Article (refereed)

Whitaker, J.; Chaplow, J.S.; Potter, E.; Scott, W.A.; Hopkin, S.; Harman, M.; Sims, I.; Sorokin, N.. 2009 The comparative toxicity to soil invertebrates of natural chemicals and their synthetic analogues. *Chemosphere*, 76 (3). 345-352. 10.1016/j.chemosphere.2009.03.060

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

J. Whitaker[a*], J.S. Chaplow[a], E. Potter[a], W.A. Scott[a], S. Hopkin[b], M. Harman[c], I. Sims[d] and N. Sorokin[e]

(a) Centre for Ecology & Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster, LA1 4AP, UK.

(b) Division of Zoology, School of Animal & Microbial Sciences, University of Reading, Reading, RG6 6AJ, UK.

(c) WRc PLC, Blagrove Park, Swindon, SN5 8YF, UK.

(d) Syngenta, Jealott's Hill International Research Centre, Bracknell, RG42 6EY, UK.

(e) Reckitt Benckiser, Dansom Lane, Hull, HU8 7DS, UK.

*Corresponding author. Tel.: +44 1524 595888; fax +44 1524 61536.

E-mail address: jhart@ceh.ac.uk

Abstract

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

Keywords: anthraquinone, napthoquinone, ecotoxicity, risk assessment

1. Introduction

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

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

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

2.1 Test species

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

2.2 Chemicals

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

97 Aldrich, UK. Emodin (1,3,8-trihydroxy-6-methylanthraquinone) was obtained from
98 Xtrasynthase 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 ± 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^{-1} d. wt for all tests,
108 except the enchytraeid and predatory mite tests with the napthoquinones where the range was
109 4 to 512 mg kg^{-1} (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^{-1} or 26.5 ml kg^{-1} 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^{-1} 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

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

2.5 Statistical analysis

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

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 (± 73) (mean (± SE)) juveniles in the controls and 1273 (± 107) in the solvent controls, whilst
209 for *E. fetida*, values were 31 (± 3) and 63 (± 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

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

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

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

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_{50} value of 182 (± 44) mg kg^{-1}
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_{50} values of 182 (± 44) mg kg^{-1} and >512 mg kg^{-1} for emodin and
269 quinizarin respectively (Table 2).

4. Discussion

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

4.1 Toxicity measurements

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

There is a similar paucity of data for anthraquinone effects on soil organisms, although emodin has been shown to possess allelopathic, phytotoxic, anti-microbial and insecticidal 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^{-1}, 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-naphthaquinone, act
360 as redox cyclers creating oxidative stress within the organism. Studies with quinones,
361 including 1,4-naphthaquinone, 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-naphthaquinone 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

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

Conclusion

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

Acknowledgements

This work was funded by the American Chemistry Council (grant number EEE-00-05).

References

Banerjee, S., Magdum, S., Kalena, G.P., Banerji, A., 2001. Insect growth regulatory activity of naturally occurring quinones and their derivatives in *Dysdercus koenigii* Fabr. (Hem., Pyrrhocoridae). J. Appl. Entomol. 125, 25-30.

Basu, S., Ghosh, A., Hazra, B., 2005. Evaluation of the antibacterial activity of *Ventilago madraspatana* Gaertn., *Rubia cordifolia* Linn. and *Lantana camara* Linn.: Isolation of emodin and physcion as active antibacterial agents. Phytother. Res. 19, 888-894.

Bierkens, J., Klein, G., Corbisier, P., Van den Heuvel, R., Verschaeve, L., Weltens, R., Schoeters, G., 1998. Comparative sensitivity of 20 bioassays for soil quality. Chemosphere 37, 2935-2947.

Cortet, J., Joffre, R., Elmholt, S., Coeurdassier, M., Scheifler, R., Krogh, P.H., 2006. Interspecific relationships among soil invertebrates influence pollutant effects of phenanthrene. Envir. Toxic. Chem. 25, 120-127.

DEFRA, 2006. REACH Partial Regulatory Impact Assessment after Common Position. Department for Environment, Food and Rural Affairs, May 2006.

ECB, 2004. European Chemical Substances Information System (ESIS) http://ecb.jrc.it/. European Chemicals Bureau, Ispra, Italy.

