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Assessing toxicity of metal contaminated soil from glassworks sites with a battery of biotests

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Abstract

The present study addresses toxicological properties of metal contaminated soils, using glassworks sites in south-eastern Sweden as study objects. Soil from five selected glassworks sites as well as from nearby reference areas were analyzed for total and water-soluble metal concentrations and general geochemical parameters. A battery of biotests was then applied to assess the toxicity of the glassworks soil environments: a test of phytotoxicity with garden cress (*Lepidium sativum*); the BioToxTM test for toxicity to bacteria using *Vibrio fischeri*; and analyses of abundancies and biomass of nematodes and enchytraeids. The glassworks- and reference areas were comparable with respect to pH and the content of organic matter and nutrients (C, N, P), but total metal concentrations (Pb, As, Ba, Cd and Zn) were significantly higher at the former sites. Higher metal concentrations in the water-soluble fraction were also observed, even though these concentrations were low compared to the total ones. Nevertheless, toxicity of the glassworks soils was not detected by the two *ex situ* tests; inhibition of light emission by *V. fischeri* could not be seen, nor was an effect seen on the growth of *L. sativum*. A decrease in enchytraeid and nematode abundance and biomass was, however, observed for the landfill soils as compared to reference soils, implying *in situ* toxicity to soil-inhabiting organisms. The confirmation of *in situ* bioavailability and negative effects motivates additional studies of the risk posed to humans of the glassworks villages.

Keywords: metal contamination, glassworks sites, enchytraeids, biotests, bioavailability

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Introduction

There are many sites that have been contaminated by metals released from past industrial activities, and metal contamination threatens the well-being of all components of the biosphere. Even though it is well known that high total concentrations may not always translate into high mobility, bioavailability and toxicity (Kördel et al. 2013; McLaughlin et al. 2000), risks at contaminated sites are often assessed based on analyses of total concentrations, sometimes complemented with weaker extractions that dissolve only the potentially bioavailable fraction. However, the potential for environmental hazards is better understood when chemical analyses are complemented with biotests, as organisms are only sensitive to the truly bioavailable fraction of metals (Garcia-Lorenzo et al. 2009; Römbke et al. 2005; Karjalainen et al. 2009). Biological tests could also integrate the effects of mixtures and their bioavailability and therefore provide a useful tool for site-specific assessment of actual ecological risks.

The long-lasting production of glass in south-eastern Sweden is one example of industrial activity, where the local soil environment has become severely contaminated over time, and where adverse health effects are now seen among local residents. Better understanding of soil toxicity properties is thus highly relevant at the Swedish glassworks sites. The main contamination occurred during the 1970s and earlier, when unsorted waste and crushed glass were thrown in a pile near the glassworks (Falk et al. 2005). A compilation of data from previous site investigations, available from the Kalmar and Kronoberg County Administrative Boards (2016), reveals maximum total concentrations in glassworks soils of the region (or rather soil with a varying mix of glass waste) of 16 900 mg kg⁻¹ Pb, 180 mg kg⁻¹ Cd and 2600 mg kg⁻¹ As. It has also been shown that soils of private gardens around the glassworks may contain metal concentrations of the same magnitude as the glassworks properties, that there is a positive correlation between metal contamination and metal concentration in homegrown vegetables, and that consumption of these vegetables is a risk factor (Augustsson et al. 2015; Uddh-Söderberg et al. 2015). Recent findings also imply that residents living near glassworks in the area are at an increased risk of developing cancer (Nyqvist et al. 2017).

When turning from soil contamination to toxicology, a battery of toxicity tests with species of varying sensitivities and exposure pathways is recommended (Karjalainen et al. 2009). The suitability of biotests, such as the Phytotoxkit (to test plants) and Microtox®/BioTox™ (bacterial

test), in the assessment of toxicity of bottom sediments, composts, sewage sludge, and for example mining activity contaminated soils, has been proven in several studies (Boularbah et al. 2006a, b; Czerniawska-Kusza and Kusza 2011; Mamindy-Pajany et al. 2011; Loureiro et al. 2005; Dubova and Zarina 2004). Plants are essential primary food producers of ecosystems and thus it is important to identify the magnitude of the toxic effects on plants (Garcia-Lorenzo et al. 2009). Also, bacteria play a crucial role, being decomposers in the environment (Kahru et al. 2005). Other key organisms are enchytraeids and nematodes (Didden and Römbke 2001). Especially enchytraeids are sensitive to environmental stresses and the presence and species composition of enchytraeid worms have therefore been suggested for use as indicators of metal toxicity (Kapusta and Sobczyk 2015).

The aim of this study was to evaluate toxicity of glasswork contaminated soil. It was done by two *ex situ* tests: using a) a test of phytotoxicity with garden cress (*Lepidium sativum*), and b) the BioTox™ test for toxicity to bacteria using the bioluminescent bacterium *Vibrio fischeri* as test organism. A bioassay was also performed by measuring the abundancies of soil-inhabiting nematodes and enchytraeids. In contrast to standardised laboratory tests, the latter approach reflects the *in situ* situation of the soil animals and the effect of their exposure to the contaminant metals.

Materials and methods

Study region

Five typical glassworks sites in the glassworks region of south-eastern Sweden were selected for the present study: Johansfors, Bergdala, Kosta, Orrefors and Målerås (Fig. 1). Production at all these sites has included artistic and crystal glass. The major raw materials in the glass production were silica quartz, calcite, feldspar and oxides of several metals, such as As, Cd, Co, Cr, Cu, Ni, Pb, Sb and Zn (Hermelin and Welandar 1986; Magnusson 1971; Månsson and Carlsson 2002). The volume of contaminated soil and landfill materials at the sites varies from a few thousand cubic metres to approximately 100 000 m³, with high concentrations in particular of Pb and As (Fanger et al. 2004; Bergelin et al. 2006; Håkansson and Ländell 2006; Werkelin and Gustavsson 2006). Production is still running in Bergdala, Kosta, Målerås and Johansfors, but only on a small scale at the latter. The factory in Orrefors was closed down in 2013. However, most of the landfills were decommissioned in the late 1970s and have since been untouched while natural vegetation has been established and soils formed. The dominating tree species of the region are spruce and pine, and the natural soils typically show a podzolized profile. The quaternary deposits are dominated by sandy tills with a

102 mineralogy that reflects the local granitic bedrock. One particularly relevant feature in this area is
 103 the relatively high geogenic concentrations of Pb (SGU 2014). Mean January and July temperatures
 104 in the study area are -2.0°C and 17.0°C , respectively, and the mean annual precipitation is
 105 approximately 700 mm (based on data from 2006 to 2015; SMHI 2016).
 106

107
 108 Fig. 1. Location of the five glassworks sites in southeastern Sweden.

