1 **Anti-androgens act jointly in suppressing spiggin concentrations in**

2 **androgen-primed female three-spined sticklebacks – prediction of**

3 **combined effects by concentration addition**

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ABSTRACT

Increasing attention is being directed at the role played by anti-androgenic chemicals in endocrine disruption of wildlife within the aquatic environment. The co-occurrence of multiple contaminants with anti-androgenic activity highlights a need for the predictive assessment of combined effects, but information about anti-androgen mixture effects on wildlife is lacking. This study evaluated the suitability of the androgenised female stickleback screen (AFSS), in which inhibition of androgen-induced spiggin production provides a quantitative assessment of anti-androgenic activity, for predicting the effect of a four component mixture of anti-androgens. The anti-androgenic activity of four known anti-androgens (vinclozolin, fenitrothion, flutamide, linuron) was evaluated from individual concentration-response data and used to design a mixture containing each chemical at equipotent concentrations. Across a 100-fold concentration range, a concentration addition approach was used to predict the response of fish to the mixture. Two studies were conducted independently at each of two laboratories. By using a novel method to adjust for differences between nominal and measured concentrations, good agreement was obtained between the actual outcome of the mixture exposure and the predicted outcome. This demonstrated for the first time that androgen receptor antagonists act in concert in an additive fashion in fish and that existing mixture methodology is effective in predicting the outcome, based on concentration-response data for individual chemicals. The sensitivity range of the AFSS assay lies within the range of anti-androgenicity reported in rivers across many locations internationally. The approach taken in our study lays the foundations for understanding how androgen

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42 receptor antagonists work together in fish and is essential in informing risk

43 assessment methods for complex anti-androgenic mixtures in the aquatic

44 environment.

45 *Keywords:* Anti-androgen; *Gasterosteus aculeatus*; Mixture effects; Concentration

46 addition; Pesticides; Endocrine disruption.

47

1. Introduction

49 Considerable attention and concern has been focused on contaminants in the

50 aquatic environment that interfere with the functioning of the vertebrate

51 reproductive system (endocrine disrupting chemicals: EDCs), the most-documented

52 of which are EDCs that target estrogen-dependent pathways (Sumpter and Johnson,

53 2008). However, chemicals that interact with other elements of the reproductive

54 endocrine system are of equal interest. In particular, EDCs with anti-androgenic

55 properties are believed to be ubiquitous within the aquatic environment (Hill et al.,

56 2010; Johnson et al., 2007; Urbatzka et al., 2007) and may be important contributors

57 to reproductive dysfunction in aquatic animals (Jobling et al., 2009). Nonetheless,

58 the biological significance of anti-androgenic contaminants is not yet fully

59 understood. Relatively little is known about the disposition and identity (although

60 see Rostkowski et al., 2011) of anti-androgenic EDCs or the extent of their effects on

61 aquatic wildlife. These knowledge gaps highlight a need for further investigation and

62 assessment.

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64   The aquatic environment is a chemically complex medium in which individual

65   contaminants may be present at low concentrations yet still contribute to joint

66   effects on organisms as part of the overall assemblage of chemicals. In this context,

67   without the ability to extrapolate likely combined effects, reference data derived

68   from single-agent exposure studies are uninformative regarding the overall risk and

69   potential adverse effects for exposed animals (Kortenkamp, 2007). Thus, there is a

70   need to develop and refine methods that allow the prediction of effects of chemical

71   mixtures on target organisms in the aquatic environment. To date, most studies

72   using fish as an environmentally relevant model target organism to investigate

73   mixture effects of EDCs, have focused on chemicals with estrogenic modes of action

74   (Brian et al., 2005; Correia et al., 2007; Jukosky et al., 2008; Thorpe et al., 2001;

75   Zhang et al., 2010). The purpose of the present study was to extend this approach to

76   investigate the use of single agent concentration-response data to predict the effects

77   on a relevant fish model of a mixture of chemicals with anti-androgenic properties.

