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3   **Fate, Uptake and Distribution of Nanoencapsulated Pesticides in Soil-Earthworm**

4   **Systems and Implications for Environmental Risk Assessment**

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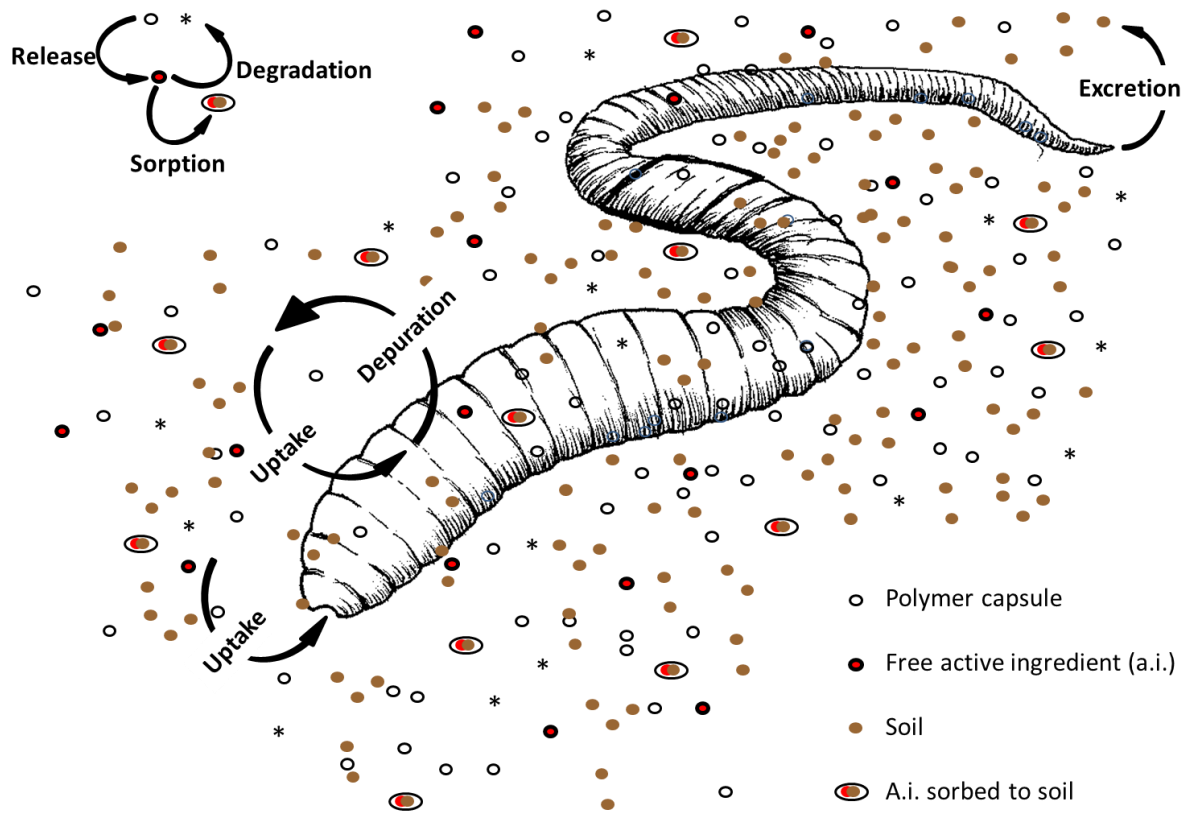
12   **Abstract**

13   Nanopesticides are novel plant protection products offering numerous benefits. As

14   nanoparticles behave differently from dissolved chemicals, environmental risks of these

15   materials could differ from conventional pesticides. Here we used soil-earthworm systems to

16   compare the fate and uptake of analytical grade bifenthrin to that of bifenthrin in traditional and

17   nano-encapsulated formulations. Apparent sorption coefficients for bifenthrin in the nano-

18   treatments were up to 3.8 times lower than in the non-nano treatments whereas dissipation

19   half-lives of the nano-treatments were up to two time longer. Earthworms in the nano-

20   treatments accumulated around 50% more bifenthrin than those in the non-nano treatments.

21   In the non-nano treatments, most of the accumulated material was found in the earthworm

22   tissue while in the nano-treatments, the majority resided in the gut. Evaluation of toxicokinetic

23   modelling approaches showed that models incorporating the release rate of bifenthrin from

24   the nanocapsule and distribution within the earthworm provided the best estimations of uptake

25   from the nanoformulations. Overall, our findings indicate that the risks of nanopesticides may

26 be different from conventional formulations. The modelling presented here provides a starting

27 point for assessing risks of these materials but needs to be further developed to better

28 consider the behaviour of the nanoencapsulated pesticide within the gut system.

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31

32   Graphical abstract

Release   *
Degradation
Sorption

Depuration
Uptake
Uptake

Excretion

○ Polymer capsule

● Free active ingredient (a.i.)

• Soil

◉ A.i. sorbed to soil

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34

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Introduction

Recently, novel pesticide products have been developed that employ nanotechnology (Kah et al., 2013; Kah, 2015). These so called 'nanopesticides' comprise either nanoparticulate forms of a pesticide active ingredient or nanocapsules containing an active ingredient (a.i.). Nanopesticides offer a range of advantages over conventional pesticides in that they may increase efficacy of the a.i. and/or enhance the environmental and human health safety profiles of the products (Kah et al., 2013; Kookana et al., 2014). However, there is recognition that the application of nanotechnology could also have negative and unanticipated impacts on the environment so it is also possible that nanopesticides could pose a greater risk than equivalent conventional pesticide products. The applicability of existing environmental risk assessment approaches for pesticides to nanoformulations has also been questioned (Kah, 2015).

One group of organisms that will be exposed to nanopesticides are terrestrial invertebrates such as earthworms. Earthworms are known to bio-magnify inorganic and organic soil contaminants, including pesticides, polycyclic aromatic hydrocarbons, brominated flame retardants, and metals (Heikens et al., 2001; Matscheko et al., 2002; Langdon et al., 2005). Earthworms being at the base of a food chain hold an integral position. Uptake and accumulation of contaminants into earthworms not only poses a risk to the earthworm directly, but bioaccumulation and contaminant transfer through the food chain to top predators such as birds has the potential to result in secondary poisoning (Spurgeon and Hopkin, 1996).

Data for other non-pesticide nanoparticles shows that these materials can be taken up by earthworms (Kwak and Youn-Joo, 2005). Investigations determining distribution of nanoparticles show that highest concentrations of accumulated materials are associated with the earthworm gut (Unrine et al., 2010; Waissi-Leinonen et al. 2012). Adverse effects have also been reported in earthworms following exposure to carbon-based and metal and metal oxide nanoparticles (Kwak and Youn-Joo, 2005; Scott-Fordsmand et al. 2008).

