

## Article (refereed)

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# **The comparative toxicity to soil invertebrates of natural chemicals and their synthetic analogues**

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1 **Abstract**

2 The introduction of REACH (Registration, Evaluation and Authorisation of Chemicals),  
3 requires companies to register and risk assess all substances produced or imported in volumes  
4 of >1 tonne per year. Extrapolation methods which use existing data for estimating the effects  
5 of chemicals are attractive to industry, and comparative data are therefore increasingly in  
6 demand. Data on natural toxic chemicals could be used for extrapolation methods such as  
7 read-across. To test this hypothesis, the toxicity of natural chemicals and their synthetic  
8 analogues were compared using standardised toxicity tests. Two chemical pairs: the  
9 naphthoquinones, juglone (natural) and 1,4-naphthoquinone (synthetic); and anthraquinones,  
10 emodin (natural) and quinizarin (synthetic)) were chosen, and their comparative effects on the  
11 survival and reproduction of collembolans, earthworms, enchytraeids and predatory mites  
12 were assessed. Differences in sensitivity between the species were observed with the  
13 predatory mite (*Hypoaspis aculeifer*) showing the least sensitivity. Within the chemical pairs,  
14 toxicity to lethal and sub-lethal endpoints was very similar for the four invertebrate species.  
15 The exception was earthworm reproduction, which showed differential sensitivity to the  
16 chemicals in both naphthoquinone and anthraquinone pairs. Differences in toxicity identified  
17 in the present study may be related to degree of exposure and/or subtle differences in the  
18 mode of toxic action for the chemicals and species tested. It may be possible to predict  
19 differences by identifying functional groups which infer increased or decreased toxicity in one  
20 or other chemical. The development of such techniques would enable the use of read-across  
21 from natural to synthetic chemicals for a wider group of compounds.

22

23

24 **Keywords:** anthraquinone, naphthoquinone, ecotoxicity, risk assessment

## 25 **1. Introduction**

26 It is estimated that of the 100,000 chemical substances registered in the European market  
27 before 1981, one third still lack information on their intrinsic properties and only 140 have  
28 been singled out for risk assessment (DEFRA, 2006). The introduction of REACH  
29 (Registration, Evaluation and Authorisation of Chemicals) in 2008, requires companies to  
30 register and risk assess all substances produced or imported in volumes of >1 tonne per year  
31 (30,000 substances), however, the available data are often insufficient to assess the range of  
32 chemicals currently in use. Integral to REACH is a requirement to reduce the need for further  
33 toxicity testing. Consequently, methods which use existing data for estimating the effects of  
34 substances are attractive to industry: for example Quantitative Structure-Activity  
35 Relationships (QSARs), and read-across. Such approaches rely on the principle that the  
36 chemical structure of a substance determines its behaviour and effects.

37

38 Data on natural plant toxins could potentially be used for read-across type methods. Plants  
39 produce and release into the environment an enormous range of chemicals including:  
40 phenolics, terpenoids and alkaloids (38,000 characterised) (Langenheim, 1994; Harborne and  
41 Baxter, 1996). Data on the fate and effects of these natural toxins could provide insight into  
42 the potential impacts of structurally similar synthetic chemicals. Previous work, using  
43 literature data, has shown strong similarities in the fate and effects of natural plant toxins and  
44 synthetic analogues in soils (Sorokin, 2007; Sorokin and Whitaker 2008). In order to confirm  
45 the suitability of using data on natural toxins for read-across methods, we made experimental  
46 comparisons of the toxicity of two chemical pairs on the survival and reproduction of four  
47 groups of soil invertebrates: earthworms; collembolans; enchytraeids; and predatory mites  
48 using standard ecotoxicity tests. In comparing the toxicity to four representative groups of soil

49 invertebrates we aimed to determine whether read-across could be used to assess the toxicity  
50 of chemicals in soil ecosystems.

51

52 Natural chemicals produced by plants were chosen with properties relevant to the risk  
53 assessment of synthetic chemicals i.e. presence in soil, persistence in soil and potential  
54 toxicity to a range of soil organisms. In addition, natural chemicals were chosen so that  
55 suitable synthetic analogues could be selected, representative of chemicals for which risk  
56 assessments may need to be carried out. Based on these criteria two natural chemicals were  
57 chosen (Table 1). Juglone (5-hydroxy-1,4-naphthoquinone), a naphthoquinone identified in  
58 the walnut genus *Juglans* (Willis, 2000), and emodin (1,3,8-trihydroxy-6-  
59 methylantraquinone), an anthraquinone identified in at least 94 plant species (Izhaki, 2002).  
60 Synthetic analogues for each chemical were then chosen: for juglone, 1,4-naphthoquinone;  
61 and for emodin, 1,4-dihydroxyanthraquinone (known as quinizarin) (ECB, 2004). Both  
62 synthetic chemicals have wide industrial uses including chemical synthesis, textile processing,  
63 biocide manufacture and in the colouring industries (IUCLID, 2000).