78

79   In order to retain relevance to real-world exposure scenarios we adopted an *in vivo*

80   assay system that utilises unique features of the three-spined stickleback

81   (*Gasterosteus aculeatus* L.). The stickleback is ubiquitous in northern latitudes and

82   widely employed in ecological, ecotoxicological and behavioural investigations

83   (Katsiadaki et al., 2007; Pottinger et al., 2002, 2011, 2013; Sanchez et al., 2008). Male

84   sticklebacks synthesize an androgen-dependent glycoprotein (spiggin) which is used

85   to glue together the structural components of the nest (Jakobsson et al., 1999; Jones

86   et al., 2001). Androgen-inducible spiggin is also present in the kidney of females but

87 normally at very low levels and this feature has been exploited to provide a bioassay

88 for EDCs with anti-androgenic activity (Katsiadaki et al., 2002). Priming females by

89 exposure to a standardised concentration of androgen in order to stimulate the

90 synthesis of spiggin provides a sensitive *in vivo* quantitative assay system for the

91 detection and evaluation of anti-androgenic EDCs (Jolly et al., 2009; Katsiadaki et al.,

92 2006). The use of females, in which spiggin levels are normally low, provides a

93 relatively constant baseline from which consistent androgen-induced spiggin levels

94 can be achieved. This would not be possible using males in which the annual cycle of

95 endogenous androgen causes large inter-individual fluctuations in kidney spiggin

96 content. The use of females also reduces the likelihood that non-receptor mediated

97 mechanisms, for example those acting on steroid synthesis, might affect spiggin

98 levels; in females the synthesis and accumulation of spiggin is primarily a direct

99 consequence of an androgen receptor-mediated process (Olsson et al., 2005).

100 Because of this, the AFSS is an *in vivo* assay with a sound mechanistic basis that

101 specifically identifies androgen receptor antagonists.

102

103 This series of studies was designed to evaluate whether the joint effects of a mixture

104 of anti-androgens on spiggin synthesis in female sticklebacks could be predicted

105 accurately from knowledge of the individual potencies of each component of the

106 mixture. The concept of concentration addition (CA), which is applicable to mixtures

107 of chemicals with a common mode of action (Drescher and Boedeker, 1995), was

108 favoured as the prediction model. In the first instance our intention was to validate

109 the usefulness of CA, rather than to study environmentally relevant mixtures.

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110 Accordingly, the following androgen receptor antagonists (Kang et al., 2004;

111 Lambright et al., 2000; Sebire et al., 2009; Tamura et al., 2001; Wong et al., 1995)

112 were selected: fenitrothion [0,0-dimethyl 0-(4-nitro-m-tolyl) phosphorothioate] an

113 organophosphate insecticide; vinclozolin [(RS)-3-(3,5-dichlorophenyl)-5-methyl-5-

114 vinyl-1,3-oxazolidine-2,4-dione] a non-systemic dicarboximide fungicide, linuron [3-

115 (3,4-dichlorophenyl)-1-methoxy-1-methylurea] a substituted urea herbicide, and

116 flutamide [2-methyl-N-[4-nitro-3-(trifluoromethyl) phenyl] propanamide], a non-

117 steroidal anti-androgenic therapeutant. The potency of each anti-androgen in

118 countering androgen-induced spiggin synthesis in female sticklebacks was evaluated

119 singly and these data were used to predict the outcome of a series of combined

120 exposures in which all four anti-androgens were present in a mixture at ratios

121 proportional to their expected individual potencies. Using this fixed-ratio mixture

122 design, the predictive power of CA was assessed by comparing the predicted anti-

123 androgenicity of the four compounds with that observed. Because differences

124 between nominal and measured concentrations of the anti-androgens in the test

125 mixtures changed the original mixture composition, in each mixture concentration

126 the assumption of a common mixture ratio between the compounds and test

127 concentrations was unavoidably violated. This would have resulted in restricting the

128 comparative mixture assessment to only the analytically determined mixture

129 concentrations, thereby discarding one of the biggest advantages of fixed-ratio

130 mixture designs - the capacity to assess concentration ranges of the mixture that

131 were not directly tested. We overcame these limitations in this study by estimating

132 varying mixture ratios that allowed us to expand the traditional concentration-

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133 response analysis established for fixed-ratio mixtures to more complex mixture

134 compositions.

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136 **2. Materials and methods**

137 *2.1. Chemicals*

138 Analytical grade flutamide (FL), fenitrothion (FN), vinclozolin (VZ) and

139 dihydrotestosterone (DHT) were obtained from Sigma-Aldrich (Gillingham, UK) and

140 linuron (LN) was purchased from QMX Laboratories (Thaxted, UK). All chemicals used

141 in the study were matched across laboratories by batch number and were of high

142 purity (≥99%). All other chemicals were obtained from Sigma-Aldrich unless

143 otherwise stated.

