hornum
259 2012), in which the only soil contaminant present was copper. The study found that earthworm biomass
260 and population density decreased when the total soil copper content exceeded 300 mg kg^{-1} . Both the
261 Danish study and the current study analyzed soils in which the metal has been present for several
262 decades. The consistency of our results with previous studies provides robust support for the validity of
263 our findings.

264

265 While laboratory ecotoxicology studies – such as this one – provide valuable insights, caution should
266 be taken when extrapolating their results directly to natural ecosystems (Forbes et al. 2019). This is
267 particularly true for mobile organisms, such as earthworms, as laboratory settings limit their ability to
268 escape contaminated environments (Alcívar et al. 2021). However, avoidance response assays provide
269 a good proxy for natural conditions, in which mobile organisms can actively avoid unfavorable habitats
270 (Jutfelt et al. 2017).

271

272 Although copper-based pesticides effectively control crop diseases, this study suggests potential
273 adverse effects on earthworm populations in vineyards and orchards. Specifically, our results suggest
274 that earthworm biomass and population density may decrease when total soil copper content exceeds

275 ~250 mg kg⁻¹. However, future field studies similar to the field study of Holmstrup and Hornum (2012)
276 are needed to confirm this assumption. A decline in earthworm biomass and population density may
277 lead to long-term degradation of soil ecological properties and agricultural productivity. Indeed,
278 earthworms play an important role in the ecosystem (Latif et al. 2017), contributing to various
279 processes, such as soil structure formation and nutrient cycling (Blouin et al. 2013)

280

281 **Conclusion**

282 This study establishes copper toxicity thresholds for earthworm avoidance in agricultural soils
283 impacted by copper-based pesticides. Notably, these thresholds were comparable to those observed in
284 agricultural soils impacted by mining activities. This finding suggests a potentially universal effect of
285 copper contamination on earthworm behavior across different sources of contamination, namely
286 copper-based pesticides and mining activities.

287

288 The novel contribution of this study is underscored by the fact that only a handful of previous studies
289 have estimated copper toxicity thresholds for earthworms in real-world contaminated soils. As such,
290 this study provides valuable insights into the enduring impact of copper-based pesticides on
291 earthworms in agricultural soils, and represents an important advancement in our understanding of this
292 critical issue.

293

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297

298 **Author contribution**

299 Jorge Tomás Schoffer: investigation, methodology, data curation, formal analysis, writing, funding
300 acquisition; Fiama Solari: investigation; Lucas Petit-dit-Grézériat: investigation, validation; Céline
301 Pelosi: validation, funding acquisition; Rosanna Ginocchio: validation, recourses, funding acquisition;
302 Carolina Yáñez: conceptualization, validation, writing; Pilar Mazuela: validation, recourses; Alexander
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309

310 **Data availability**

311 The data is available in the Supplementary Table 2.

312

313 **Declarations**

314 Ethical approval: Not applicable.

315 Consent to participate: Not applicable.

316 Consent for publication: Not applicable.

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

319

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456

457 **Table 1.** The effective concentration at 50% (EC₅₀) of copper that causes a toxic effect in earthworms. We focused on studies that used
 458 anthropogenically contaminated soils rather than artificially contaminated (metal-spiked) soils.

Study	Soil origin	Copper source	Species tested	Test duration (days)	Endpoint	Total Cu EC ₅₀ (mg kg ⁻¹)
Scott-Fordsmand et al. (2000)	Denmark	Timber treatment (CuSO ₄)	<i>Eisenia fetida</i>	21	Reproduction	517
				21	NRR-time	163
Van Zwieten et al. (2004)	Australia	Copper-based pesticides	<i>Eisenia fetida</i>	2	Avoidance	236*
Maraldo et al. (2006)	Denmark	Timber treatment (CuSO ₄)	<i>Enchytraeus crypticus</i>	14	Reproduction	439
Konečný et al. (2014)	Zambia	Mining activities	<i>Enchytraeus crypticus</i>	28	Reproduction	351
Delgadillo et al. (2017)	Chile	Mining activities	<i>Eisenia fetida</i>	2	Avoidance	213
Mirmonsef et al. (2017)	Denmark	Timber treatment (CuSO ₄)	<i>Aporrectodea tuberculata</i>	21	Reproduction	220

459 NRR-time = neutral-red retention time

460 * data for farm 2 vs. farm 2 control

461 **Table 2.** General physicochemical properties of the background and agricultural soils under study.
 462 Effective concentration at 50% (EC₅₀) values for total metal contents are also shown for invertebrate
 463 responses in real-world contaminated soils, based on the review of Santa-Cruz et al. (2021). An EC₅₀
 464 value was not available for cadmium.