64

## 65 **2. Material and Methods**

66 Standardized methods have been developed for assessing chemical toxicity to soil  
67 invertebrates. To address the objectives of this study we chose the collembolan reproduction  
68 test (ISO, 1999); the earthworm reproduction test (OECD, 2004a); the enchytraeid  
69 reproduction test (OECD, 2004b); and a modified predatory mite reproduction test (Hamers  
70 and Krogh, 1997). Since the completion of these experiments, OECD have published  
71 Guideline 226 for testing chemical effects on reproduction of predatory mites (OECD, 2008),  
72 however our method differs from this guideline in a number of ways. In addition, a number

73 of minor deviations from published protocols were necessary in order to complete this wide  
74 range of tests, all deviations are detailed in the methods below.

75

### 76 *2.1 Test species*

77 All cultures were kept at  $20 \pm 1$  °C with a 16:8 h photo-period prior to testing. *Folsomia*  
78 *candida* (Collembola: Isotomidae) obtained from Reading University, were cultured on a  
79 moist substrate of plaster of Paris/charcoal (8:1) and fed dried bakers yeast. Synchronised  
80 cohorts for testing were obtained by transferring several hundred adults to clean culture  
81 vessels for 2-3 days. Adults were then removed to allow eggs laid by the females to hatch.  
82 *Eisenia fetida* (Lumbricidae: Oligochaeta) were purchased from Aquaculture Supplies, UK.  
83 They were kept in commercial culture soil at ~35% soil moisture content and were fed dried  
84 and ground rabbit manure as required. Cultures of *Enchytraeus albidus* (Enchytraeidae:  
85 Oligochaeta) were also purchased from Aquaculture Supplies, UK in commercial culture  
86 medium and fed ground rolled oats as required. Cultures of *Hypoaspis aculeifer* Canestrini  
87 (Gamasida: Laelapidae) were purchased from Biological Crop Protection Ltd., UK. They  
88 were cultured under the same conditions as *F. candida*, and were fed with *F. candida* or  
89 *Folsomia fimetaria* L. (Collembola: Isotomidae) as required. Synchronised cultures for testing  
90 were obtained by transferring several hundred adults to clean culture vessels at a ratio of 10  
91 females: 5 males for 3 days. Adults were then removed to allow eggs laid by the females to  
92 hatch.

93

### 94 *2.2 Chemicals*

95 Juglone (5-hydroxy-1,4-naphthoquinone), 1,4-naphthoquinone, quinizarin (1,4-  
96 dihydroxyanthraquinone), acetone and dichloromethane (DCM) were obtained from Sigma-

97 Aldrich, UK. Emodin (1,3,8-trihydroxy-6-methylantraquinone) was obtained from  
98 Xtrasyntase France. All reagents were of 95 % purity or greater.

99

### 100 *2.3 Test soil and treatments*

101 All tests were conducted in standard OECD artificial soil (pH  $6 \pm 0.5$  (pH-KCl) (OECD,  
102 2004a). Soil moisture content was maintained at 35% maximum water holding capacity  
103 (WHC, d. wt.) for tests with *E. fetida* and *F. candida* and 50 % maximum WHC for tests with  
104 *E. albidus* and *H. aculeifer*. Range-finding mortality tests were conducted with each chemical  
105 and each test species to determine a suitable concentration range for the reproduction tests.  
106 Eight concentrations with a factor of two spacing were used in each test, with three replicates  
107 per test concentration. Concentrations ranged from 8 to 1024 mg kg<sup>-1</sup> d. wt for all tests,  
108 except the enchytraeid and predatory mite tests with the naphthoquinones where the range was  
109 4 to 512 mg kg<sup>-1</sup> (range adjusted due to range-finding test results). These concentrations were  
110 nominal as we did not analyse the treated soil to determine the actual concentrations due to  
111 the complexity of analysing these compounds. In addition controls and solvent controls were  
112 prepared with either three (mites), six (collembola and earthworm) or eight (enchytraeid)  
113 replicates.