62 To date, the focus of research into bioconcentration and impacts of nanoparticles on

63 earthworms has been on metals and metal oxides (Kwak and Youn-Joo, 2005), carbon

64 nanotubes (Petersen et al., 2008, Petersen et al., 2011, Scott-Fordsmand et al. 2008) and

65 fullerenes (Li et al. 2010, Kelsey and White 2013). To the best of our knowledge, no-one has

66 explored the uptake of nanopesticides, even though it is inevitable that earthworms will be

67 exposed to these products during use. Therefore, here we investigate the effects of

68 nanoencapsulation on the fate, uptake, depuration and distribution of a pesticide a.i. in soil-

69 earthworm systems. The nanoencapsulated materials used in the study were developed by

70 Vive Crop Protection Inc and comprise bifenthrin encapsulated in a polymer nanoparticle with

71 the aim to better target the active ingredient to the pest species. We compare the fate, uptake

72 and distribution of the analytical grade a.i. with that of conventional and nanoformulated

73 products for the two earthworm species *Eisenia fetida* and *Lumbricus terrestris*. The findings

74 are used to explore the suitability of existing and novel toxicokinetic models to better

75 characterise the environmental risks of nanoencapsulated substances in the future.

76 **Materials and methods**

77 *Chemicals, soils and organisms*

78 Analytical PESTANAL® grade bifenthrin was purchased from Sigma-Aldrich (Dorset, UK),

79 formulated bifenthrin (Capture LFR) was obtained from FMC Corporation (Philadelphia, USA).

80 Two nanoencapsulated formulations of bifenthrin (Nano A and B) were obtained from Vive

81 Crop Protection Inc. (Toronto, Canada). The precise make-up of these materials is proprietary

82 but both formulations employ an acrylate copolymer to encapsulate the bifenthrin but contain

83 different co-formulants. Acetonitrile (99.9%) was purchased from Fisher Scientific

84 (Loughborough, UK). Details of the bifenthrin treatments are provided in the Supporting

85 Information.

86 A sandy loam soil was obtained from Landlook (Midlands, UK). Prior to use, the soil was air

87 dried, sieved to ≤ 2 mm to ensure homogeneity within the soil matrix and stored at room

88 temperature. Characteristics of the study soil are provided in the Supporting Information.

89   *Eisenia fetida* and *Lumbricus terrestris* were obtained from Blades Biological Ltd. (Kent, UK).

90   The earthworms were cultured in a medium of horse manure and peat (50:50) for *E. fetida*,

91   and in moist soil for *L. terrestris*. They were kept moist with deionized water under laboratory

92   conditions (20 ± 3 °C). The horse manure used in this culture was collected from horses that

93   were not under medication to avoid any toxic effects on the earthworms. *E. fetida* were fed

94   twice weekly with homogenized mashed potato powder which was added to the surface of the

95   culture and *L. terrestris* were fed with dead birch leaves distributed on the surface of the moist

96   soil.

97   *Uptake and depuration studies*

98   Uptake and depuration experiments followed OECD Guideline 317 'Bioaccumulation in

99   Terrestrial Oligochaetes' and used only *E. fetida* (OECD, 2010). Experiments were performed

100   in glass jars at a concentration of 10 µg/g of active ingredient where each jar contained 50 ±

101   1 g of test soil and kept in an incubator at 20 ± 2 °C, using a 16:8 light/dark cycle. Assuming

102   a mixing depth of 20 cm, concentrations expected in the environment from the use of Capture

103   would be expected to range from 35 - 100 µg g^{-1}. The test concentration was one order of

104   magnitude lower than the concentration we used previously to assess the toxicity of the

105   different bifenthrin treatments to *E. fetida*. At 100 µg g^{-1}, no mortality, a slight increase in

106   growth and a small decrease in cocoon production were observed (Anuar, unpublished data).

107   Before the earthworms were exposed to the different treatments, they were acclimated to the

108   experimental conditions in the incubator for 48 h using non-treated soil. The different bifenthrin

109   treatments were then mixed with the soil using deionized water as solvent carrier to achieve

110   a moisture content between 60-70% of the maximum water holding capacity (MWHC). Treated

111   soil was left for 24 h before adding the earthworms.

112   For each bifenthrin treatment (analytical grade, conventional and two nanoformulations), 45

113   glass jars of treated soil were prepared. At the start of the uptake phase, one mature adult *E.*

114   *fetida* with a visible clitellum was added to each glass jar. Glass jars were then covered with

115   garden fleece (to prevent earthworms from escaping while allowing sufficient air supply to be

116   maintained) attached with an elastic band. The uptake phase of the experiment lasted for up

117   to 21 d with triplicate samples being taken at 0 and 6 h and 1, 3, 7, 10, 14, 21 d. *E. fetida* in

118   the remaining glass jars were then transferred to clean soil for up to another 21 d of depuration

119   with samples being taken at 6 h and 1, 3, 7, 10, 14, 21 d after transfer. At each time point in

120   both phases, the earthworm weight and mortality were recorded. Soil moisture content in each

121   glass jars was monitored throughout both phases, and adjusted, where necessary, by adding

122   deionized water so that it remained between 60-70% of the MWHC. The pH of the soils was

123   measured at the beginning and end of the uptake phase and at the end of the depuration

124   phase. Earthworms were fed weekly with mashed potato powder.

125   Once samples were collected, earthworms were removed, rinsed with deionized water, blotted

126   dry, weighed and then placed for 48 h on moist filter papers to allow the earthworms to purge

127   their gut contents (Dalby et al., 1996). The moist filter papers were changed twice a day (in

128   the morning and evening). The earthworms were then frozen prior to analysis. Soil samples

129   were taken for chemical analysis and to extract soil pore water.

130   *Distribution of bifenthrin in earthworms*

131   The distribution of bifenthrin following exposure to the different treatments was assessed using

132   both *E. fetida* and *L. terrestris*. Experiments were performed at the same concentration and

133   conditions as used in the uptake and depuration studies. *E. fetida*, were exposed to 50 ± 1 g

134   of soil treated with each treatment or soil only while *L. terrestis* were exposed to 350 ± 5 g of

135   treated soil or soil only. The duration of the uptake phase was 10 d while the depuration phase

136   lasted for 7 d. There were six replicates per treatment and sampling point. Soil, faeces and

137   earthworm samples were taken at the end of each phase for analysis.

138   The removed earthworms were placed on a dissecting tray with their dorsal side facing

139   upwards. Using a pair of dissecting scissors, an opening cut was made below the clitellum. A

140   straight line cut was made from the opening cut down to the posterior. The cut was made

141   carefully and not too deeply to avoid damage to the internal organs. The skin was pulled apart

142   using forceps and pinned back using dissecting pins. The earthworms were then separated

143   into skin, gut and other tissue (hereafter referred to as 'tissue') for *L. terrestris*. Separation of

144   *E. fetida* tissues proved challenging so it was only possible to separate these samples into gut

145   + tissue and skin. Prior to analysis, samples were washed with distilled water and centrifuged

146   at 3000 rpm for 15 min. Samples and washing water were analysed separately for bifenthrin

147   residues.