Georges, K., Jayaprakasam, B., Dalavoy, S.S., Nair, M.G., 2008. Pest-managing activities of plant extracts and anthraquinones from *Cassia nigricans* from Burkina Faso. Bioresource Technol. 99, 2037-2045.

Hamers, T., Krogh, P.H., 1997. Predator-prey relationships in a two-species toxicity test system. Ecotox. Envir. Safe. 37, 203-212.

417 Harborne, J.B., Baxter, H., 1996. Dictionary of Plant Toxins. John Wiley & Sons, London.

418 Hejl, A.M., Koster, K.L., 2004. Juglone disrupts root plasma membrane H^+-ATPase activity
419 and impairs water uptake, root respiration, and growth in soybean (*Glycine max*) and corn
420 (*Zea mays*). J. Chem. Ecol. 30, 453-471.

421 ISO, 1999. Soil quality - Inhibition of Reproduction of Collembola (*Folsomia candida*) by
422 Soil Pollutants. ISO/DIS 11267. International Standardization Organisation, Geneva,
423 Switzerland, pp. 1–16.

424 IUCLID, 2000. IUCLID Chemical Datasheet. European Commission, European Chemicals
425 Bureau.

426 Izhaki, I., 2002. Emodin - a secondary metabolite with multiple ecological functions in higher
427 plants. New Phytol. 155, 205-217.

428 Jensen, J., Krogh, P.H., Sverdrup, L.E., 2003. Effects of the antibacterial agents tiamulin,
429 olanquindox and metronidazole and the anthelmintic ivermectin on the soil invertebrate
430 species *Folsomia fimetaria* (Collembola) and *Enchytraeus crypticus* (Enchytraeidae).
431 Chemosphere 50, 437-443.

432 Langenheim, J.H., 1994. Higher-plant terpenoids - a phytocentric overview of their ecological
433 roles. J. Chem. Ecol. 20, 1223-1280.

434 Lee, H.S., 2008. Acaricidal activity and function of mite indicator using plumbagin and its
435 derivatives isolated from *Diospyros kaki* Thunb. roots (Ebenaceae). J. Microbiol. Biotechn.
436 18, 314-321.

437 Mahoney, N., Molyneux, R.J., Campbell, B.C., 2000. Regulation of aflatoxin production by
438 naphthoquinones of walnut (*Juglans regia*). J. Agr. Food Chem. 48, 4418-4421.

OECD, 2004a. OECD Guidelines For The Testing Of Chemicals. Test No. 222: Earthworm Reproduction Test (*Eisenia fetida* / *Eisenia andrei*). Organization for Economic Co-operation and Development, Paris, France.

OECD, 2004b. OECD Guidelines For The Testing Of Chemicals: Test No. 220: Enchytraeid Reproduction Test. Organization for Economic Co-operation and Development, Paris, France.

OECD, 2008. OECD Guidelines For The Testing of Chemicals. Test No. 226: Predatory Mite (*Hypoaspis* (Geolaelaps) *aculeifer*) Reproduction Test In Soil. Organization for Economic Co-operation and Development, Paris, France.

Pernin, C., Ambrosi, J.P., Cortet, J., Joffre, R., Le Petit, J., Tabone, E., Torre, F., Krogh, P.H., 2006. Effects of sewage sludge and copper enrichment on both soil mesofauna community and decomposition of oak leaves (*Quercus suber*) in a mesocosm. Biol. Fert. Soils 43, 39-50.

Schultz, T.W., Sinks, G.D., Cronin, M.T.D., 1997. Quinone-induced toxicity to Tetrahymena: structure-activity relationships. Aquat. Toxicol. 39, 267-278.

Scott-Fordsmand, J.J., Maraldo, K., van den Brink, P.J., 2008. The toxicity of copper contaminated soil using a gnotobiotic Soil Multi-species Test System (SMS). Environ. Int. 34, 524-530.

Sorokin, N., 2007. The fate and effects of natural chemicals and their structural analogues in the terrestrial environment. PhD thesis, School of Biological Sciences. University of Reading, p. 253.

Sorokin, N., Whitaker, J. 2008. The impacts of selected natural plant chemicals on terrestrial invertebrates. In: Karlovsky, P. (Ed.) Secondary Metabolites in Soil Ecology, Soil Biology vol. 14, Springer, pp255-268.