114

#### 115 *(a) Soil treatments for collembola, earthworm and predatory mite tests*

116 Test substances were dissolved in a 50:50 (v:v) mixture of DCM and acetone to produce stock  
117 solutions corresponding to the highest test dosage. Test soils were prepared by mixing the  
118 appropriate amount of stock solution into the soil. Solvent controls (50:50, acetone: DCM)  
119 were prepared in the same manner using the highest volume of solvent used to generate the  
120 test soils (50 ml kg<sup>-1</sup> or 26.5 ml kg<sup>-1</sup> for *H. aculeifer*). After mixing, the solvent was

121 evaporated for 24 h after which the water content of the soil was adjusted to 35 % maximum  
122 WHC (50 % for *H. aculeifer*).

123

124 *(b) Soil treatments for enchytraeid test*

125 Test substances were dissolved in acetone in a stock solution corresponding to the highest test  
126 dosage. For this test the treated soil was prepared individually in each test vessel. Sand (1 g)  
127 was placed in each screw top glass jar (~200 ml vol.), appropriate volumes of the test  
128 solutions were then added to the sand and allowed to evaporate for 24 h. In addition, to the  
129 test soils, controls, and solvent controls (acetone) were prepared in the same manner using the  
130 highest volume of solvent used to generate the test soils (500 ml kg<sup>-1</sup> acetone). Half-  
131 moistened soil (20 g d. wt) was then added to each jar and mixed thoroughly. After mixing,  
132 the moisture content of the soils was adjusted to 50 % maximum WHC.

133

134 *2.4 Ecotoxicity tests*

135 *(a) Collembola reproduction test*

136 Tests were carried out in 100 ml glass jars filled with 30 g (w. wt.) of test soil. Fifteen, 10-  
137 12 d old *F. candida* were placed into each vessel with 2 mg of bakers yeast. Test vessels were  
138 maintained at 20 ± 1 °C with a 16:8 h photo-period and moisture levels were adjusted twice  
139 weekly. After 14 d, 2 mg of bakers yeast was added to each vessel and after 28 d the test was  
140 terminated. Jars were flooded with deionised water, stirred, and the contents transferred to  
141 200 ml glass containers. A small quantity (~2 ml) of black Indian ink was added to each jar to  
142 increase the definition of the collembolans for counting. Digital photographs were taken and  
143 the number of surviving adults and juveniles quantified by manual counting and marking of  
144 photographs.

145



146 (b) *Earthworm reproduction test*

147 Flat-bottomed glass jars (1 L) were filled with 800 g (w. wt.) of test soil and eight adult  
148 *E. fetida* (400 ± 100 mg) with a pronounced clitellum were placed into each vessel. After  
149 24 h, 0.5 g of dried, ground rabbit manure was added to each vessel and weekly thereafter.  
150 Vessels were sealed with netting and elastic bands, maintained at 20 ± 1 °C with a 16:8 h  
151 photo-period and moisture levels adjusted three times per week. After 28 d, adult earthworms  
152 were dry sieved from the test vessels using a 10 mm sieve, and the number of surviving adults  
153 was recorded. The sieved soils were then returned to the vessels with additional food (0.5 g)  
154 and left for a further 28 d. After this time each vessel was wet sieved using 1 and 0.5 mm  
155 sieves and the number of juvenile worms counted.

156

157 (c) *Enchytraeid reproduction test*

158 Prior to the test, approximately 1000 adult *E. albidus* (approx 1 cm long with eggs in the  
159 clitellum region) were acclimated in OECD artificial soil for 24 h. Test soils were prepared in  
160 each test vessel (20 g d. wt.) as described above. Autoclaved, ground rolled oats (50 mg)  
161 were mixed into the soil in each vessel and 10 adult *E. albidus* added. Test vessels were then  
162 covered with parafilm, maintained at 20 ± 1 °C with a 16:8 h photo-period and moisture levels  
163 adjusted weekly. On days 7, 14, 21 and 35, 25 mg of rolled oats were added to each vessel.  
164 On day 21, all adult worms were removed and counted and any changes in adult behaviour  
165 were observed. After 42 d the test was terminated newly hatched worms stained using the  
166 Bengal staining method (OECD, 2004b) and counted under a microscope (10x mag.).

167

168 (d) *Predatory mite reproduction test*

169 This method was based on the method of Hamers and Krogh (1997) which has since been  
170 modified and published as OECD (2008). Containers consisting of a Perspex cylinder (5.5 cm

171 high, 6 cm diameter) with a 1 mm mesh base were filled with 60 g (w. wt.) of test soil. On day  
172 0, 100 collembolan prey (16-19 d old *F. fimetaria*) were introduced to each test vessel and  
173 allowed to disperse for 1 h. Ten female and 5 male *H. aculeifer* (16-19 d old) were allocated  
174 to each test vessel and the vessels were then covered with parafilm, maintained at  $20 \pm 1$  °C  
175 with a 12:12 h photo-period and moisture levels adjusted weekly. Collembolan prey were fed  
176 with 15 mg bakers yeast every 7 days and mites were fed young ~20 d old *F. fimetaria* twice  
177 per week. On day 21 the test was terminated and test vessels transferred to high gradient  
178 extractors for 48 h (25 watt bulb). Extracted animals were collected in universal tubes with a  
179 moist plaster of paris/charcoal base, frozen at -18 °C and counted within 48 h.