144

145 *2.2. Fish*

146 Sticklebacks were obtained from a supplier (Moore & Moore Carp, Reading, UK; CEH

147 Lancaster) or captured by beach seine in Oslo fjord (Drøbak Research Station;

148 University of Bergen). At both Lancaster and Bergen, the fish were subsequently kept

149 in glass aquaria supplied with a constant flow-through of water and fed five times

150 weekly with frozen bloodworm. Because of the requirement that the test fish exhibit

151 low levels of endogenous spiggin, only female fish were selected for these studies.

152 Males were identified by inspection of iris and oesophageal colour (immature males

153 exhibit traces of blue and red respectively) and separated from the females. For a

154 period of at least one month prior to the exposure studies the sticklebacks were

155 acclimated to the temperature (Lancaster: $15 \pm 2^{o}C$; Bergen: $16 \pm 2^{o}C$) and

156 photoperiod (12h light:12h dark) under which the studies were conducted.

157

158 *2.3. Experimental design*

159 Single agent and mixture studies were performed in parallel at two laboratories

160 (Centre for Ecology & Hydrology, Lancaster, UK, and Department of Biology,

161 University of Bergen, Norway) over a period of three years. The *in vivo* exposures

162 closely followed procedures outlined in the OECD Guidance Document 148 (OECD,

163 2011). The exposure system comprised the required number of 30 L (working

164 volume) glass aquaria each supplied with a constant inflow of untreated raw water

165 (100 mL/min; PVC tubing, Portex; 5 mm i.d.; Lancaster: lake water; Bergen:

166 seawater) via peristaltic pumps (Watson Marlow 505S; Marprene tubing, 6.4 mm

167 i.d.) with twin head cassettes. The performance of each of the pumps was checked

168 twice weekly by timing the delivery of 100 mL of water into a volumetric flask. Each

169 tank was aerated throughout the study period via a single airstone. Working

170 solutions of the test compounds were formulated in methanol and held in 1.0 L glass

171 bottles. A multi-channel peristaltic pump (Watson Marlow 205U; 0.76 mm i.d. PVC

172 manifold tubing) delivered the test compound solution from the stock bottle to the

173 aquaria via silicone tubing through a three-way connector inserted immediately

174 downstream of the raw water pump head. A pumped delivery rate of 100 µL/min for

175 the chemical stock was maintained resulting in a concentration of methanol in the

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176 exposure tanks of 0.1%. The stock solutions of test compound, either single

177 chemicals or mixtures, were formulated at 1000-fold the nominal concentration

178 required in the exposure tanks. The flow rates of the multi-channel pump were

179 validated twice-weekly by determining the weight of solvent delivered into pre-

180 weighed vials during a defined period of time. Water temperature was held within

181 the range required using thermostatically controlled water heaters. The temperature

182 within each exposure tank was logged at 30 min intervals via temperature probes

183 attached to a 10-channel data logger, downloaded at weekly intervals to a computer.

184 Water quality measurements (pH, dissolved O_2) were taken at weekly intervals with

185 portable metering systems to ensure that study conditions met the requirements

186 laid out in the OECD Guidance Document (OECD, 2011).

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188 *2.4. In vivo exposure studies*

189 For each study, the exposure system was set up and run with the test chemical for

190 one week to equilibrate the system before the fish were added. Each tank was

191 populated with 10 - 15 female sticklebacks (according to the test requirements) from

192 a stock population that had been acclimated to the experimental conditions of

193 temperature and photoperiod. A series of single chemical concentration-response

194 exposures were conducted first, the results of which were used to design the final

195 four component mixture exposure study.