148   *Sample extraction and HPLC analysis*

149   Soil (5 ± 0.5 g) was extracted by adding 15 mL acetonitrile and then shaking the mixture on

150   an orbital shaker (250 oscillations min^{-1}) at room temperature (20 ± 2 °C) for 2 h. Samples

151   were then allowed to settle and 2 mL aliquots of supernatant were taken for analysis. Soil pore

152   water was obtained by placing 10 ± 1 g of soil into a glass syringe with a layer of 3 cm of glass

153   wool inserted into the bottom. The syringe was inserted into a glass centrifuge tube and

154   centrifuged for 20 min at 2016 g to separate soil and soil pore water. The difference in density

155   of the polymer capsules and water is small so centrifugation would not be expected to affect

156   the recovery of the bifenthrin from the nano-treatments compared to the non nano-treatments

157   (Kah et al., 2016).

158   Earthworm samples were homogenized for 5 minutes using a LabGen Series 7 homogenizer

159   with 5 mL of acetonitrile. The suspension was transferred, with rinsing using an additional 5

160   ml of acetonitrile, to a glass vial. The extracts were centrifuged for 20 min at 2016 g. The

161   samples were then filtered using 0.45 µm nylon filters and a 2 mL aliquot of the supernatant

162   was taken for further analysis.

163   Soil and earthworm extracts and pore water were analysed using High-performance Liquid

164   Chromatography (HPLC; Perkin Elmer, Flexar) coupled with photodiode array detection. More

165   detail on the methods used are provided in the Supporting Information. The limits of detection

166   and quantification were 1.2 and 3.7 ng mL^{-1} for the analytical grade, 1.5 and 4.7 ng mL^{-1} for

167   the conventional formulation, 1.9 and 5.9 ng mL^{-1} for nano A and 2.1 and 6.5 ng mL^{-1} for nano

168   B. Recoveries for analytical method ranged from 90-107% for water, 88-103% for soil, 84-

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169   102% for *E. fetida* and 90-107% for *L. terrestris* and recoveries of the filtration method ranged

170   from 87-100% (see supporting information).

171   *Data analysis*

172   <u>Determination of sorption coefficient, k_d</u>

173   Sorption coefficient, k_d values were calculated at each time point (Equation 1) where: C_{water}

174   and C_{soil} are the concentrations of bifenthrin in soil pore water ($\mu g\ mL^{-1}$) and soil ($\mu g\ g^{-1}$),

175   MWHC is the maximum water holding capacity of the soil (%), and %water is the moisture

176   content of the soil (%). Averages of k_d-values were then determined.

177     $k_d = \dfrac{C_{soil}}{C_{water}*(\frac{\%water}{MWHC})} - 1$ (1)

178   <u>Kinetic modelling</u>

179   We wanted to evaluate whether data on the uptake and depuration characteristics could be

180   used to inform the uptake and depuration of bifenthrin resulting from exposure to Capture LFR

181   and the two nano formulations. Three models were explored with increasing complexity. Data

182   from the analytical grade bifenthrin treatment was always used to parameterise the models.

183   Model 1 was the first order one compartment toxicokinetic model outlined by Ashauer *et al.*

184   (2010) (Equation 2).

185     $\dfrac{dC\text{organism}}{dt} = k\text{in} * C\text{water}\,(t) - k\text{out} * C\text{organism}\,(t)$ (2)

186   Where: $C_{organism}$ is the internal concentration ($\mu g\ g^{-1}$); C_{water} is the concentration in the pore

187   water ($\mu g\ mL^{-1}$); and k_{in} and k_{out} are the uptake rate constant ($mL\ g^{-1}\ h^{-1}$) and the depuration

188   rate constant (h^{-1}), respectively.

189   Model 2 was designed for estimating uptake of an active ingredient from a nonencapsulated

190   treatment. This model is an adaptation of Model 1 modified to account for the release of

191   bifenthrin from the polymer capsules into the soil pore water (Equation 3).

192     $\dfrac{dC\text{organism}}{dt} = k\text{in} * C\text{water}_2\,(t) - k\text{out} * C\text{organism}\,(t)$ (3.1)

193   with

194    $\frac{dC\text{water}_2}{dt} = (C\text{water}\,(t) - C\text{water}_2\,(t)) * k\text{r}$ (3.2)

195   Where: $C\text{water}_2$ is the concentration of the compound in the pore water released from the

196   nanoformulation ($\mu g\ g^{-1}$) and k_r is the release rate of the nanoformulation (h^{-1}). The release

197   rate can be calculated by comparing the degradation rate of the bifenthrin a.i. with the

198   degradation rate of bifenthrin in the nanoencapsulated formulation (Kah et al., 2016). A full

199   description of the approach for estimating release rate is provided in Kah et al. (2016).

200   Model 3 was used for the distribution studies. This model extends either Model 1 or 2 to

201   account for the distribution of the compound in gut, skin and tissue (Equations 4).

202    $C\text{skin} = C\text{organism}\,(t) * a$ (4.1)

203    $C\text{tissue} = C\text{organism}\,(t) * b$ (4.2)

204    $C\text{gut} = C\text{organism}\,(t) - C\text{skin} - C\text{tissue}$ (4.3)

205   Where: Cskin, Cgut and Ctissue are the concentration of the compound in skin, gut and tissue

206   ($\mu g\ g^{-1}$), and a and b are distribution coefficients between the total internal concentration and

207   the skin, the total internal concentration and the tissue. The distribution coefficients are

208   obtained using studies on analytical grade a.i..

209   <u>Statistical analysis</u>

210   Statistical analysis was performed using SigmaPlot (Version 13.0; Systat Software, San Jose,

211   CA). Data were tested performing one-way - or two-way- Analysis of Variance (ANOVA) via

212   the Holm-Sidak pairwise comparison method with the Shapiro-Wilk test for normality of data

213   and the Brown-Forsythe test for equal variance of data. Modelling was conducted in

214   OpenModel V 2.4.2. (http://openmodel.info/) using the Runge-Kulta (4th Order) ordinary

215   differential equation method with Monte Carlo simulations to obtain the 95% confidence

216   interval and the Nash–Sutcliffe Efficiency calculation for goodness of fit indication. Nash–

217   Sutcliffe values (hereafter called Nash index) between 0 and 1 represent an acceptable fit of

218   the model to the data.