109
 110 *Sampling and chemical analyses of soils*

111
 112 Soil samples were collected in October 2015. At each of the five selected glassworks sites, samples
 113 were taken both from the main landfill and from a nearby (within a few hundred metres) reference
 114 area. The reference area of each glassworks site was selected on-site based on the criteria: 1) similar
 115 vegetation characteristics i.e. type of ground cover and dominating tree species, and 2) similar
 116 natural soil characteristics (based on assessment in the field, e.g. a rough estimate of soil grain size,
 117 colour, approximate content of organic matter (OM) etc.). At each study site, five samples
 118 (approximately 5 L each) were collected from the landfill area, and five were taken from the
 119 reference area. This gave in total 50 samples, each taken from a unique hand dug pit as a composite
 120 sample from 0–20 cm depth (after the upper vegetative layer of loose litter and mosses had been
 121 removed). Samples were homogenized thoroughly but gently by hand and stored at 4°C until
 122 analysis. During the first week following sampling, a subset of the (unsieved) fresh material was

123 used for determination of nematodes and enchytraeids (see *Toxicity studies*). The soil moisture (dry
124 matter = DM) was determined after drying 20 g of soil at 105°C for 24 h (Standard ASTM D2216,
125 ASTM 2010). The remaining material was air dried and sifted through a sieve with a 2 mm mesh. In
126 soil prepared this way, basic soil properties were determined. Soil OM content was measured as the
127 ignition loss (4 h, 550°C; Radojević and Bashkin 2006). Electrical conductivity (EC) was measured
128 using the WTW Cond330i meter, and pH was determined on a 1:2.5 (v/v) soil:distilled water
129 suspension with a Mettler Delta 340 pH meter according to the ISO 10390 standard (ISO 2005).
130 Total nitrogen (tot-N) and carbon (tot-C) were analysed using a LECO CNS-analyser (Table 1).

131

132 The total concentrations of phosphorus (tot-P) and metal(loid)s (Ag, As, Ba, Cd, Co, Cr, Cu, Hg,
133 Ni, Pb, V, Sn, Mo, Sb, Zn) in the < 2 mm soil fraction were determined with inductively coupled
134 plasma sector field mass spectrometry (ICP-SFMS) at the commercial Swedish laboratory ALS
135 Scandinavia. The ICP-SFMS analyses followed the protocols of SS EN ISO 17294-1 and the US
136 Environmental Protection Agency's (EPA's) method 200.8. Before analysis, soil samples were
137 dried at 50°C and a 0.5 g sub-portion of the dried material was digested with 5.0 mL concentrated
138 nitric acid (HNO₃) and 0.5 mL hydrogen peroxide (H₂O₂) in closed Teflon vessels in a high-
139 pressure microwave oven. The received concentrations can be viewed as total, or pseudo-total. To
140 get an efficient extraction of Sb, the addition of hydrochloric acid (HCl) is needed (Hjortenkrans et
141 al. 2009). This element was therefore determined after an aqua regia extraction, where a 0.5 g sub-
142 portion of the material was digested using 7.5 mL concentrated HCl and 2.5 mL concentrated
143 HNO₃. All chemicals used were of analytical grade quality.

144

145 Extracts of water-soluble metals (referred to as H₂O-metals throughout the paper) were obtained
146 after a multi-step procedure (Loureiro et al. 2005). Firstly, 10 mL of the air-dried and sieved
147 (< 2 mm) soil was mixed with 40 mL of distilled water (18.2 MΩ/cm² Milli-QTM water) and shaken
148 mechanically (150 rpm) for 24 h in the dark (Loureiro et al. 2005). The suspension was then
149 centrifuged (20 min, 4600 g) and filtered through glass microfibre using vacuum filtration apparatus
150 (Whatman GF/C Ø 47 mm, 1.2 µm porosity). The collected elutriate was divided in half and water
151 extractable metals (Cd-112, Co, Cr-52, Cu, Mn, Ni, Zn-64, P, V, As, Pb-sum) were determined
152 from one half. The other half was processed further to get elutriates for the BioToxTM test. The
153 concentrations of soluble metals were analysed with inductively coupled plasma mass spectrometry
154 (ICP-MS) in the Almalab of the University of Helsinki. Prior to the ICP-MS analyses, 0.1 mL
155 concentrated HNO₃ was added to 5 mL elutriates.

156

157 *Toxicity studies*

158

159 Phytotoxicity to garden cress (*L. sativum*) was evaluated by the biomass production (root and shoot
160 weight) following the methodology of OECD (2006), with some minor modifications. Seeds were
161 rinsed and imbibed in distilled water for 8 h prior to planting in 300 mL pots with moist sample
162 soil (sieved to < 2 mm). Fifteen seeds were planted per pot, evenly distributed over the surface of
163 the soil. Each pot was then placed on a Petri dish, to which irrigation water (distilled water) was
164 added daily. The temperature in the cultivation room was 20°C and the relative humidity 35%.
165 Samples were illuminated for 16 h/day with warm fluorescent light of a photon flux density of
166 approximately 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were harvested after 21 days. The soil was then carefully
167 removed, and root and shoot parts were separated and rinsed in distilled water prior to drying at
168 70°C for 24 h. Three pots were planted for each landfill and reference soil.