461 Sverdrup, L.E., Krogh, P.H., Nielsen, T., Stenersen, J., 2002. Relative sensitivity of three
462 terrestrial invertebrate tests to polycyclic aromatic compounds. Envir. Toxic. Chem. 21, 1927-
463 1933.

464 U.S. Environmental Protection Agency. 2006. Estimation Programs Interface for Windows
465 (EPI Suite). http://www.epa.gov/opptintr/exposure/

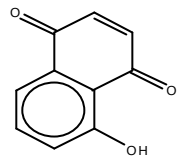
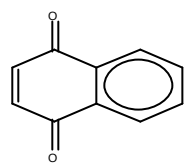
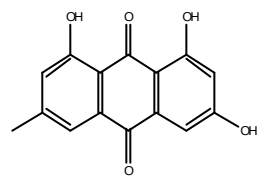
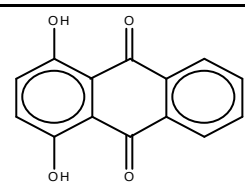
466 Willis, R.J., 2000. Juglans spp, juglone and allelopathy. Allelo. J. 7, 1-55.

467 Wright, D.A., Dawson, R., Cutler, S.J., Cutler, H.G., Orano-Dawson, C.E., 2007. Screening
468 of natural product biocides for control of non-indigenous species. Envir. Technol. 28, 309-
469 319.

470

471 .

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

Compound	CAS no.	Structure	MW (g)	S*w* (mg l^{-1})[a]	log K_{ow}[a]
Naphthoquinones					
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
Anthraquinones					
Emodin (1,3,8-trihydroxy-6-methylanthraquinone)	518-82-1		270.24	2.04 (19°C)	4.01
Quinizarin (1,4-dihydroxy-anthraquinone)	81-64-1		240.22	3.34	3.94

[a] Model predictions generated using EPIWIN - Estimation Programs Interface for Windows (U.S. Environmental Protection Agency, 2006).

Table 2. The effects of exposure to natural and synthetic naphthoquinones and anthraquinones on survival and reproduction of *Folsomia candida* (28 d exposure), *Eisenia fetida* (56 d exposure), *Enchytraeus albidus* (42 d exposure) and *Hypoaspis aculeifer* (21 d exposure) cultured in OECD artificial soil (OECD, 2004a). Estimated LC_{50}/EC_{50} (SE) reported as appropriate. Data were subject to analysis of variance (General 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 difference in dose-response relationship between sets of chemicals.

Chemical	*Folsomia candida*		*Eisenia fetida*		*Enchytraeus albidus*		*Hypoaspis aculeifer*	
	p	LC_{50}/EC_{50} (se)	p	LC_{50}/EC_{50} (se)	p	LC_{50}/EC_{50} (se)	p	LC_{50}/EC_{50} (se)
Adult survival								
Juglone	<0.0001	124 (34)	n/a	385[a]	<0.0001	133 (4)	0.001	857 (192)
1,4-Naphthaquinone	<0.0001	75 (15)	n/a	385[a]	<0.0001	148 (14)	0.001	606 (143)
Emodin	0.136	404 (212)	n/a	>512[b]	0.035	n/a	0.149	>1024
Quinizarin	0.527	>512	n/a	>512[b]	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	
Reproduction								
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	
Juvenile/ adult ratio								
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	