196

197 *2.4.1. Single chemical exposures*

198 All studies were carried out using the same protocol in which groups of female

199 sticklebacks were exposed to DHT (5 µg/L), a non-aromatizable androgen, to

200 stimulate spiggin synthesis both in the presence of a range of concentrations of the

201 test chemical and in the absence of the test chemical (positive control). In addition, a

202 single tank received the highest concentration of test chemical in the absence of DHT

203 (negative control) and water-only (absolute control) and methanol-only (solvent

204 control) control tanks were also included. Each single compound was tested at a

205 minimum of seven concentrations, with nominal concentrations ranging from 0.1 to

206 250 µg/L for FN, 5 to 250 µg/L for FL, 0.25 to 250 µg/L for LN and 0.25 to 500 µg/L for

207 VZ. Single compound tests for FN were conducted only at Lancaster. FL was tested at

208 both Lancaster and Bergen and VZ and LN were tested only at Bergen. In each

209 individual study, one tank per treatment group was used based on the assumption

210 that the standardised and closely controlled experimental conditions would minimise

211 between-tank variation, other than that arising from the treatment. Additional

212 confidence was provided by the replication of studies across two laboratories. All

213 single compound data were then pooled for further data analysis.

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215 *2.4.2. Four component mixture exposures*

216 The mixture study comprised a series of tanks receiving each chemical singly at both

217 the IC50 and IC50/10 together with tanks receiving a mixture of all four anti-

218 androgens at mixture ratios proportional to their individual potencies and ranging

219 from the IC50 to the IC50/100 (fixed-ratio mixture design, see Table S1 for details).

220 Water-only (absolute control), methanol-only (solvent control) and DHT-only

221 (positive control) treatments were also included. The range of dilutions was based on

222 the concentration range described by the additivity prediction, such that the mixture

223 was expected to inhibit completely the androgenic effect of DHT. The four-

224 component mixture study was conducted in both the Bergen and Lancaster

225 laboratories.

226

227 *2.5. Sampling procedure*

228 At the end of each study (day 21) the fish were killed immediately by immersion in a

229 lethal dose of sedative (2-phenoxyethanol; 1:1000), and stored individually at -20°C

230 in labelled 12 mL polypropylene centrifuge tubes (Sarstedt). All the fish from a single

231 tank were processed before disturbing the second tank. Kidneys were dissected from

232 part-thawed carcasses, placed in 2 mL screw-capped cryovials (Nalgene, VWR

233 International), and stored at -20°C until required for assay. Water samples (1000 mL)

234 were taken from each tank at time 0 and at 7, 14 and 21 days after the start of the

235 study. These were collected by immersing bottles (Nalgene HDPE; VWR

236 International) directly in the tanks and were stored at -20°C before extraction.

237 Extraction was accomplished by pumping the water sample (at 10-20 mL/min)

238 through a methanol-conditioned, distilled water-washed, solid phase extraction

239 (SPE) cartridge (Sep-Pak C18; Waters Ltd, UK) with an inline 0.45 µm pre-filter (Pall

240 Gellman Acrocap, Pall Life Sciences). Air-purged cartridges and filters were labelled,

241 wrapped in parafilm, and stored at -20°C until being despatched (on dry ice) to the

242 receiving laboratories for chemical analysis.

243

244 *2.6. Analytical procedures*

245 Water sample extracts and kidney spiggin concentrations were analysed at the Cefas

246 laboratories. Kidney spiggin content was measured using a specific ELISA and is

247 reported as arbitrary spiggin units per gram body weight (U/g bw; Katsiadaki et al.,

248 2002). Concentrations of DHT in aquarium water extracts were determined with an

249 established radioimmunoassay procedure (Katsiadaki et al., 2002; Scott et al., 1984).

250 Concentrations of FL, LN, FN and VZ in the extracts of exposure tank water were

251 determined as described previously (Katsiadaki et al., 2006). In brief, concentrated

252 methanolic SPE derived extracts were analysed by high performance liquid

253 chromatography with electro spray ionization and selected ion mass spectrometry

254 (HPLC-ESI-SIM-MS). Quantities of the target chemicals were determined by external

255 calibrations, using a series of calibration (n=6) solutions prepared from the same

256 stock chemicals used for the exposure studies. The performance of the SPE

257 procedure was assessed prior to the start of the studies by extracting six replicate

258 solutions each containing a mixture of FL, LN, FN and VZ at 100 µg/L each. The

259 extracts were then analysed as described above and percent recoveries calculated.