169

170 To assess toxicity to bacteria, the BioTox™ test was performed using freeze-dried *V. fischeri* (strain
171 NRRL B-11177, Aboatox Oy, Finland), which were exposed to elutriates obtained from water
172 extraction of the sample soils. The acidity of the elutriates was adjusted to pH 6.5–7.0 with a
173 phosphate buffer and the salinity was adjusted to 2% with sodium chloride (NaCl). Elutriates were
174 stored (max 2 days) at 4°C up to the testing time. Luminescence (RLU) was measured before and
175 after 15 min incubation of the bacterial suspension with the sample elutriates, according to the
176 ISO 11348-3 (1998) standard. The 2% NaCl with phosphate buffer addition was used as a control
177 liquid. Toxicity results were expressed as RLU inhibition percent (INH%). Each analysis was
178 performed on duplicate samples.

179

180 Luminescence inhibition of *V. fischeri* was calculated as follows:

181

182
$$KF = \frac{IC15}{IC0}$$

183
$$INH\% = 100 - \frac{IT15}{KF \times IT0} \times 100$$

184

185 where:

186

187 KF = correction factor

188 IC15 = luminescence intensity of control solution after contact time (15 min) in RLU

189 IC0 = initial luminescence of control sample in RLU

190 IT15 = luminescence intensity of test sample after contact time (15 min) in RLU

191 IT0 = initial luminescence intensity of test sample in RLU

192

193 In our study, nematodes and enchytraeids were extracted from non-sieved soil samples, as sieving
194 may hurt soil organisms. Glass pieces larger than approximately 4 mm were, however, gently
195 removed by hand. Nematodes were extracted from 5 to 10 g and enchytraeids from 30 to 50 g of
196 fresh soil samples using the wet funnel methods by Sohlenius (1979) and O'Connor (1955),
197 respectively. The number of nematodes and enchytraeids were counted under a binocular-
198 microscope. In addition, enchytraeids were classified into size classes according to length (0–2, 2–
199 4, 4–6, 6–8, 8–10, 10–12 and > 12 mm), and the total biomass of enchytraeids per sample was
200 calculated according to Abrahamsen (1973). All the nematodes and enchytraeids data were
201 expressed per g of soil OM. The presence of glass remains, which is a distinctive feature of the
202 landfill soil samples, might inevitably dilute all natural soil components – both minerogenic and
203 organic. By presenting the nematodes and enchytraeids data per g soil OM, these dilution effects are
204 reduced when interpreting toxicity effects.

205

206 *Statistical analyses*

207

208 The normality of data was analysed using Kolmogorov–Smirnov and Shapiro–Wilk tests and the
209 homogeneity of variances with Levene's test. The data were not always normally distributed and
210 the variances were sometimes heterogeneous, and thus the data that required so were log
211 transformed to meet the requirements of parametric tests.

212

213 The main aim of the study was to explore whether the biological responses (number of nematodes,
214 number and biomass of enchytraeids, luminescence inhibition of *V. fischeri*, and shoot and root
215 growth of *L. sativum*) differ between glasswork landfills (Johansfors, Kosta, Målerås, Orrefors,
216 Bergdala) and reference sites (Johansfors ref., Kosta ref., Målerås ref., Orrefors ref., Bergdala ref.).
217 Mean values per site based on five replicate soil samples collected from each sampling site were
218 used as tested values. In all analyses concerning soil parameters (total and H₂O extractable metal
219 concentrations, nutrient concentrations, pH, EC) and biota (plants, number of nematodes per g OM,
220 number and biomass of enchytraeids per g OM), comparisons between landfill and reference sites
221 were performed using a paired sample *t*-test. As the luminescence inhibition of *V. fischeri* data was
222 not normally distributed even after transformation, paired comparisons were performed using the

223 non-parametric Wilcoxon's test. In addition, the differences in measured parameters between single
224 glasswork landfills and their nearby reference areas were analyzed using independent *t*-test.

225

226 Multiple regression analysis (with stepwise correction) was used to explore whether the abundances
227 of soil fauna within the sites are influenced by measured soil variables. Response variables were the
228 root and shoot biomass of *L. sativum*, the number of nematodes, and the biomass of enchytraeids.
229 Soil metal and nutrient concentrations, moisture, OM, pH and EC were used as predictor variables.
230 In the regression analyses the five samples per site were used as replicates despite the fact that
231 they do not represent independent sample units. This makes 25 (5*5) tested values for both
232 glassworks and reference sites (altogether 50 samples). Thus, *p*-values should be considered with
233 caution. All tests were run using IBM SPSS Statistics 23 (SPSS Inc.).

234

235 Results

236

237 *Soil parameters*

238

239 Each landfill site was largely contaminated with various metals, as expected. Average
240 concentrations for each sampling site are presented in Table 1, and a more detailed compilation is
241 provided in Supplementary material I. Pb together with As, Ba, Cd and Zn were the major
242 contaminants, with mean concentrations at all glassworks sites exceeding the guideline values of
243 Swedish legislation for less sensitive land use (Swedish EPA 2009). Also concentrations of Cu and
244 Sb were found in elevated mean concentrations, exceeding the guideline values for sensitive land
245 use (Swedish EPA 2009). Overall, however, a huge variation in total metal concentrations was
246 observed for the landfill samples, which can be explained by the large heterogeneity of the
247 deposited waste materials: for example, the concentration of Pb varied from 161 to 38 000 mg kg⁻¹,
248 As from 64 to 7790 mg kg⁻¹, Ba from 30 to 3560 mg kg⁻¹, Cd from 0.2 to 62.8 mg kg⁻¹, and Zn
249 from 45 to 1050 mg kg⁻¹ (see Supplementary material I). Nevertheless, considering the whole data
250 set, positive correlations between all the contaminant metals (Pb, As, Ba, Cd, Zn and Sb) were
251 observed, implying a common source (the glass waste). Pearson's correlations (two-tailed) were
252 found in the range 0.53–0.88, with all *p*-values < 0.001. As expected, since elevated concentrations
253 are due to disposal of glass waste materials, significantly higher concentrations at the glassworks
254 sites than at the reference areas were also found for all six metals (all *t*-test, *p* > 0.05).