219   **Results and discussion**

220   <u>Fate of bifenthrin in soil</u>

221   Generally, throughout the uptake phase, there was a decrease in concentration of bifenthrin

222   in the soil and soil pore water which was associated with an increase in the concentration of

223   bifenthrin in the earthworms (Data are summarised in the Supporting information). At the end

224   of the uptake phase, 65-82% of bifenthrin was extractable and associated with the soil

225   particles, 16-33% had dissipated/degraded, around 1% was present in the pore water and <

226   1% was taken up by the earthworms (Figure 1 in the Supporting Information). Apparent half-

227   lives for bifenthrin in the different treatments increased in the order analytical grade bifenthrin

228   – Capture LFR – Nano B – Nano A. The observed DT_{50} for the analytical grade bifenthrin is at

229   the lower end of the values reported in the field for bifenthrin and is lower than reported in

230   laboratory studies (Pesticide Properties Database, 2017). Half-lives are also lower than those

231   obtained by Kah *et al.* (2016) in similar investigations into the differences in persistence of

232   analytical grade bifenthrin and the a.i. in Capture LFR and nanoencapsulated treatments,

233   although the order of half-lives is the same (Kah et al., 2016). Release rates and associated

234   release times (RT_{50}) for the nanoformulations (Table 1) are lower than those found by Kah *et*

235   *al.* (2016). Overall, these results indicate that even traditional formulations can affect the

236   persistence of an active ingredient but this impact is more enhanced in the nano-encapsulated

237   treatments, possibly due to the nanocapsules 'shielding' the unreleased a.i. from the

238   degrading microbes.

239   Sorption coefficients (k_d), based on the soil and soil-pore water concentrations ranged from

240   154 to 585 L kg^{-1} and increased significantly in the order Nano A = Nano B < Capture LFR <

241   analytical grade bifenthrin (Table 1; Two-Way ANOVA Holm-Sidak method P < 0.001).

242   Sorption coefficients are lower than previously reported for the a.i. which range from 882 to

243   6000 mL g^{-1} in different soil types (Pesticides Property Database, 2017). Sorption coefficients

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244   are also lower than those observed by Kah et al. for bifenthrin a.i. and bifenthrin in traditional

245   and nanoencapsulated formulations (Kah et al., 2016). The mismatch is possibly explained by

246   the fact that we derived k_d values based on pore water measurements, which is arguably more

247   realistic than the batch equilibrium approach employed in previous studies. The differences

248   might be explained by dissolved organic carbon in the pore water which may act as an

249   additional sink for the bifenthrin or due to differences in the nature of the organic carbon in the

250   soils used in the different studies.

251   The observations for Capture LFR demonstrate that even traditional co-formulants can affect

252   the distribution of the bifenthrin in soils although the effect is more enhanced in the nano-

253   encapsulated treatments. Other studies have explored the effects of formulation on pesticide

254   behaviour. In studies with chlorsulfuron, co-formulants reduced sorption (Foldenyi et al., 2013)

255   while studies with propyzamide (Khan and Brown, 2017) showed sorption to increase and

256   studies with triticonazole, cyprodinil, propetamphhos and fludioxinil showed sorption to

257   increase (Beigel et al., 1998; Beigel and Barriuso, 2000; Garcia-Ortega et al., 2006; Pose-

258   Juan et al., 2011). The impacts of co-formulants therefore likely depend on the active

259   ingredient and the nature of the co-formulants used in a product. The observed reduction in

260   the k_d values for the nano-encapsulated materials is likely due to a combination of the co-

261   formulant effects and the fact that the polymer capsule shielded the bifenthrin from sorption

262   sites on the soil surface.

263   <u>Uptake and depuration behaviour</u>

264   *Uptake and depuration in* E. fetida

265   No mortality was recorded and the studies passed the validity criteria (based on earthworm

266   growth and mortality) according to the principles outlined in the OECD 317 (OECD, 2010).

267   Lower uptake and depuration was seen for bifenthrin in the analytical and Capture LFR

268   treatments compared to the two nanoformulation treatments (Figure 1). At the end of the

269   depuration phase, in the analytical grade and Capture LFR treatments, bifenthrin was still

270   detectable in the earthworms whereas for the two nanoformulation treatments, it was not

271 detectable. The pattern of uptake and depuration between the non-nano and nano treatments

272 was also different. The non-nano exposures were characterised by a steady uptake and

273 elimination of bifenthrin over time whereas in the nano treatments, an initial rapid period of

274 uptake or elimination was observed and this then tailed off (Figure 1).

275 The first order one-compartment model (Model 1) was successfully fitted to the data from the

276 analytical grade bifenthrin treatment (Nash index = 0.94) obtaining an uptake- and depuration

277 - rate constant of 0.222 ± 0.009 mL g^{-1} h^{-1} and 0.0036 ± 0.0002 h^{-1}. Use of the uptake and

278 depuration rates in the model to simulate the uptake and depuration bifenthrin from the

279 Capture LFR formulation worked well (Nash index = 0.68) but failed to acceptably simulate

280 the uptake of Nano A and Nano B (Nash index < -0.01) (Figure 1). The results for the Capture

281 modelling do, however, suggest that it may be possible to extrapolate from studies into the

282 uptake of analytical grade materials to estimate uptake of a.i.'s from traditional formulations.

283 Model 2, which incorporates the release rate of bifenthrin from the nanocapsule,

284 underestimated uptake and depuration of the bifenthrin from the two nano treatments (Nash

285 index < 0; Figure 1). Closer inspection of the simulation however revealed that this model

286 more accurately simulated the internal concentration at the end of the depuration phase. The

287 differences in kinetic patterns and model fits suggests that the nanoencapsulated bifenthrin

288 was accumulated *via* a different mechanism than in the analytical grade material and Capture

289 LFR treatments. As previous studies with earthworms have shown that other nanoparticles

290 accumulate in the earthworm gut rather than the actual tissue (Unrine et al., 2010; Waissi-

291 Leinonen 2012), we performed studies to explore whether there were any differences in the

292 distribution of the bifenthrin in earthworms exposed to the different treatments. Here, we not

293 only used *E. fetida* but also *L. terrestris* due to its larger size and consequent ease of handling.

294 *Distribution studies*

295 Concentrations of bifenthrin in *L. terrestis* were significantly greater than in *E. fetida* for all

296 treatments (P < 0.001). Within each species, uptake was significantly different between the

297 nano formulated and non-nano treatments, while within the non-nano and nano treatments no

298 significant difference in uptake was observed (P<0.001). These differences in uptake might

299 be explained by differences in the way the two earthworm species process soil organic matter,

300 their ecological strategy and/or lipid content (Kelsey et al., 2005). *Eisenia fetida* is smaller than

301 *L. terrestris* and is an epigeic species living primarily at or near the soil surface and consumes

302 coarse particulate organic matter and surface litter. *L. terrestris* is an anecic species that lives

303 in deep burrows and comes to surface to feed on surface litter [Bouche, 1983]. Interestingly,

304 the interspecies difference in uptake that we see is the opposite to that observed in a similar

305 study using pharmaceuticals covering a range of physico-chemical properties (Carter et al.,

306 2016).