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261 *2.7. Concentration-response analysis*

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262   To account for inter-study variation in absolute spiggin levels, spiggin concentrations

263   (U/g bw) were log-transformed and then standardised to the mean values of the

264   positive DHT-stimulated controls and the solvent controls (unstimulated baseline

265   spiggin concentration). By this scaling approach, the absolute effects scale was

266   normalised to relative effects between 0 and 1. The median inhibitory concentration

267   (IC50) of DHT was that which produced a $\log_{10}$-transformed spiggin inhibition which

268   was median in relation to the DHT controls (maximum spiggin concentration) and

269   solvent controls (minimum spiggin concentration). Concentration response data

270   analysis was based on the geometric mean of concentrations of the test chemicals

271   that were measured at intervals (7d, 14d, 21d) during the three-week exposure

272   period. We determined concentration–response curves for each of the four

273   chemicals using pooled data from the exposures conducted by the two participating

274   laboratories. To account for the intra- and inter-experimental variability associated

275   with this nested data scenario, we used the generalised, nonlinear mixed modeling

276   approach in which both fixed and random effects are permitted to have a non-linear

277   relationship with the effect end point (Vonesh and Chinchilli, 1997). A shift

278   parameter was included in the non-linear regression model as a random effect which

279   accounts for a shift of the whole curve based on the $\log_{10}$-transformed concentration

280   scale. Furthermore, a best-fit approach was adopted, in which different regression

281   models were fitted independently to the same pooled data set, and the best fit was

282   selected on the basis of statistical criteria (Scholze et al., 2001). This approach was

283   implemented using the NLMIXED function of the SAS statistical software package

284   (SAS Institute, Cary, USA).

285

286   *2.8. Mixture prediction*

287   Following the logic of Berenbaum (1985), and as described by Faust et al. (2001),

288   under the assumption of concentration addition (CA) contours of constant effect X

289   are planar such that

290    $$\sum_{i=1}^{n} \frac{c_i}{EC_{Xi}} = 1 ,$$ (1)

291   where, for a combination of n components, c_i is the concentration of the ith

292   component in the mixture concentration $c_{mixture} = (c_1,...,c_n)$ that produces the effect X,

293   and EC_i^X is the concentration of the ith component that produces the same

294   magnitude of effect. The effect concentration EC_i^X is derived from the inverse of

295   the regression function which describes the observed concentration effect data of

296   the ith component (Table 1). The nonlinear regression models used in the best-fit

297   approach assumes that the expected mixture effect *X* at given mixture concentration

298    $c_{mixture}$ can only be calculated by solving iteratively Equation 1. A fixed-ratio mixture

299   design simplifies this implicit equation by re-arranging Equation 1 into an explicit

300   form that allows the calculation of the effect concentration at given mixture

301   concentration (Faust et al. 2001):

302    $$c_{mixture} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{Xi}} \right)^{-1} ,$$ (2)

303   where p_i is the is the ratio of the ith component in the mixture and the sum of all p_i

304   equals 1. This equation also allows interpolative calculations for untested

305   concentration ranges, similar to the common concentration-response regression

306   analysis for single components. The prerequisite is that the relative composition

307   remains unchanged at every mixture concentration, i.e., all test concentrations of

308   the mixture have something in common functionally. However, a common problem

309   with exposures in aquatic flow-through test systems is that the measured

310   concentrations do not always closely correspond to nominal concentrations.

311   Consequently, an identical relative composition at every mixture concentration can *a*

312   *priori* not be assumed, and therefore the calculation of effect concentrations

313   according to Equation 2 is *a priori* not feasible. For the mixture assessment this

314   means, in the worst-case, that only observed and predicted responses at mixture

315   concentrations for which measured concentrations are available can be compared.

316   Effect concentrations corresponding to values between measured concentrations

317   cannot be assessed. To overcome this limitation, we developed a methodology that

318   under certain assumptions about the functional relationship between measured and

319   nominal concentrations allows the prediction of mixture effects at untested

320   concentrations. To better understand the fixed-ratio design and its meaning for our

321   proposed method, we have illustrated in Figure 1 how concentrations of two single

322   components can be combined in a mixture: showing the range of all possible

323   combinations of test concentrations that can be tested as a mixture. The fixed-ratio

324   design limits them to those pairs which follow a straight line with zero origin (Figure

325   1, Line A), i.e. the compounds in the mixture are characterised by a consistently

326   identical ratio. If the measured concentrations are not the same as those planned,

327   three cases can be identified that would still allow a mixture assessment for untested

328   concentrations: (i) all single compound concentrations measured in the mixture