255

256 However, only a small part of the analysed total metals were found to be easily water-soluble
257 (Table 2). Of the potential contaminant metals, water-soluble concentrations were determined for
258 As, Cd, Pb and Zn (but not Ba and Sb). Pb and As were significantly higher in water extracts from
259 landfill soils than from reference areas (*t*-test, $p = 0.003$ and < 0.001 , respectively), even though
260 only 0.02% and 0.38% of the total concentrations were water extractable. At the reference areas, the
261 average concentration of water extractable Pb was $40 \mu\text{g kg}^{-1}$ and the corresponding value for As
262 was $201 \mu\text{g kg}^{-1}$ (Table 2 and Supplementary material II). The water extractable concentrations at
263 the landfill sites were $447 \mu\text{g kg}^{-1}$ (Pb) and $989 \mu\text{g kg}^{-1}$ (As), i.e. concentrations were on average
264 only 11 and 5 times (respectively) higher in water extracts derived from the landfill soils. The
265 average concentration of Cd in the water extracts was ca 20 times higher in landfill sites (ca
266 $30 \mu\text{g kg}^{-1}$) compared to reference sites (ca $1.5 \mu\text{g kg}^{-1}$), but due to the high variation the difference
267 was not statistically significant (paired *t*-test, $p = 0.113$). The concentrations of Zn-64 did not vary
268 between landfill and reference sites (paired *t*-test, $p > 0.05$), but this was also the metal with the
269 least obvious contamination impact.

270

271 Comparing the other investigated soil geochemical parameters between the landfill and reference
272 areas, significant differences were observed for pH in Johansfors (independent *t*-test, $p < 0.05$). In
273 addition, soil OM and nutrient contents varied between sites but no clear trend was shown (paired *t*-
274 test, $p > 0.05$) (Table 1). The EC was significantly different, being ca 50% higher in landfill sites
275 (paired *t*-test, $p = 0.003$) (Table 1), but overall, the conductivity of all soils was quite low.

276

277 In summary, although both the basic soil geochemistry and metal concentrations were highly
278 variable, significant differences were seen only for the metal concentrations and EC.

279 **Table 1.** Soil metal and nutrient (N, C, P) concentrations (mg kg⁻¹; mean ± standard error; n = 5) and characteristics at landfill and reference sites. Electric conductivity (EC
280 in ms cm⁻¹ and the dry- and organic matter content (DM and OM) in g g⁻¹. The first, and lowest, of the two generic guideline values (mg kg⁻¹) of the Swedish EPA (2009)
281 corresponds to the maximum permissible metal concentration in areas with sensitive land use. Concentrations above this level may, among other negative effects, result in
282 negative impacts on soil ecosystem functions. The higher value represents the corresponding figure for land used for industrial purposes (where some reduction in soil
283 functions is accepted). Values in bold highlight metal concentrations that exceed the generic guideline values for sensitive land use.
284

	Guideline	Bergdala	Bergdala ref.	Johansfors	Johansfors ref.	Kosta	Kosta ref.	Målerås	Målerås ref.	Orrefors	
As	10/25	180±82	3.0±0.53	2200±1400	11±1.2	380±99	2.5±0.50	500 ±190	8.4 ± 2.3	170 ± 37	2.7 ± 0.49
Ba	200/300	180±57	34±4.8	1300±640	51±9.1	470±88	41±5.7	1100 ±530	91 ± 15	220 ± 50	120 ± 21
Cd	0,5/15	13±6.0	0.23±0.03	4.4±1.8	0.18±0.02	6.5±4.0	0.16±0.02	5.5±2.2	0.38 ± 0.08	19 ± 11	0.30 ± 0.30
Cu	80/200	24±2.7	27±19	62±9.1	8.4±0.48	140±75	8.4±1.2	42±14	21 ± 2.2	48 ± 16	16 ± 1.7
Pb	50/400	800±380	19±2.4	12000 ±6900	62±13	7900±2100	30±5.5	1200±670	100 ± 26	7000 ± 2200	45 ± 12
Sb	12/30	28±7.2	0.58±0.15	6.9±2.9	1.0±0.21	31±5.7	0.41±0.05	17±5.9	1.4 ± 0.34	3.4 ± 1.0	0.41 ± 0.03
Zn	250/500	330±100	26±7.8	430±180	20±3.7	350±42	36±4.8	340±85	170 ± 41	420 ± 170	72 ± 7.6
Tot-P	-	510±43	360± 65	3800±1700	280±49	1100±1200	680±100	3100±1300	680 ± 74	420 ± 80	1800 ± 240
Tot-N	-	3500±830	2300±280	1700±920	2100±730	3300±430	2200±370	1900±370	2800 ± 360	700 ± 290	2900 ± 200
Tot-C	-	73000±19000	35000±5900	42000±25000	57000±23000	82000±1400	32000±4200	68000±14000	47000±5600	14100 ± 4900	40000 ± 1900
C:N	-	21±0.56	15±1.1	24±1.3	27±1.5	25±2.6	14±1.1	36±12	17 ± 0.31	20 ± 5.2	14 ± 0.64
EC	-	0.12±0.01	0.04±0.01	0.09±0.02	0.04±0.01	0.10±0.02	0.06±0.01	0.14±0.01	0.07±0.01	0.06±0.01	0.05±0.01
DM	-	0.85±0.02	0.86±0.02	0.91±0.04	0.82±0.02	0.79±0.02	0.88±0.02	0.78±0.03	0.82±0.02	0.90±0.02	0.86±0.01
OM	-	0.13±0.03	0.06±0.01	0.10±0.07	0.09±0.02	0.11±0.02	0.06±0.01	0.11±0.01	0.07±0.02	0.02±0.01	0.06±0.003
pH	-	5.9±0.05	5.9±0.09	7.1±0.04	5.3±0.50	6.2±0.10	6.0±0.11	6.7±0.09	6.7±0.12	5.9±0.08	5.9±0.10

Table 2. Concentrations of H₂O extractable metals (mean± standard error), expressed per kg dry weight of the soil (µg kg⁻¹).