307 Significant differences were also seen in the depuration of bifenthrin by the two species (P <

308 0.001). For the analytical grade and Capture LFR treatments, *L. terrestris* still contained 57 -

309 59% of the accumulated bifenthrin after the 7 d depuration phase while concentrations in

310 *E.fetida* were significantly lower (43-47%; P < 0.001). Depuration of bifenthrin from the two

311 nano treatments was faster with *L. terrestris* containing 20-22% of accumulated bifenthrin after

312 7 d depuration while *E. fetida* contained only 10-13% of the accumulated mass. Bifenthrin from

313 the nanoformulations was eliminated 2.8 and 4 times more quickly than from the non-nano

314 treatments (P < 0.001).

315 In *L. terrestris*, following the uptake phase, concentrations of bifenthrin from the analytical

316 grade and Capture LFR treatments was significantly higher (P < 0.01) in the tissue compared

317 to the gut and skin which had similar (P = 0.6) bifenthrin concentrations (Figure 2). In contrast,

318 for the nanoformulation treatments significantly (P < 0.001) higher concentrations were

319 observed in the gut of the earthworms compared to the skin and tissue. Concentrations in skin

320 and tissue were also significantly different (P < 0.001). The concentration in the gut of the

321 nano- exposed animals was significantly higher (P < 0.001) than the non-nano exposed

322 earthworms even though the concentration of bifenthrin in the soil was the same. A significant

323 difference was also observed between the two nanoformulations (P = 0.019). This might

324 indicate that the earthworms are selectively consuming the polymer capsules and/or that the

325 capsules are becoming 'trapped' in the gut of the animal and are eliminated more slowly than

326 the bulk soil. This seems to be nanoformulation specific. While it is not known whether

327 earthworms are able to select finer material from coarser particles, there is a body of evidence

328 indicating that they do exhibit preferences for different food types (Curry and Schmidt, 2007).

329 At the end of the 7 d depuration phase, for the analytical grade and Capture LFR treatments,

330 highest concentrations of bifenthrin in *L. terrestris* were seen in the tissue while for the

331 nanoformulation treatments highest concentrations were seen in the gut (Figure 2). The

332 observation that nanopesticide treatments result in highest bifenthrin concentrations in the gut

333 are similar to findings from a previous study into the uptake and distribution of C60 and Au

334 nanoparticles into earthworms (Unrine et al., 2010; Waissi-Leinonen 2012; Petersen et al.,

335 2008, 2011). We found that gut associated bifenthrin was generally less eliminated *via* the gut

336 for the non-nanoformulation treatments compared to the nanoformulated treatments (Figure

337 3). Furthermore, a temporal shift in elimination of the nanoformulated bifenthrin occurred. Gut

338 associated elimination of bifenthrin was greatest for the non-nanoformulation treatments whilst

339 the earthworms were still in soil during the elimination phase of the experiment whilst

340 elimination for the nanoformulated treatments was greatest when the organisms were on the

341 filter paper after the elimination phase of the experiment.

342 Unfortunately, it was not practically possible to separate out the internal organs of *E. fetida*

343 from the tissue so we could only distinguish between bifenthrin in the skin and in tissue

344 combined with the gut (Figure 2). For the analytical grade and Capture LFR treatments,

345 concentrations in the gut + tissue and in the skin were significantly different after the uptake

346 phase (Figure 2; P < 0.001) with higher concentrations being seen in the skin. For the

347 nanoformulation treatments, concentrations of bifenthrin accumulated in the gut combined

348 with the internal organs were significantly higher than in the skin (P < 0.001). Following the

349 depuration phase, concentrations of bifenthrin in the gut + tissue and skin in all treatments

350 were similar (Figure 2) and not significantly different within all treatment (P > 0.1) except for

15

351   the analytical grade treatment (P = 0.003). As we were unable to fully characterise the

352   distribution of the a.i. in *E. fetida* our modelling efforts focused on the *L. terrestris* studies.

353   Model 1, and combinations of models 1 and 3 and models 2 and 3 were used to simulate

354   uptake and depuration in the different *L. terrestris* treatments (Figure 4). Model 1 performed

355   very well for estimating the concentrations of the a.i. in the whole organism for the analytical

356   grade and Capture LFR treatments (Nash Index > 0.90). Predictions for the nano treatments

357   were also good (Nash Index > 0.48) although the model overestimated whole organism

358   concentrations at the end of the depuration phase (Figure 4). When model 1 was combined

359   with model 3 to simulate distribution of the a.i. between gut- skin- and remaining-tissues, good

360   predictions were obtained for the analytical grade and Capture LFR treatments for all tissues

361   (Nash Index > 0.76) and for the skin in the two nano treatments (Nash Index > 0.39).

362   Underestimates of concentrations in the gut and overestimates of concentrations in the tissue

363   by a factor of 8-11 were obtained using a combination of models 1 and 3 for both nano

364   treatments (Nash Index < 0.05). The fact that the two models worked well for estimating

365   behaviour in the Capture treatment is encouraging and suggests that estimates of uptake and

366   distribution based on analytical grade material can be used to extrapolate to behaviours in

367   traditional formulations. The approach worked less well for the nanoformulations so we then

368   extended the modelling to factor in the effect of the release rate from the capsule.

369   Incorporation of the a.i. release from the nanocapsule into the modelling of the

370   nanoformulations (i.e. model 2 and model 3 were used) resulted in predictions that fitted the

371   whole organism data and the skin data well (Nash Index > 0.58). This approach

372   underestimated concentrations in the gut at the end of the uptake phase while predictions of

373   concentrations at the end of the depuration phase were close to the measured data. This is a

374   direct result of the model assumption that compound distribution between different tissues

375   (skin, gut and remaining tissue) is instantaneous and fixed by distribution factors and a

376   temporal change in gut clearance between nano and non-nano formulations (Figure 3).

377   Nonetheless, inclusion of the release rate resulted in better predictions, compared to the

378 approach not considering release, of internal tissue concentrations of the a.i. with

379 concentrations being a factor of 3.8 (nano A) and 5.1 (nano B) of measured data at the end of

380 the uptake phase and within a factor of 5.7 (nano A) to 7.5. (nano B) at the end of the

381 depuration phase. While the predictions were not perfect, these results indicate that to model

382 internal tissue exposure, which will likely represent the toxicologically important fraction of the

383 accumulate a.i., it is necessary to factor in the release rate of the a.i. from the nanocapsule

384 into the toxicokinetic modelling.