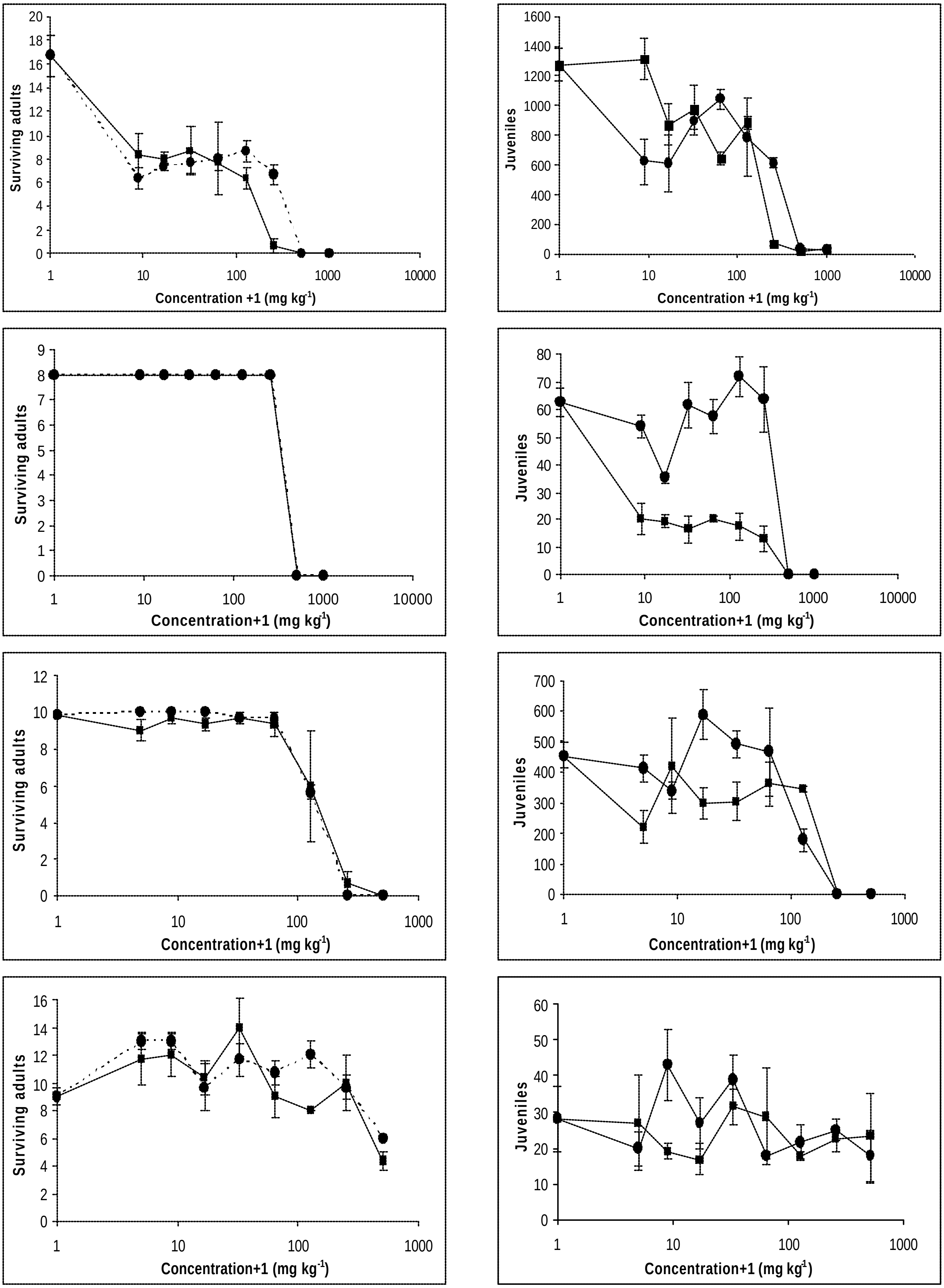
n/a: model did not converge or gave zero or positive dose-response relationship.

[a] adult survival was 100% at concentration 256, 0% at 512, value given is midpoint, model fit and SE ill-defined.