180

### 181 *2.5 Statistical analysis*

182 Average adult survival, reproduction (number of juveniles) and juvenile/adult ratios were  
183 calculated at each treatment concentration. Generalised linear models were used to model  
184 each of these variables as a function of concentration (SAS 9.1). For adult survival a logit  
185 link function was used, since the counts had both an upper and lower bound, with a binomial  
186 error. A log link transformation was used for the juveniles and juvenile/adult ratios, as these  
187 were both counts with no upper limit, with a Poisson error. In all cases allowance was made  
188 for over-dispersion. Initially models were fitted to the control data to determine whether there  
189 was a significant effect of the solvent carrier, on adult survival or reproduction. Effect  
190 estimates (LC<sub>50</sub>, effective concentration causing a 50 % reduction in survival; EC<sub>50</sub>, effective  
191 concentration causing a 50 % reduction in reproduction) were calculated as functions of  
192 model parameters and their standard errors (SE). Likelihood ratio tests were used to compare  
193 dose response effects across chemicals (SAS v. 9.1).

194

## 195 **3. Results**

196 3.1 Control treatments

197 Average adult survival in the controls (non-solvent and solvent) for collembola, earthworms  
198 and enchytraeids was greater than 95 % (data not shown). Adult control survival was lower  
199 for the predatory mite test at 73 % and 60 % for non-solvent and solvent controls respectively,  
200 which is below the 20 % mortality limit in the OECD guideline published recently. This may  
201 be due to difficulties with the extraction of these organisms from soil. The number of adults  
202 surviving was not significantly affected by the type of control treatment in all experiments  
203 ( $p > 0.05$ , data not shown), and the number of enchytraeid and predatory mite juveniles were  
204 also not significantly different between the control and solvent control treatments ( $p > 0.05$ ).  
205 In contrast, the number of juvenile collembola and earthworms were significantly different for  
206 the two control types, with higher numbers of juveniles in the solvent control compared with  
207 the ordinary control ( $p < 0.001$ , data not shown). For *F. candida*, there was an average of 678  
208 ( $\pm 73$ ) (mean ( $\pm$  SE)) juveniles in the controls and 1273 ( $\pm 107$ ) in the solvent controls, whilst  
209 for *E. fetida*, values were 31 ( $\pm 3$ ) and 63 ( $\pm 5$ ) juveniles for the control and solvent controls  
210 respectively. This shows that the presence of the solvent stimulated reproduction in these two  
211 species. Non-solvent controls were therefore excluded from the statistical analysis of juvenile  
212 data for these two species. For consistency they were also excluded from analysis of adult  
213 survival. Non-solvent controls and solvent controls were pooled for analysis of the  
214 enchytraeid and predatory mite data as no solvent effect was observed for these species.

215

216 3.2 Toxicity of naphthoquinones

217 Exposure to juglone and 1,4-naphthoquinone significantly reduced survival and reproduction  
218 of *F. candida*, *E. fetida*, and *E. albidus*, with clear dose-response relationships observed (Fig.  
219 1a-c, Table 2). The exception was the predatory mite *H. aculeifer* which had lower sensitivity  
220 to the two chemicals compared to the other species, with no significant effect of either

221 chemical on reproduction (Fig. 1d, Table 2). The juvenile/adult ratio was not significantly  
222 affected by juglone for the four species tested, indicating that where adults survived they  
223 reproduced at the same rate, irrespective of exposure concentration (Table 2). In contrast,  
224 when exposed to 1,4-naphthoquinone, adult reproduction rates (juvenile:adult ratio) of all  
225 species except *F. candida* decreased significantly with increasing concentration (Table 2).