385 <u>Implications for risk and a potential modelling approach</u>

386 We have demonstrated that nanoencapsulation will affect the behaviour and uptake of

387 pesticides in soil. For bifenthrin, it will decrease the sorption of the active ingredient to soils,

388 increase the apparent persistence of the compound and alter the uptake behaviour of the

389 active ingredient into earthworms and the subsequent distribution. Consequently, the risk of

390 nanoencapsulated bifenthrin to earthworms will be different from a conventional product.

391 Whether nanoencapsulation increases, decreases or has no effect on risk is difficult to

392 establish at this stage. While nanoencapsulated bifenthrin is taken up more quickly by the

393 earthworms, from the *L. terristris* studies, it appears that the majority of the bifenthrin taken up

394 is contained in the gut so the internalised concentration is lower than in earthworms exposed

395 to the analytical grade substance and a conventional formulation. If less active ingredient is

396 internalised, one would assume that less of the active ingredient will reach the site of toxic

397 action so the effects of the nanoformulation will be lower. However, nanoencapsulation also

398 increases the apparent persistence of the active ingredient which will lengthen the exposure

399 duration of the earthworms to the active ingredient in the nanoformulation compared to a

400 conventional ingredient. The increased efficacy of the nanoformulation compared to

401 conventional formulations could mean that application rates to field are decreased which will

402 also affect risk. The risks to birds and mammals feeding of earthworms could also be altered.

403 If a nanoformulation is applied at the same rate as a conventional product then the oral

404 exposure of these organisms will increase but differences in the bioaccessibility of

405 nanoencapsulated bifenthrin compared to free bifenthrin could be lower meaning less is

406 internalised. Again the duration of exposure will increase.

407 To answer some of these questions around the implications of changes in fate and uptake or

408 effects, a toxicokinetic toxicodynamic modelling approach is probably required (Ashauer and

409 Escher, 2010). In Figure 5, we present a conceptual model, based on our experimental

410 findings and investigations into the performance of the different toxicokinetic modelling

411 approaches, that could be used to model the toxicokinetics of a nanoencapsulated active

412 ingredient. The model assumes sorption to soil is instantaneous following release of pesticide

413 from the capsule and that it is the free (i.e. dissolved pore water) pesticide that is taken up into

414 the earthworm tissue – this assumption is supported by our distribution studies in *L. terrestris*

415 and the testing of Models 2 and 3. The internal concentration in the earthworm tissue over

416 time, needed for toxicokinetic toxicodynamic modelling of the effects, are then calculated

417 based on the release rate from the capsule, the soil-water distribution coefficient and the

418 uptake and depuration rates of the free active ingredient into/out of the earthworm. To estimate

419 oral exposure of birds and mammals, the mass concentration of the active ingredient in the

420 gut also needs to be considered and this is estimated based on the feeding rate of the

421 earthworm on whole soil and on the nanoparticles.

422 We believe that this conceptual model is a useful first step towards developing improved

423 environmental risk assessment approaches for estimating the uptake and effects of

424 nanoencapsulated pesticides in earthworms. The approach might also be applicable to other

425 materials (e.g. nanoencapsulated pharmaceuticals) and other organisms. In the future, we

426 recommend that the model be further parameterised for bifenthrin. We also recommend that

427 studies of the type reported here are done on a wider range of organisms using other pesticide

428 active ingredients with different persistence and physico-chemical properties contained in a

429 wider range of nanocarrier materials in order to evaluate the broader applicability of the model.

430 **Acknowledgements**

Data Availability

The experimental data on which this manuscript is based can be obtained, on request, from the corresponding author.

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520

521 Table 1: Sorption coefficients (k_d), dissipation half-lives (DT50), release half times (RT50),

522 bioconcentration factors (BCFs) and rates for release, uptake and depuration (k) for the

523 different bifenthrin treatments studied in the *Eisenia fetida* and *L. terrestris* studies.

Endpoint	Study	Unit	Bifenthrin	Capture LFR	Nano A	Nano B
k_d	Uptake and depuration	L Kg^{-1}	550 ± 21	394 ± 15	186 ± 4	233 ± 17
k_d	Distribution study *E.fetida*	L Kg^{-1}	494 ± 71	371 ± 47	154 ± 7	163 ± 9
k_d	Distribution study *L. terrestris*	L Kg^{-1}	585 ± 92	488 ± 32	274 ± 42	251 ± 31
DT$_{50}$	Uptake and depuration	d	25 - 27	33-35	49 - 50	38 - 40
Release rate (k_r)	Uptake and depuration	h^{-1}	NA	NA	0.104 ± 0.008	0.182 ± 0.016
RT$_{50}$	Uptake and depuration	d	NA	NA	6 - 7	3 - 4
k_{in} *E.fetida*	Uptake and depuration	LKg^{-1}h^{-1}	0.222 ± 0.009	NA	NA	NA
k_{out} *E.fetida*	Uptake and depuration	h^{-1}	0.0036 ± 0.0002	NA	NA	NA
BCF *E.fetida*	Uptake and depuration	-	61.7	NA	NA	NA
k_{in} *L. terrestris*	Uptake and depuration	LKg^{-1}h^{-1}	0.7021 ± 0.0336	NA	NA	NA
k_{out} *L. terrestris*	Uptake and depuration	h^{-1}	0.0033 ± 0.0003	NA	NA	NA
BCF *L. terrestris*	Uptake and depuration	-	212	NA	NA	NA

524 [1] Determined from data presented here with additional data on soil concentrations over time in four other soils
525 (unpublished).

526

23

Figure Legends

Figure 1: Measured (dots) and predicted (lines) total internal concentration of different formulations of bifenthrin in *E. fetida* over time using Model 1 (black) and Model 2 (grey). Dotted lines indicate the 95% confidence interval for the predictions. Model parameterisation was conducted with data from bifenthrin a.i..

Figure 2: Proportion of total internal concentration of different formulations of bifenthrin in earthworms after 10 d uptake and 7 d depuration as average ± SD in relation to the internal concentration at the end of the uptake phase.

Figure 3: Proportion of the gut concentration at the end of the uptake phase of different formulations of bifenthrin recovered from *L. terrestris* faeces samples as average ± SD.

Figure 4: Measurements (dots) and predictions from different model combinations (lines) of internal concentration of different formulations of bifenthrin in whole organism and different compartments of *L. terrestris* over time. Grey backgrounds indicate that predictions account for the released fraction of bifenthrin from the nanoformulation. Dotted lines indicate the 95% confidence intervals for the predictions.