	Bargdala	Bergdala	Johansfors	Johansfors	Kosta	Kosta	Målerås	Målerås	Orrefors	Orrefors
	ref.			ref.		ref.		ref.		ref.
	H ₂ O extractable metals in soils (µg kg ⁻¹)									
As	794 ± 222	208 ± 14	1072 ± 333	226 ± 28	1482 ± 498	228 ± 21	1214 ± 301	205 ± 24	382 ± 36	139 ± 5.6
Cd-112	29 ± 8.3	1.2 ± 0.18	1.5 ± 0.60	0.83 ± 0.12	9.9 ± 6.2	0.89 ± 0.12	0.6 ± 0.18	0.70 ± 0.18	111 ± 91	3.8 ± 2.0
Pb-sum	152 ± 41	13 ± 4.1	1006 ± 376	68 ± 31	544 ± 147	36 ± 11	20 ± 6.4	53 ± 29	512 ± 369	27 ± 10
Zn-64	462 ± 169	195 ± 50	530 ± 325	651 ± 107	106 ± 34	262 ± 63	356 ± 70	465 ± 55	511 ± 246	224 ± 72

Plant and microbe toxicity

In the plant toxicity test with *L. sativum*, the results varied greatly between sampling sites. In Bergdala and Orrefors, for example, there was a lack of difference between biomass production of plants growing on soils from the contaminated glassworks areas and those from reference areas (Fig. 2; paired *t*-test, $p > 0.05$). In Kosta, the plants cultivated in the landfill soil samples had a higher *root* biomass than those grown in the reference soils (independent *t*-test, $p > 0.05$) (Fig 2a), whereas in Malerås, the *shoot* biomass of plants growing in soil from the landfill was reduced (independent *t*-test, $p < 0.05$) (Fig 2b). When analysing all sites together, no difference between landfill and reference sites were observed. The same results applied both to the shoot biomass and the root biomass (paired *t*-test, $p = 0.933$, $p = 0.384$).

However, regression analysis showed a positive correlation between *shoot* weight and soil H₂O-Pb ($R^2 = 0.300$, $F = 13.047$, $p = 0.028$) and P concentrations ($R^2 = 0.519$, $F = 9.018$, $p = 0.030$).

Fig. 2. Biomass production of a) roots and b) shoots of *L. sativum* at the soils from the glassworks landfill areas (grey) and those from reference areas (white). Each column represents mean values of five samples (with $\pm$ standard error bars, $n = 5$). An asterisk denotes statistical significance between the glassworks soil samples and reference samples ($p < 0.05$).

Table 3. Results of linear regression analysis (Stepwise model).

Variable	Predictors in model	β	p -value	R^2	R
Number of enchytraeids	H ₂ O-Pb	-0.450	0.005	0.202	0.450
Biomass of enchytraeids	H ₂ O-Pb	-0.408	<0.001	0.286	0.535
	H ₂ O-As	-0.343	<0.001	0.388	0.623
Number of nematodes	Tot-Pb	-0.279	0.050	0.078	0.279
	Tot-As	0.755	0.004	0.206	0.453
Shoot weight of <i>L. sativum</i>	H ₂ O-Pb	0.548	0.028	0.300	0.548
	Tot-P	0.468	0.030	0.519	0.720

In the BioTox™ tests, the elutriates from both the metal contaminated landfill and reference soils exposed very low toxicity to *V. fischeri* with no differences between reference and landfill sites (Wilcoxon, all sites, $p = 0.345$). In addition, high variation between sampling sites was observed (Fig. 3). Out of the tested landfill sites, only the landfill soil from Kosta reduced light emission of

322 *V. fischeri* in comparison to its reference soil (independent *t*-test, $p = 0.038$). Both landfill and
 323 reference soils from Målerås were biostimulative, increasing the light emission of *V. fischeri*; the
 324 soils from the landfill more so than reference soils (independent $p = 0.008$). No statistically
 325 significant correlation between H₂O extracted metals and INH% was shown in the regression
 326 analysis (Stepwise model).

330
 331 **Fig. 3.** The luminescence inhibition percent (INH%) of water elutriates from glassworks landfill
 332 soils (grey) and from reference areas (white) for *V. fischeri*. Mean $\pm$ standard error, $n = 5$. Negative
 333 values indicate increased light emission/biostimulation. An asterisk denotes statistical significance
 334 (with $p < 0.05$).

335 336 337 *Soil organisms*

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 339 In the soil samples from the contaminated landfill sites, 13 out of 25 samples were totally lacking
 340 enchytraeids. In comparison, only one sample from the reference areas was without enchytraeids.
 341 The number and biomass of enchytraeids were significantly reduced in landfill sites of Bergdala,
 342 Johansfors and Kosta (*t*-test, $p < 0.05$). (Fig. 4). Consequently, the total biomass of enchytraeids
 343 (expressed per g OM) was significantly lower in landfill sites (paired *t*-test including all sites,

344 $p = 0.016$), being on average $17.7 \mu\text{g g}^{-1}$ OM in the landfill soils and $44.1 \mu\text{g g}^{-1}$ OM in the
 345 reference soils. Also average biomass of enchytraeid individuals was reduced in glasswork landfill
 346 sites (average weight $17.8 \mu\text{g}$) compared to reference sites (average $28.1 \mu\text{g}$), with the exception of
 347 Bergdala (paired t -test including Bergdala $p = 0.337$, excluding Bergdala $p = 0.011$).
 348