226

227 When comparing the relative effects of the natural (juglone) and synthetic (1,4-  
228 naphthoquinone) naphthoquinones on mortality and reproduction, we found that results were  
229 generally consistent across the four species and two endpoints, with little significant  
230 difference in effect of the two chemicals (Table 2). The exceptions to this were earthworm  
231 reproduction, which was significantly more sensitive to 1,4-naphthoquinone compared to  
232 juglone ( $p < 0.0001$ ) and collembolan survival which was also more sensitive to 1,4-  
233 naphthoquinone (Table 2). These differences are reflected in the  $EC_{50}$  values for earthworm  
234 reproduction of  $239 (\pm 55)$  and  $99 (\pm 24)$   $mg\ kg^{-1}$  and the  $LC_{50}$  values for collembolan  
235 survival of  $124 (\pm 34)$  and  $75 (\pm 15)$   $mg\ kg^{-1}$  for juglone and 1,4-naphthoquinone respectively,  
236 illustrating the higher toxicity of 1,4-naphthoquinone in these instances (Table 2).

237

238 A comparison of the  $LC_{50}$  and  $EC_{50}$  values was conducted to examine the relative sensitivity  
239 of the two endpoints tested. This comparison revealed that the relative sensitivity of survival  
240 and reproduction was consistent within the species tested but varied interspecifically (Table  
241 2). For *F. candida*, reproduction was less sensitive than survival for both naphthoquinones,  
242 whilst for *E. fetida* and *E. albidus* the opposite was true.

243

244 *3.3 Toxicity of anthraquinones*

245 Overall, the anthraquinones were considerably less toxic to the four species than the  
246 naphthoquinones. Despite this, some interspecific variation in sensitivity of both mortality and  
247 reproduction was revealed in the results. The only significant finding was that emodin  
248 significantly inhibited reproduction of *E. fetida*, with an EC<sub>50</sub> value of 182 ( $\pm$  44) mg kg<sup>-1</sup>  
249 (Table 2, Fig. 2b). In contrast adult *E. fetida* were insensitive to anthraquinone exposure in  
250 terms of survival. This finding was reflected in the significant effect of emodin on the  
251 juvenile/adult ratio. Of the four species tested, *E. albidus* was the least sensitive to the two  
252 anthraquinones, with no significant effect on survival or reproduction (the apparent significant  
253 positive dose response relationship for adult survival under exposure to emodin is an artefact  
254 of the very small variation in survival rates for this species) (Table 2, Fig. 2c). Results from  
255 the predatory mite tests showed no significant effect of either chemical on survival or  
256 reproduction. Examination of the plotted data (Fig. 2d) shows very similar response to the  
257 two chemicals, however, the variability of the dataset makes it difficult to draw firm  
258 conclusions.

259

260 Conclusions on the relative toxicity of emodin (natural) and quinizarin (synthetic) were  
261 generally consistent across the four species tested. The two chemicals had similar toxicity to  
262 each other with respect to adult survival and reproduction for three out of four species (Table  
263 2). The exceptions to this were effects on adult survival of *E. albidus* and reproduction of  
264 *E. fetida*. For *E. albidus*, the apparent increased survival under increasing doses of emodin is  
265 clearly erroneous and Figure 2c illustrates negligible effects of the two chemicals. Sensitivity  
266 of earthworm reproduction was significantly different for the two chemicals, with  
267 reproduction more sensitive to emodin than quinizarin ( $p < 0.0001$ ). This finding was  
268 confirmed by the EC<sub>50</sub> values of 182 ( $\pm$  44) mg kg<sup>-1</sup> and  $>512$  mg kg<sup>-1</sup> for emodin and  
269 quinizarin respectively (Table 2).

270

#### 271 **4. Discussion**

272 This study compared the toxicity of two chemical pairs from natural and synthetic origins to  
273 test the hypothesis that “chemicals which are structurally similar will have similar toxic  
274 effects”. Overall, the experiments produced consistent results which revealed that within the  
275 chemical pairs, toxicity to lethal and sub-lethal endpoints was similar for the four invertebrate  
276 species tested. The exception to this rule was earthworm reproduction, which showed  
277 differential sensitivity to the chemicals in both naphthoquinone and anthraquinone pairs. The  
278 study also revealed that at the highest concentrations tested naphthoquinones were  
279 significantly more toxic than the anthraquinones to survival and reproduction, for three of the  
280 four species tested.

281

##### 282 *4.1 Toxicity measurements*

283 There is very little data available on the toxicity of naphthoquinones and anthraquinones to  
284 soil organisms, and there is no information on their toxicity to soil invertebrates.  
285 Naphthoquinones, particularly juglone, have been reported to have phytotoxic and anti-  
286 microbial properties (Mahoney et al., 2000; Willis, 2000; Hejl and Koster, 2004), and are  
287 toxic to a range of aquatic organisms including invertebrates (Wright et al., 2007).  
288 Naphthoquinones have also been tested for their efficacy as control agents for invertebrate  
289 crop pests, as they act as growth-disruptors to above-ground invertebrates (Banerjee et al.,  
290 2001; Lee, 2008).