Figure 5: Conceptual model for estimating residues of active ingredients contained in nanoencapsulated formulations in terrestrial invertebrates over time.
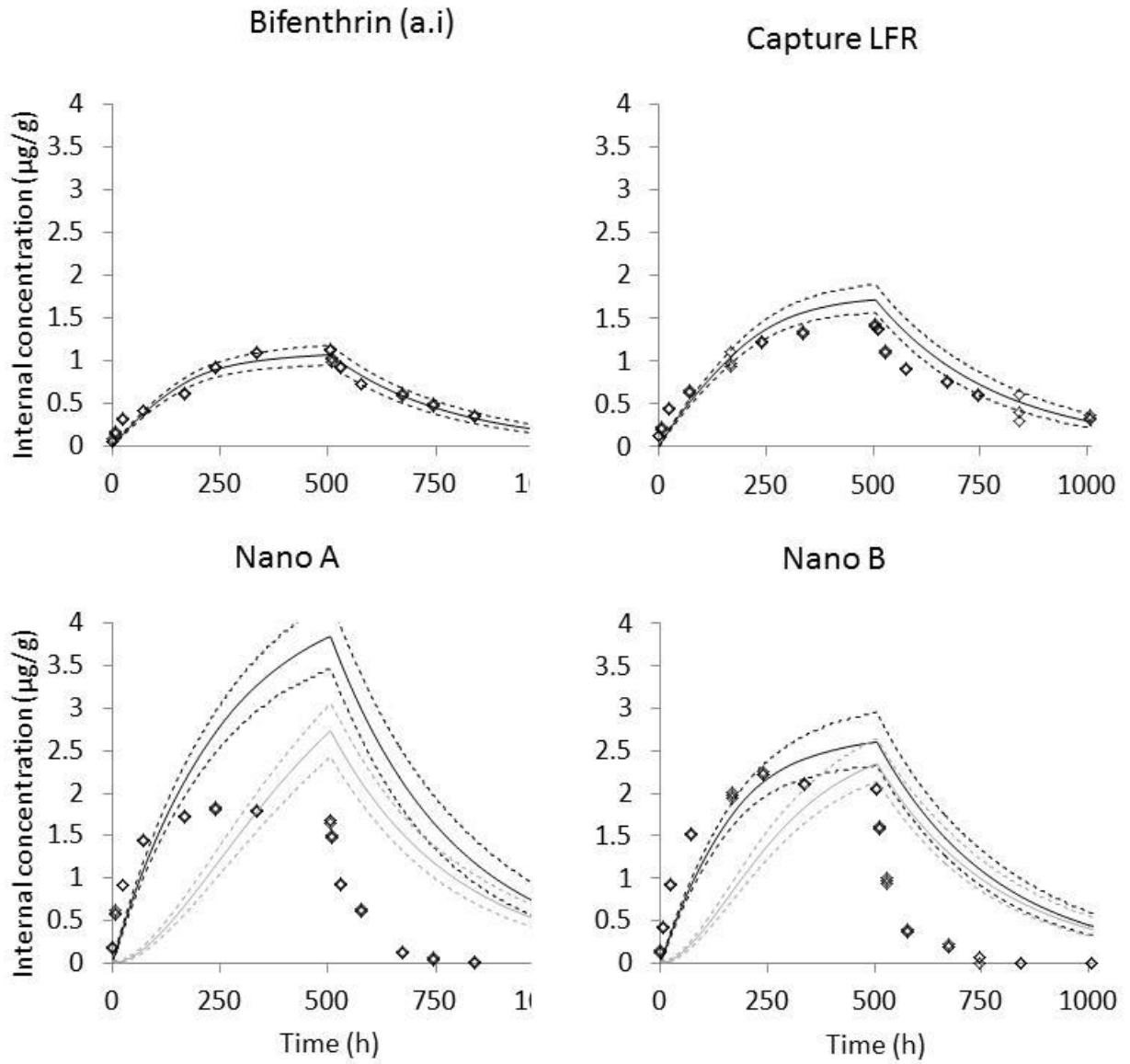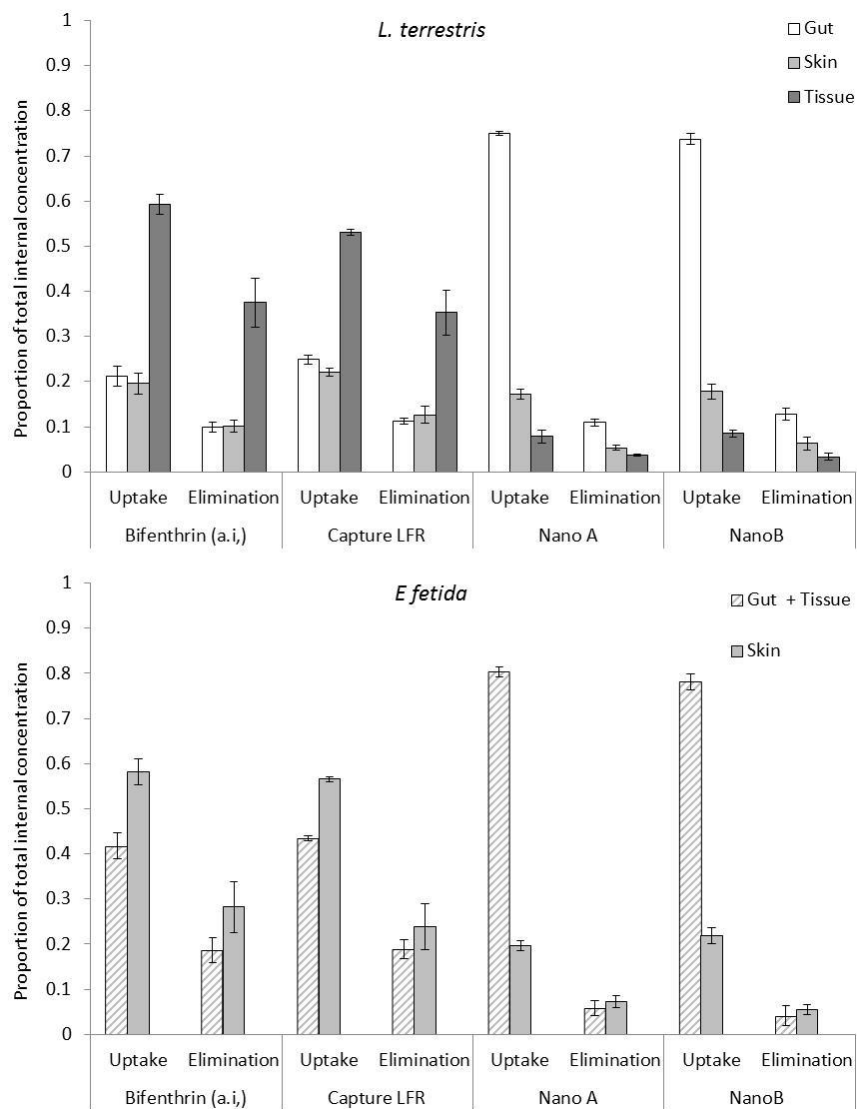
Figure 1