349 According to the regression analysis (stepwise; Table 3), none of the following analysed parameters
 350 could alone explain the difference in number and biomass of enchytraeids: 1) total concentrations of
 351 different metals (neither of As, Ba, Cd, Pb, Zn, Sb or any other of the metals listed in
 352 Supplementary material I), 2) total concentrations of nutrients (N, C or P), or 3) soil characteristics
 353 (pH, dry or OM content, EC). The multiple regression analysis showed that 20.2% and 28.6% of the
 354 observed variation in number and biomass (respectively) of enchytraeids could be explained by
 355 $\text{H}_2\text{O-Pb}$ (number: $R^2 = 0.202$, $F = 8.870$, $p = 0.005$ and biomass: $R^2 = 0.286$, $F = 18.425$,
 356 $p > 0.001$). In addition, ca 10% of reduced enchytraeid biomass can be explained by $\text{H}_2\text{O-As}$
 357 (increase in the coefficient of determination by 10%; $R^2 = 0.388$, $F = 14.242$, $p < 0.001$).
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 361 **Fig. 4.** Number of enchytraeids per g soil OM from the glassworks landfill areas (grey) and those
 362 from reference areas (white). Mean $\pm$ standard error, $n = 5$. Asterisks denote statistical significance (
 363 * <0.05 , ** <0.01).
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Also the number of nematodes tended to be reduced in the landfill soils, but the difference was statistically significant only in Målerås (independent *t*-test, $p = 0.038$; Fig. 5). When analyzing all sites together, no statistically significant difference between landfill and reference soils was observed (paired *t*-test, $p = 0.087$). The average number of individuals in landfill soils was 54.9 g^{-1} OM, compared to 74.4 individuals per g OM in reference sites (Fig. 5). According to the regression analysis (Table 3), 27.9% of the observed variation in the numbers of nematodes could be explained by tot-Pb ($R^2 = 0.078$, $F = 4.060$, $p = 0.050$). In addition, 22% of the reduction in nematode numbers could be explained by tot-As (increase in the coefficient of determination $R^2 = 0.276$, $F = 6.084$, $p = 0.004$).

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Fig. 5. Number of nematodes per g soil OM from the glassworks landfill areas (grey) and those from reference areas (white). Mean $\pm$ standard error, $n = 5$. An asterisk denotes statistical significance (with $p < 0.05$).

387 Discussion

388

389 The assessment of potential risks to ecological functions and humans living around contaminated
390 sites is a task that is as important as it is complex and challenging. In Sweden alone, there are
391 approximately 85,000 potentially contaminated sites. Since remediation of all these sites is not
392 feasible in the near future, addressing the national goal of a non-toxic environment requires
393 understanding of actual risks for humans and ecosystems around contaminated sites. One way of
394 achieving this, in Sweden as well as elsewhere, may be to include relevant biotests in generic risk
395 assessment methodologies. Several studies have shown that biotests give a better indication of metal
396 bioavailability and ecological risks than chemical analyses alone (Kahru et al. 2005; Zhang et al.
397 2013). The tests used and evaluated in the present study have all been proven useful in other
398 studies:

399

400 Firstly, the biomass production (root and shoot weight) of garden cress (*L. sativum*) has been found
401 a suitable indicator for the evaluation of potential phytotoxicity to vascular plants due to its well-
402 documented sensitivity to metal contaminants (Baderna et al. 2015; Gill et al. 2012; Montvydiene
403 and Marciulioniene 2004; Vasile et al. 2013). Secondly, the measurement of luminescence
404 inhibition in *V. fischeri* by the BioTox™ method is a well established and standardized approach.
405 Briefly, the metabolic pathway responsible for light emission by the bacterium is intrinsically linked
406 to cellular respiration, and any disruption of normal cellular metabolism causes a decrease in light
407 production (Parvez et al. 2006). Finally, enchytraeid worms (mostly omnivorous) and nematodes
408 (covering several trophic positions) are often selected as test biota for toxicity tests (Kapusta and
409 Sobczyk 2015). They are present in a wide range of ecosystems, occur abundantly, play a key role
410 in the functioning of soil ecosystems, are easy to use, collect and culture, come into contact with a
411 variety of stress factors (the soil solution, the solid phase, and the gaseous phase in soil), and are
412 sensitive to environmental stresses (Didden and Römbke 2001; Römbke and Moser 2002). The
413 indicator capacity of enchytraeids and also nematodes has been proved in several laboratory
414 (Didden and Römbke 2001) and field experiments (Hui et al. 2009; Höss and Williams 2009;
415 Selonon et al. 2014).

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417 When applied to the five glassworks sites of this study and their reference sites, we saw that the
418 different tests were not consistent, and that there was a large variability between sites. For example,
419 the number of enchytraeids and nematodes was consistently lower at the landfill areas (Fig 4+5),
420 indicating *in-situ* toxicity to soil inhabiting organisms. But the *L. Sativum* biomass production (Fig

2) and luminescence inhibition in *V. fischeri* (Fig. 3) were higher at some of the glassworks sites compared to the corresponding reference sites and lower at others, resulting in no overall statistical difference. Similar site specific inconsistencies between different tests were observed by Karjalainen et al. (2009) regarding CCA-contaminated soil samples. Thus, our results clearly demonstrate that risk assessments of contaminated sites are often complicated due to several influencing factors in the field.

Chosing relevant biotests for assessing soil toxicity

Most previous studies have focused on the impact of single pollutants (Kapusta and Sobczyk 2015). In reality, many contaminated sites are affected by mixtures of pollutants. Considering landfill sites, the composition of the waste material may also be quite different even in two adjacent soil volumes – something which was clearly manifested by the large variability in, for example, total metal concentrations at single landfill sites of our study. In addition, different subsamples - even from the same site - may differ substantially when it comes to basic geochemical properties (i.e. redox potential, pH, average grain size, content of sorbants such as organic particles or Al/Fe/Mn hydroxides). Large variabilities between subsamples regarding leachability/bioavailability and toxicity are therefore to expect. From such a perspective, the consistent decline in enchytraeid and nematode abundance and size constitutes a relatively robust indication of the toxic properties of the glassworks soils, despite the low number of samples analyzed. The lack of measurable effects using the acute toxicity tests with bacteria and plants, however, indicated that these tests were not appropriate for the studied environment. As concluded by Waara et al. (2009), the detectability of toxicity in challenging environments depends on site specific conditions and the test organism chosen.