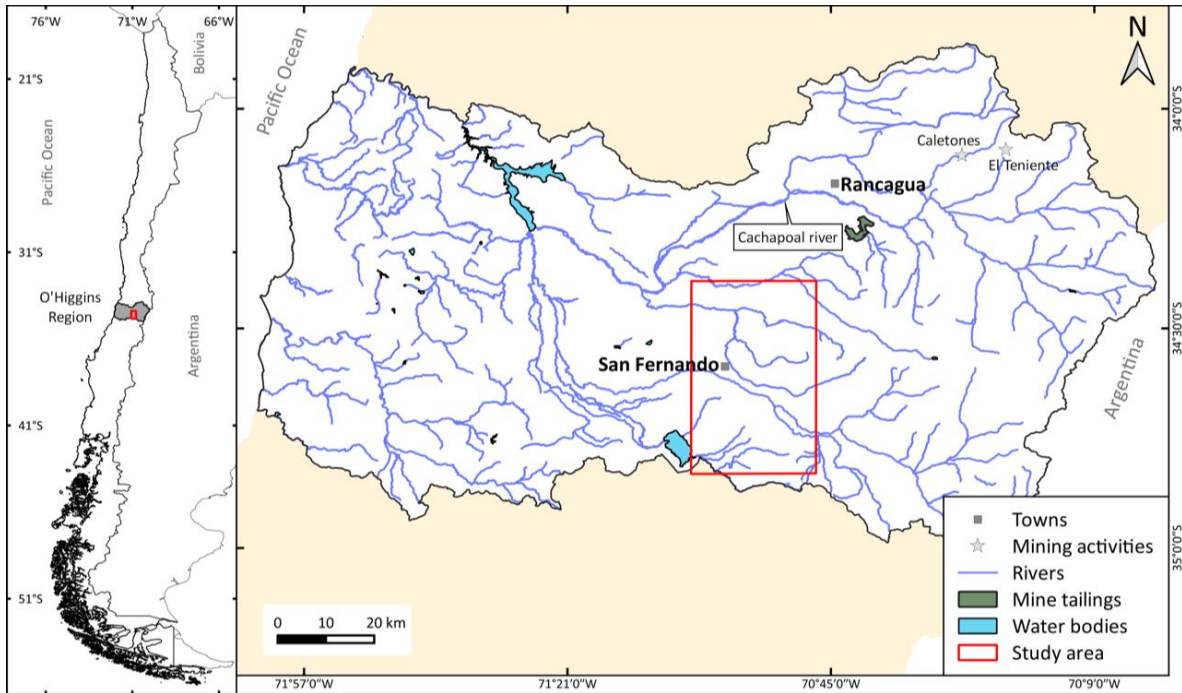
Soil property	Unit	Background soils (n = 10)		Agricultural soils (n = 40)		EC ₅₀ , mg kg ⁻¹
		Median (range)	Mean ± SD	Median (range)	Mean ± SD	
EC, water 1:5	dS m ⁻¹	0.12 (0.06-0.94)	0.23 ± 0.26	0.17 (0.06-0.82)	0.21 ± 0.14	–
pH, water 1:5	–	6.5 (5.9-8.3)	6.6 ± 0.71	6.3 (5.0-7.8)	6.4 ± 0.71	–
Organic matter*	%	4.3 (1.8-17)	5.9 ± 4.0	3.4 (2.1-5.8)	3.6 ± 1.0	–
Sand	%	36 (24-45)	35 ± 7.5	27 (12-47)	29 ± 8.8	–
Clay*	%	13 (10-25)	14 ± 4.3	20 (8-28)	19 ± 4.7	–
Silt	%	50 (43-62)	52 ± 6.4	54 (36-70)	53 ± 7.3	–
Total Cu*	mg kg ⁻¹	38 (23-56)	37 ± 9.5	118 (24-566)	146 ± 114	282
Total Zn*	mg kg ⁻¹	63 (38-98)	67 ± 19	104 (41-265)	136 ± 59	1912
Total As*	mg kg ⁻¹	9.5 (3.6-16)	9.87 ± 4.7	14 (8-23)	14 ± 4.1	22
Total Pb*	mg kg ⁻¹	36 (28-44)	36 ± 4.4	39 (26-155)	41 ± 19	1092
Total Cd	mg kg ⁻¹	1.7 (1.4–2.2)	1.8 ± 0.24	2.0 (0.74-6.6)	2.1 ± 0.86	n.a.