Figure 2

Figure 3

Figure legend:
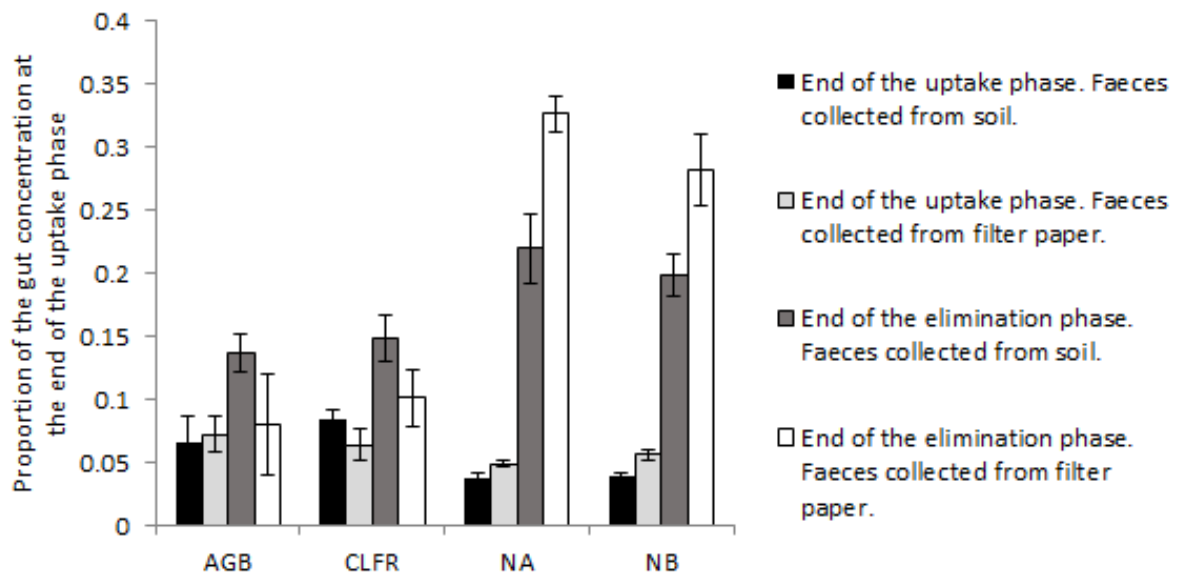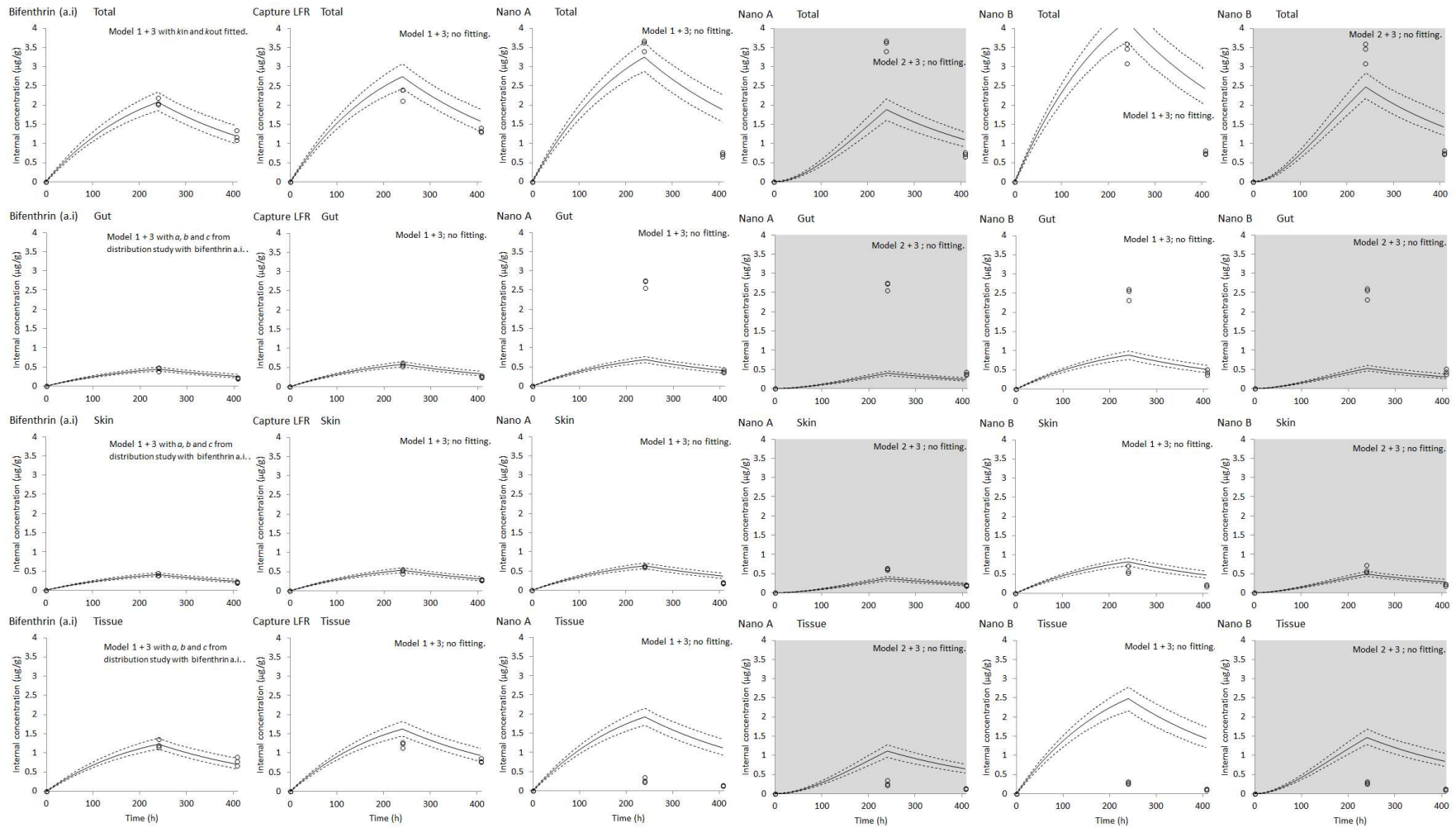- ■ End of the uptake phase. Faeces collected from soil.
- □ End of the uptake phase. Faeces collected from filter paper.
- ▨ End of the elimination phase. Faeces collected from soil.
- □ End of the elimination phase. Faeces collected from filter paper.

Figure 4

Figure 5.

K_R = release rate from capsule (d^{-1})
K_d = soil-water distribution coefficient (l kg^{-1})
K_{in} = uptake rate into tissue (d^{-1})
K_{out} = elimination rate from tissue (d^{-1})
$K_{F-capsule}$ = feeding rate on capsule (d^{-1})
$K_{DEF-capsule}$ = defecation rate of capsule (d^{-1})
K_{F-soil} = feeding rate on soil (d^{-1})
$K_{DEF-soil}$ = defecation rate of soil (d^{-1})