Toxicity not implied by *V. fischeri* or *L. sativum*

One possible explanation for the lacking inhibition in both *V. fischeri* luminescence and in *L. sativum* growth is obviously that the glassworks soils don't leach metal contaminants to such a degree that the soil pore water becomes toxic enough. In favor of this hypothesis are the results which showed a low extractability when the highly polluted soils were shaken with distilled water.

However, one must also consider the possibility of these tests not being suitable for the evaluation of toxicity of glassworks contaminated soils. Regarding the evaluated plant growth, the positive

455 connection observed between soil metal contamination (in particular of Pb) and shoot growth of
456 *L. sativum* was contrary to what would be expected if soil contamination was limiting plant growth.
457 However, the increase in shoot weight at moderately elevated bioavailable concentrations is
458 probably due to the hormesis effect. Hormesis, which is a dose–response relationship that is
459 characterized by low-dose stimulation and high-dose inhibition, has previously been observed in
460 plants growing on metal contaminated soils (Wang et al. 2010; Calabrese and Blain 2009). Baderna
461 et al. (2015), for example, investigated the phytotoxicity of different metals (As, Cd, Cr, Pb, Hg, Ni
462 and Zn) alone and in mixtures using *L. sativum* and two others plants, and found a clear hormesis
463 dose–effect relationship. As they highlighted, the biostimulation observed at moderately elevated
464 bioavailable concentrations may be a potential alert flag because it could be the initial adaptive
465 response to low doses of one or several toxicants, where the initial stimulation could turn into
466 strong toxicity at longer exposure times or higher toxicant doses.

467

468 Regarding the lacking effect on *V. fischeri* luminescence, the first important note to make is that
469 water extracts produced in batch leaching tests may not adequately mirror the true pore water
470 composition of the site under investigation. Dissolved metal concentrations may be significantly
471 higher in the field (Petänen et al. 2001; Petänen and Romantschuk 2003), for example due to longer
472 contact times and lower L/S ratios. In addition, while the leachability was low with distilled water
473 in our study, a sequential extraction of samples from the fine fraction (< 2 mm) of another
474 glassworks landfill of the region showed that 15–35% of the total Pb concentrations and 40–60% of
475 the total Cd were dissolved in the first step (using 1 M CH₃COONa) (Augustsson et al. 2016). This
476 step (at least theoretically) targets metals that are relatively easily assessible; associated with pore
477 water of the fresh soil and including ions that are weakly sorbed by ion exchange and associated
478 with carbonate complexes (Hall et al. 1996; Kersten and Förstner 1989). Beside the uncertainties in
479 the representativity of the water extracts, it has also been found that bacteria may develop
480 resistance in metal-contaminated environments. Their suitability as proxies for metal contamination
481 is thus indistinct. For example, tests made at a heavily Pb contaminated shooting range in southern
482 Finland showed that the frequency of Pb resistant bacteria increased in metal polluted soils, but that
483 neither the bacterial numbers nor the community profile was significantly altered (Hui et al. 2012).
484 Also plants appeared to be unaffected by the high Pb levels at these sites (Hui et al. 2009, 2011,
485 2012). When compared to laboratory experiments of bacterial luminescence inhibition, however,
486 plant growth experiments (direct exposure) have been suggested much more sensitive in assessing
487 soil toxicity (Alvarenga et al. 2008). In tests using Hg- and As-sensing bacteria, the conclusion
488 made by Petänen and Romantschuk (2003) was that some soil bacteria mobilize heavy metals,

which means that soil animals ingesting these bacteria, like enchytraeids and nematodes, could become exposed. While soil microbes become resistant to heavy metals and plants are able to close them out from the root tissue, soil fauna that ingest detritus including bacterial and fungal cells become exposed to heavy metals in their food. That is another possible explanation for the response seen in enchytraeids (and nematodes), and the lack of such in our indicator bacteria.

1.2. Toxicity implied by enchytraeids and nematodes

It has, reasonably, been argued that the most relevant risk assessment should be based on the evaluation of effects in soil organisms that are exposed to contaminants in their real habitats (Markert et al. 2003). *In situ* risks are always affected by a cascade of factors (such as soil texture, geochemistry, and hydrology) that may mitigate or amplify the negative effects from contaminants.

The observed low *in situ* frequency and decreased biomass of enchytraeids (and nematodes) in the metal contaminated soils compared to reference soils, imply that the decomposer community did suffer from the heavy metal contamination. In our case, where we didn't reach the same conclusion from the tests with *V. fisheri* and *L. sativum*, it may be that enchytraeids and nematodes are more sensitive in general, or simply that they give a better reflection of the true field conditions. While the hormesis effect may complicate the interpretation of *L. sativum* experiments and soil bacteria may develop tolerance against high metal concentrations, previous studies agree on enchytraeids being sensitive to metal contamination (Salminen et al. 2001a, b; Hui et al. 2009), in particular to Pb (Didden and Römbke 2001). For example, Karjalainen et al. (2009) and Selonon (2015) observed reduced numbers of enchytraeids and nematodes extracted from metal contaminated soils compared to similar pristine forest soil in southern Finland. Also Haimi and Mätäsniemi (2002) showed that close to a metal emission source in central Finland the numbers of enchytraeids and nematodes were clearly decreased. Even though much of the Pb is relatively immobile, high tot-Pb concentrations may still be harmful to soil organisms (Selonon et al. 2012). Such an effect is suggested in our study too, since the multiple regression analysis showed a connection between soil H₂O-Pb and also H₂O-As and the numbers and biomass of enchytraeids. Also, reduced numbers of nematodes seems to be dependent on the concentrations of Pb and As in the soil. As the metals at the glassworks sites occur as complex mixtures with variable/covariable metal concentrations, we cannot determine the effect of each metal separately. Furthermore, since the concentrations of single metals had quite low explanatory power on the observed changes, the effect seen is probably a result of multiple stressors having an additive effect. One possible explanation for the larger

523 impact seen on enchytraeids than nematodes may be that the summer of 2015 was unusually dry in
524 south-eastern Sweden. Enchytraeids are more sensitive to changes in soil moisture than nematodes
525 are (Lindberg et al. 2002), and the dry conditions may have increased the enchytraeids' sensitivity
526 also for other environmental stress factors.