465 * denotes statistically significant differences between background and agricultural soils as determined
 466 by a Kruskal-Wallis test ($p \leq 0.05$). SD denotes the standard deviation. The values of pH and electrical
 467 conductivity (EC) were measured in water extracts at a soil:water ratio of 1:5.
 468 – denotes “not applicable”, whereas n.a. mean “not available”.

469 **Table 3.** The effective concentrations at 25% and 50% (EC₂₅ and EC₅₀) of soil total Cu that resulted in
 470 avoidance behavior in *E. fetida* were determined. Values in parentheses refer to the 95% confidence
 471 intervals. The study of Van Zwieten et al. (2004) did not report confidence intervals.
 472

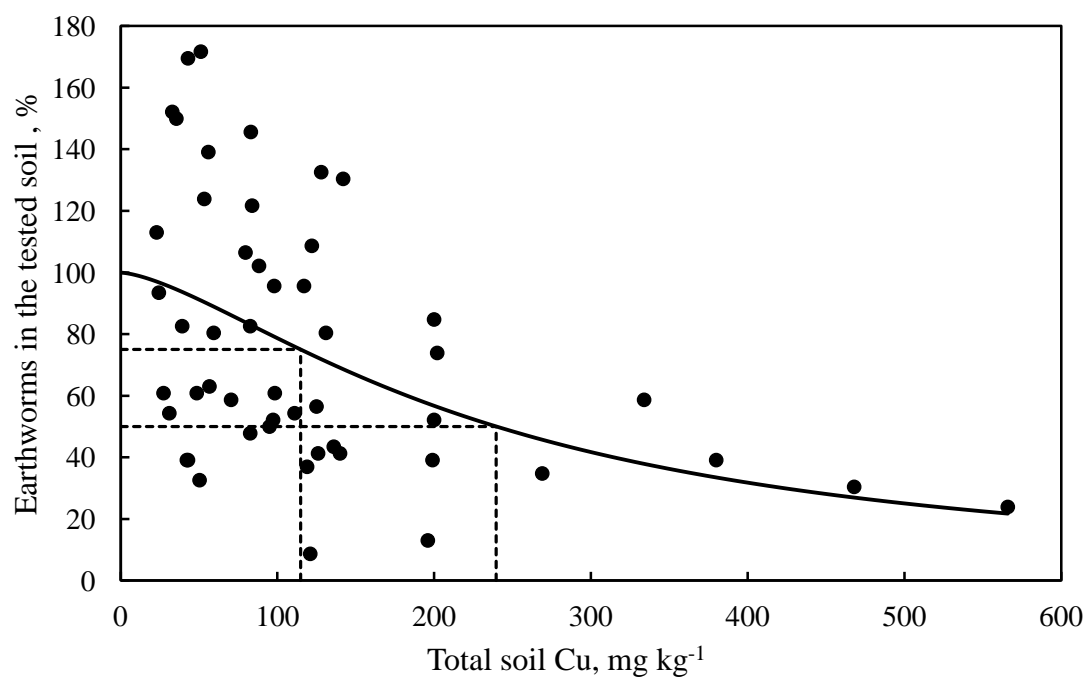
Study	Soil origin	Copper source	Total copper (mg kg ⁻¹)	
			EC ₂₅	EC ₅₀
This study	O'Higgins Region, Chile	Copper-based pesticides	115 (95-149)	240 (193-341)
Delgadillo et al. (2017)	Valparaíso Region, Chile	Copper mining activities	nr	213 (172-253)
Van Zwieten et al. (2004)	Australia	Copper-based pesticides	nr	236

473 nr = not reported.



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Fig. 1. General location of the study area in the O'Higgins Region, central Chile



477
 478 **Fig. 2.** *Eisenia fetida* response as a function of total soil Cu. The earthworm response
 479 determined as the number of earthworms present in the tested soils, expressed as a
 480 percentage of the mean number of earthworms present in the background soils. The
 481 effective concentrations at 25% and 50% (EC₂₅ and EC₅₀) are also shown.