291

292 There is a similar paucity of data for anthraquinone effects on soil organisms, although  
293 emodin has been shown to possess allelopathic, phytotoxic, anti-microbial and insecticidal  
294 properties (Izhaki, 2002; Basu et al., 2005). Toxicity data for anthraquinones tested

295 specifically on soil invertebrates could not be located, however, insecticidal activity of  
296 emodin to mosquito larvae has been demonstrated (Georges et al., 2008). Anthraquinones  
297 have also been tested for their efficacy as biocides in controlling aquatic nuisance species in  
298 ships' ballast water including aquatic plants and invertebrates. The study concluded that  
299 naphthoquinones showed higher toxicity and were toxic to a wider range of organisms than  
300 anthraquinones, which echoes the conclusions of this study (Wright et al., 2007). Whilst these  
301 findings demonstrate the biological activities of naphthoquinones and anthraquinones, the  
302 effect concentrations cannot be compared with the results of this study as they were not  
303 conducted in soil, which significantly affects the degree of exposure to which the organisms  
304 are subjected.

305

#### 306 *4.2. Interspecific variation in sensitivity*

307 In terms of the relative sensitivity of the four invertebrate species, the predatory mite  
308 *H. aculeifer* was the least sensitive to the two naphthoquinones. Exposure to both juglone and  
309 1,4-naphthoquinone resulted in 100 % mortality of collembola, earthworms and enchytraeids  
310 at nominal concentrations = 512 mg kg<sup>-1</sup>, and reproduction was similarly sensitive (Fig 1a-c).  
311 In contrast, neither chemical had significant effects on reproduction of predatory mites, and  
312 their effect on predatory mite survival did not cause 100% mortality at any concentration  
313 tested (Fig. 1d). Interspecific differences in sensitivity to the anthraquinones were less clear  
314 cut than for the naphthoquinones, with conflicting results recorded for the two endpoints.  
315 Emodin and quinizarin increased mortality of collembola and predatory mites with increasing  
316 concentration, but were of limited toxicity to adult survival of earthworms and enchytraeids  
317 (Table 2, Fig. 2). Yet, when reproductive effects were examined, earthworm reproduction  
318 was significantly reduced on exposure to emodin.

319

320 The interspecific differences in sensitivity to the two naphthoquinones may be explained by  
321 the feeding habits of the four species, and the consequent degree of soil contact which each  
322 species is subject to. *F. candida*, *E. albidus* and *E. fetida* all burrow in the soil, and ingest  
323 micro-organisms, organic matter, or soil and will therefore ingest the chemicals incorporated  
324 into the soil. In contrast, *H. aculeifer* is a predatory mite feeding on live collembola prey. Its  
325 predominant exposure routes are likely to be through the chemical residue remaining in the  
326 collembola prey, and through soil contact via absorption through thin areas of the cuticle, with  
327 negligible direct ingestion of the chemicals themselves. This difference may explain their  
328 lower sensitivity, but does not explain the differences observed in response to the  
329 anthraquinones described above. This theory could only be confirmed by further experiments  
330 analysing the uptake of chemicals within the individuals exposed. It must also be noted that  
331 the survival of predatory mites in the control and solvent controls was lower than the control  
332 survival of the other three species tested. We do not have an explanation for this poor survival  
333 rate but it is possible that high mortality in the controls could have affected the overall  
334 validity of the test for this species.

335

336 It was not possible to locate any comparative invertebrate toxicity data for naphthoquinones  
337 or anthraquinones. However, there are studies investigating the relative sensitivity of soil  
338 invertebrate species to other toxic chemicals. In most cases collembolans were found to be  
339 more sensitive than earthworms in terms of survival and reproduction with enchytraeids the  
340 least sensitive of the three to cadmium (Bierkens et al., 1998) polycyclic aromatic  
341 hydrocarbons (Sverdrup et al., 2002) and antibacterial agents (Jensen et al., 2003). As far as  
342 we know there have been no studies comparing the relative sensitivity of predatory mites with  
343 other soil invertebrates under individual species tests. However, more complex food web  
344 exposures to metal and organic contaminants have generally shown low sensitivity of



345 *H. aculeifer*, compared with enchytraeids, but similar sensitivity to collembola (Cortet et al.,  
346 2006; Pernin et al., 2006; Scott-Fordsmand et al., 2008). These studies are likely to produce  
347 different results to single species tests as species interactions and chemical toxicity to the  
348 mites prey will play a role in their sensitivity, in contrast to our test where unlimited prey  
349 were supplied.