527

528 To sum up, the enchytraeids and nematodes were in this study found to be the most reliable
529 bioindicator species. We suggest therefore – in line with Kapusta and Sobczyk (2015) – that
530 enchytraeid and nematode worm density and biomass are to be used as a proxies of soil quality in
531 metal polluted soils together with chemical analyses of the total and 'bioavailable' fraction.

532

533 Conclusions

534 In understanding health and environmental risks at contaminated sites, the issues of bioavailability
535 and toxicity are of key relevance. Despite high total concentrations of several toxic metals
536 (primarily Sb, As, Ba, Cd, Pb and Zn) at five examined glassworks sites in south-eastern Sweden,
537 the toxicity of the glassworks soils was not revealed by indirect tests; inhibition of light emission by
538 *V. fischeri* could not be seen, nor was an effect seen on the growth of *L. sativum*. That the soils can
539 be toxic to organisms was, however, shown by the decrease in enchytraeid abundance and biomass
540 in these soils compared to reference soils collected nearby. Direct observation of soil fauna is
541 therefore the better proxy for *in situ* toxicity. In this case they confirm the results from previous
542 chemical leaching tests of glassworks soils, which have indicated that a significant fraction of the
543 metals may be available for biological uptake. The confirmation of *in situ* bioavailability to soil-
544 inhabiting organisms also motivates additional studies of the risk posed to humans of the
545 glassworks villages, who consume local foodstuffs and/or drink water from private wells.

546

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551 University, Kalmar, Sweden.

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554 **Supplementary material I.** Concentrations (minimum, median and maximum values) of total
555 metals (mg kg⁻¹) in the studied glass landfill sites.

		Bergdala	Johansfors	Kosta	Målerås	Orrefors
As	min	76.4	96.6	228	64.1	73.9
	max	503	7790	764	1180	241
	median	96	473	300	331	228
Ba	min	67.3	30.2	188	337	94.1
	max	393	3560	668	3110	384
	median	143	1350	435	416	197
Cd	min	3.3	0.2	1.7	1.6	1.3
	max	36.7	8.2	22.4	13.9	62.8
	median	9.9	5.2	2.3	3.8	10.0
Co	min	1.4	3.2	3.9	0.7	1.2
	max	3.7	7.9	6.2	8.6	5.7
	median	2.2	5.4	4.9	2.9	2.8
Cr	min	4.4	3.5	8.0	3.6	2.9
	max	39.9	18.0	31.1	12.9	7.1
	median	6.2	14.2	12.7	7.3	5.8
Cu	min	16.4	31.4	39.3	15.6	17.3
	max	32.1	79.8	430	89.4	108
	median	24.0	68.2	54.5	26.7	30.8
Hg	min	< 0.1	< 0.1	0.4	< 0.1	< 0.1
	max	< 0.1	< 0.1	0.9	–	< 0.1
	median	< 0.1	< 0.1	0.5	2.0	< 0.1
Ni	min	2.8	2.7	7.1	1.4	1.8
	max	6.9	19.8	15.0	22.9	7.4
	median	4.2	8.5	11.1	7.2	4.7
Pb	min	161	981	4520	539	1530
	max	2030	38 000	16 000	4210	13 000
	median	263	4480	6240	1290	6480
V	min	6.3	7.1	10.4	1.9	2.4
	max	19.2	14.4	21.4	18.3	10.3
	median	8.7	11.4	16.6	6.6	7.0
Zn	min	142	45.0	197	101	76.9
	max	710	1050	441	589	1040
	median	301	422	354	275	419

Ag	min	0.1	0.1	0.5	0.1	0.1
	max	0.1	3.0	1.7	0.4	0.6
	median	0.1	0.9	1.3	0.2	0.2
Mo	min	0.4	0.4	1.8	0.5	0.3
	max	0.9	2.1	5.0	1.5	1.1
	median	0.6	0.9	2.4	1.0	0.8
Sb	min	15.8	1.1	16.6	2.6	0.4
	max	55.7	15.2	51.4	38.6	6.4
	median	23.2	3.7	30.2	15.4	3.8
Sn	min	1.1	1.7	3.5	1.7	1.1
	max	2.3	17.2	22.6	5.7	2.7
	median	1.6	6.0	6.0	3.9	2.0

556

557 **Supplementary material II.** Concentrations (minimum, median and maximum values) of H₂O
558 extractable metals (mg kg⁻¹) in the studied glass landfill soils.
559

		Bargdala	Johansfors	Kosta	Målerås	Orrefors
As	min	0.316	0.328	0.573	0.613	0.272
	max	1.559	1.922	3.416	2.219	0.497
	median	0.565	1.019	1.084	1.091	0.383
Cd-112	min	0.012	0.001	0.002	0.000	0.003
	max	0.059	0.003	0.034	0.001	0.475
	median	0.026	0.001	0.003	0.000	0.030
Zn-64	min	0.126	0.158	0.030	0.356	0.050
	max	0.936	1.504	0.183	0.356	1.275
	median	0.249	0.229	0.106	0.356	0.209
Pb-sum	min	0.060	0.298	0.226	0.011	0.063
	max	0.304	1.922	0.908	0.045	1.971
	median	0.143	0.902	0.435	0.014	0.101

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