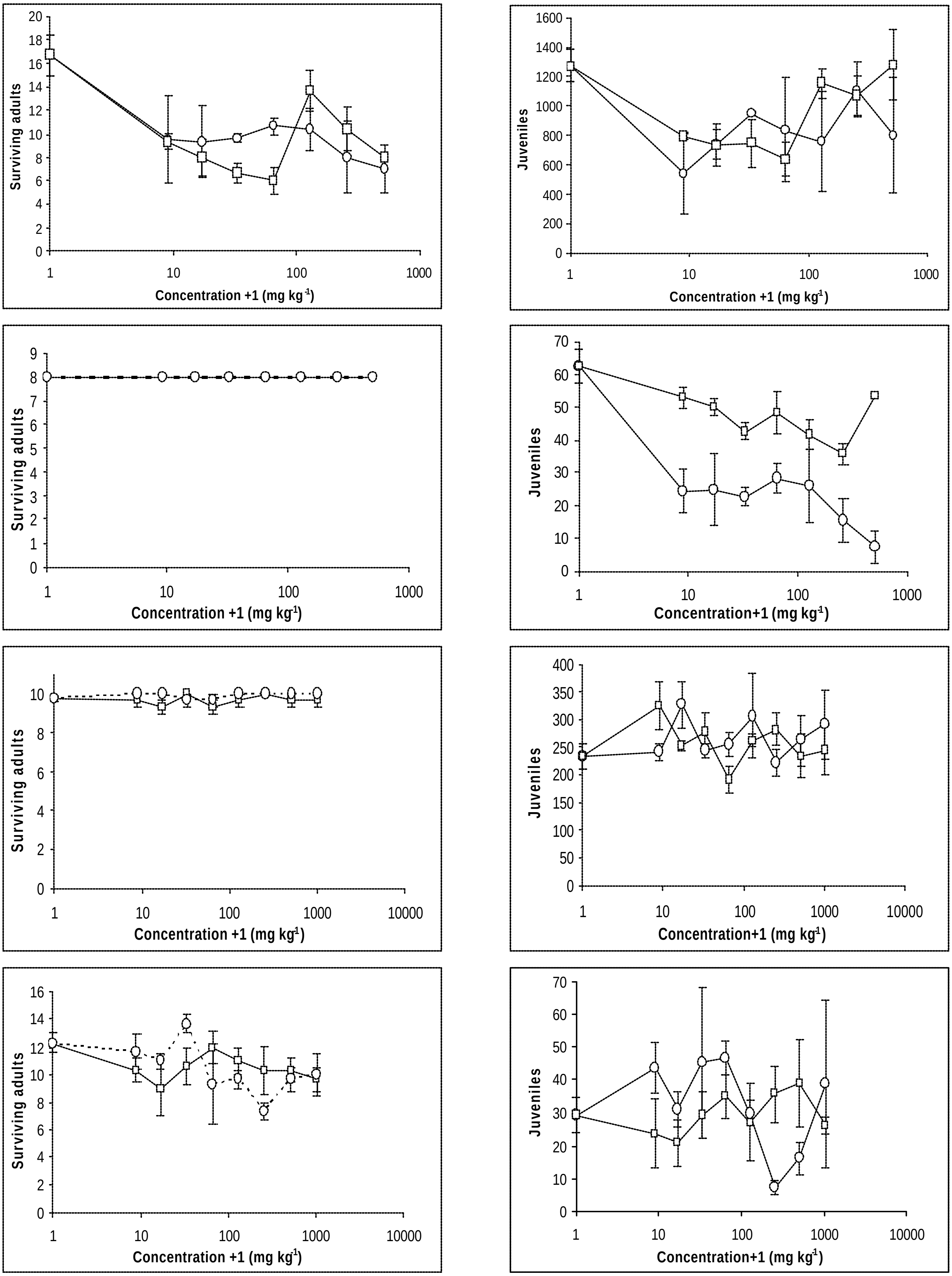
[b] no adult mortality at highest dose

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

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


Surviving adults
0
2
4
6
8
10
12
14
16
18
20
1
10
100
1000
10000
Concentration +1 (mg kg-1)
Juveniles
0
200
400
600
800
1000
1200
1400
1600
1
10
100
1000
10000
Concentration +1 (mg kg-1)
Surviving adults
0
1
2
3
4
5
6
7
8
9
1
10
100
1000
10000
Concentration+1 (mg kg-1)
Juveniles
0
10
20
30
40
50
60
70
80
1
10
100
1000
10000
Concentration+1 (mg kg-1)
Surviving adults
0
2
4
6
8
10
12
1
10
100
1000
Concentration+1 (mg kg-1)
Juveniles
0
100
200
300
400
500
600
700
1
10
100
1000
Concentration+1 (mg kg-1)
Surviving adults
0
2
4
6
8
10
12
14
16
1
10
100
1000
Concentration+1 (mg kg-1)
Juveniles
0
10
20
30
40
50
60
1
10
100
1000
Concentration+1 (mg kg-1)

498


Surviving adults
Concentration +1 (mg kg-1)
Juveniles
Concentration +1 (mg kg1)
Surviving adults
Concentration +1 (mg kg1)
Juveniles
Concentration+1 (mg kg1)
Surviving adults
Concentration +1 (mg kg1)
Juveniles
Concentration+1 (mg kg1)
Surviving adults
Concentration +1 (mg kg1)
Juveniles
Concentration+1 (mg kg1)