350

### 351 *Comparison of natural and synthetic chemical pairs*

352 The focus of this study was to compare the toxicity of structurally similar compounds.  
353 Overall, the toxicity within the chemical pairs was broadly similar for both lethal and sub-  
354 lethal endpoints. The exception was earthworm reproduction which was differentially  
355 sensitive to both the naphthoquinone and anthraquinone pairs, with the natural toxin emodin  
356 and the synthetic toxin 1,4-naphthoquinone being more toxic than their respective analogues.

357

358 For the naphthoquinones, the slight difference in the structures of the two naphthoquinones  
359 could be responsible for the difference in toxicity. Quinones, such as 1,4-naphthoquinone, act  
360 as redox cyclers creating oxidative stress within the organism. Studies with quinones,  
361 including 1,4-naphthoquinone, have shown that the addition of an electron-donating group,  
362 such as the hydroxyl group on juglone, makes the quinone a weaker oxidising agent and  
363 therefore less toxic (Schultz et al., 1997). Given that the two chemicals appear to affect  
364 survival in the same way it would appear that 1,4-naphthoquinone has a specific effect on  
365 reproduction which may be caused by the absence of the hydroxy group (Schultz et al., 1997).

366

367 The higher toxicity of emodin compared with quinizarin, to earthworm reproduction, could  
368 also be due to differences in chemical structure, with emodin containing an additional  
369 hydroxyl and methyl group. Although this is contrary to published evidence which suggests

370 that the presence of additional hydroxy groups on substituted quinones may reduce toxicity  
371 (Schultz et al., 1997). In addition, the position of the functional group on a quinone may also  
372 affect toxicity. Schultz et al. (1997) reported lower toxicity in methyl- and methoxy-  
373 benzoquinones with a 2,5-substitution than those with a 2,6-substitution. These effects were  
374 reported to be due to stereo-electronic disturbance in the 2,5-substitution, which may not have  
375 been present in the 2,6-substitution. Such effects could be responsible for the lower toxicity of  
376 quinizarin. In addition, the presence of the methyl group, as an electron accepting moiety on  
377 emodin, may also be responsible for the greater toxicity of this substance.

378

### 379 **Conclusion**

380 Data generated in this study revealed that natural and synthetic chemicals which were  
381 structurally similar, had similar toxic effects on lethal and sub-lethal endpoints for three out of  
382 four soil invertebrates tested. This would indicate that data on natural chemicals could be  
383 useful as a predictive tool for looking at chemical toxicity to a suite of species. However, the  
384 differential effect of the chemicals on earthworm reproduction demonstrates that this  
385 approach may not be protective of all species. Differences in toxicity identified in the present  
386 study may be related to degree of exposure and/or subtle differences in the mode of toxic  
387 action for the chemicals and species tested. It may therefore, be possible to predict some  
388 differences by looking at the structures of the compounds to identify functional groups that  
389 may infer increased or decreased toxicity in one or other chemical. By developing such  
390 techniques it may be possible to use read-across from natural to synthetic chemicals on a  
391 wider group of compounds.

392

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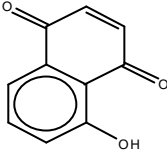
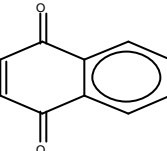
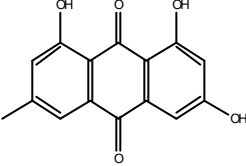
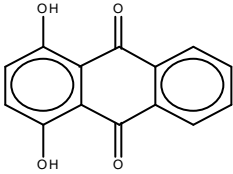
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471 .

472 **Table 1.** Key parameters of the selected natural chemicals and their respective synthetic  
 473 analogues: Chemical Abstracts Service (CAS) number, molecular weight (MW), predicted  
 474 water solubility ( $S_w$ ) and  $\log K_{ow}$ .

Compound	CAS no.	Structure	MW (g)	$S_w$ (mg l <sup>-1</sup> ) <sup>a</sup>	$\log K_{ow}$ <sup>a</sup>
<i>Naphthoquinones</i>					
Juglone (5-hydroxy-1,4-naphthoquinone)	481-39-0		174.16	5121	1.92
1,4-Naphthoquinone	130-15-4		158.16	2417 (25°C)	1.71
<i>Anthraquinones</i>					
Emodin (1,3,8-trihydroxy-6-methylantraquinone)	518-82-1		270.24	2.04 (19°C)	4.01
Quinizarin (1,4-dihydroxyanthraquinone)	81-64-1		240.22	3.34	3.94

475

476 <sup>a</sup> Model predictions generated using EPIWIN - Estimation Programs Interface for Windows  
 477 (U.S. Environmental Protection Agency, 2006).

478

479 **Table 2.** The effects of exposure to natural and synthetic naphthoquinones and anthraquinones on survival and reproduction of *Folsomia*  
480 *candida* (28 d exposure), *Eisenia fetida* (56 d exposure), *Enchytraeus albidus* (42 d exposure) and *Hypoaspis aculeifer* (21 d exposure) cultured  
481 in OECD artificial soil (OECD, 2004a). Estimated LC<sub>50</sub>/EC<sub>50</sub> (SE) reported as appropriate. Data were subject to analysis of variance (General  
482 linear model, SAS v. 9.1) and are illustrated in Figs. 1-2. Columns labelled p give significance of test for dose-response relationship, or for test of  
483 difference in dose-response relationship between sets of chemicals.

484



Chemical	<i>Folsomia candida</i>		<i>Eisenia fetida</i>		<i>Enchytraeus albidus</i>		<i>Hypoaspis aculeifer</i>	
	P	LC <sub>50</sub> /EC <sub>50</sub> (se)	P	LC <sub>50</sub> /EC <sub>50</sub> (se)	P	LC <sub>50</sub> /EC <sub>50</sub> (se)	P	LC <sub>50</sub> /EC <sub>50</sub> (se)
<b>Adult survival</b>								
Juglone	<0.0001	124 (34)	n/a	385 <sup>a</sup>	<0.0001	133 (4)	0.001	857 (192)
1,4-Naphthaquinone	<0.0001	75 (15)	n/a	385 <sup>a</sup>	<0.0001	148 (14)	0.001	606 (143)
Emodin	0.136	404 (212)	n/a	>512 <sup>b</sup>	0.035	n/a	0.149	>1024
Quinizarin	0.527	>512	n/a	>512 <sup>b</sup>	0.859	>1024	0.225	>1024
Juglone v. 1,4-Naphthoquinone	0.027		n/a		0.434		0.279	
Emodin v. Quinizarin	0.590		n/a		0.011		0.700	
All four	<0.0001		n/a		<0.0001		0.053	
<b>Reproduction</b>								
Juglone	<0.0001	192 (37)	<0.0001	239 (55)	<0.0001	79 (12)	0.055	>1024
1,4-Naphthaquinone	<0.0001	110 (16)	<0.0001	99 (24)	<0.0001	87 (17)	0.548	>1024
Emodin	0.758	>512	<0.0001	182 (44)	0.259	n/a	0.614	>1024
Quinizarin	0.192	n/a	0.426	>512	0.941	n/a	0.806	>1024
Juglone v. 1,4-Naphthoquinone	0.074		0.0002		0.854		0.756	
Emodin v. Quinizarin	0.254		<0.0001		0.381		0.824	
All four	<0.0001		<0.0001		<0.0001		0.868	
<b>Juvenile/ adult ratio</b>								
Juglone	0.922		0.224		0.106		0.987	
1,4-Naphthaquinone	0.779		0.003		0.007		0.004	
Emodin	0.178		<0.0001		0.327		0.679	
Quinizarin	0.001		0.427		0.887		0.322	
Juglone v. 1,4-Naphthoquinone	0.995		<0.0001		0.132		0.034	
Emodin v. Quinizarin	0.686		<0.0001		0.536		0.671	
All four	0.941		<0.0001		0.001		0.163	

485 n/a: model did not converge or gave zero or positive dose-response relationship.

486 <sup>a</sup> adult survival was 100% at concentration 256, 0% at 512, value given is midpoint, model fit and SE ill-defined.

487 <sup>b</sup> no adult mortality at highest dose

488 **Figure 1.** Effects of juglone --●-- and 1,4-naphthoquinone —■— (nominal concentrations)  
489 on adult survival and reproduction of (a) *Folsomia candida* (28 d exposure), (b) *Eisenia*  
490 *fetida* (56 d exposure), (c) *Enchytraeus albidus* (42 d exposure), (d) *Hypoaspis aculeifer* (21 d  
491 exposure) cultured in standard OECD artificial soil (OECD, 2004a). Data represent the mean  
492  $\pm$  SE.

493

494 **Figure 2.** Effects of emodin --○-- and quinizarin —□— (nominal concentrations) on adult  
495 survival and reproduction of (a) *Folsomia candida* (28 d exposure), (b) *Eisenia fetida* (56 d  
496 exposure), (c) *Enchytraeus albidus* (42 d exposure), (d) *Hypoaspis aculeifer* (21 d exposure)  
497 cultured in standard OECD artificial soil (OECD 2004a). Data represent the mean  $\pm$  SE.