329   deviate at all test mixtures roughly by the same factor from the planned

330   composition, in which case the relative mixture composition is unchanged and can

331   be described by the same line (Figure 1, line A) and no corrections to Equation 2 are

332   required; (ii) the concentrations of compounds measured in the mixture deviate in

333   all test mixtures by approximately the same factor, but for each compound a

334   different ratio is estimated. This still maintains a straight line (Figure 1, line B), but

335   would require the correction of the mixture ratio by replacing the original fractions p_i

336   in Equation 2 with fractions estimated from the measured concentrations; (iii) for at

337   least one mixture compound the ratio between nominal and measured

338   concentrations is not constant over all tested mixtures, but follows a functional

339   relationship (e.g., recovery rate decreases with increasing test concentrations).

340   Consequently, each mixture concentration has a different composition and the

341   fraction p_i in Equation 2 depends on the measured mixture composition. The

342   functional relationship is non-linear (Figure 1, line C). However, in this case Equation

343   2 cannot be used as each effect level X requires its own mixture composition, and

344   mixture effects can only be calculated according to Equation 1. In the present study

345   we were challenged with a mixture that deviated from linearity as illustrated in

346   Figure 1 (line C). We used a second-order regression function to describe the

347   measured concentrations in relation to the nominal values, which also allowed the

348   prediction of mixture effects for concentration ranges along the non-linear plot for

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349 which analytically determined concentrations of anti-androgen were not available.

350 Details of the regression model can be found in the Appendix. As the measured

351 concentrations were replaced by smoothed regression estimates, we additionally

352 calculated the mixture effect at the measured mixture concentration.

353 All effect concentrations of the single components are estimates and are therefore

354 subject to stochastic variability. This meant that the predicted effect concentration

355 of the mixture also had to include a measure of statistical uncertainty. This was

356 achieved by using the bootstrap method (Efron and Tibshirani, 1993), which enabled

357 approximate 95% confidence limits to be derived for the mean predicted effect. It

358 should be noted that the variation of measured concentrations observed over the

359 exposure period was not taken into account, i.e. the geometric mean of the

360 measured exposure concentration was used as a fixed value in the resampling

361 approach. Therefore the confidence limits might slightly underestimate the true

362 uncertainty.

363

364 3. Results

365 All single-agent and mixture studies ran to completion. No atypical behaviour was

366 observed among the fish during any of the studies. Mortality among the test fish did

367 not exceed 1.5% overall and there was no evidence of disease or parasite infections.

368 At Lancaster there were no significant differences between spiggin concentrations in

369 fish from the water control tanks (73 ± 10 U/g bw, n = 20), solvent control tanks (105

370 ± 17 U/g bw, n = 22) and negative control tanks (58 ± 8 U/g bw, n = 21). In studies

371   conducted at Bergen, there was a small but significant difference between spiggin

372   concentrations in fish from solvent tanks (43 ± 8 U/g bw, n = 60) and negative control

373   tanks (66 ± 6 U/g bw, n = 63) but neither were significantly different from the water

374   controls (46 ± 5 U/g bw, n = 61) (One-way ANOVA, Tukey's Test). There were no

375   significant differences between studies or laboratories in the DHT-stimulated spiggin

376   concentration measured in positive controls (overall mean = 43730 ± 3064 U/g bw, n

377   = 94). The extraction efficiency of the SPE procedure was found to be high for all

378   compounds (LN: 83.5 ± 2.2 %, FN: 94.0 ± 3.2 %, FL: 84.3 ± 3.9 %, VZ: 86.0 ± 2.0 %;

379   mean ± SEM, n = 6). For the single agent studies mean recoveries of the test

380   compounds from the exposure tanks were: LN: 43.3 ± 4.8 %, n = 12, FN: 53.9 ± 6.3 %,

381   n = 15, FL: 49.6 ± 4.2 % n = 24, VZ: 6.7 ± 1.4 %, n = 9 (mean ± SEM). The % recovery

382   values for each test compound in the mixture study are provided in Table 2 and Fig.

383   4.

384

385   *3.1. Single compound studies*

386   Repeated studies with the four compounds were performed in two separate

387   laboratories (Lancaster and Bergen) over a period of three years. Concentration-

388   response data were always in good agreement, and differences in response between

389   laboratories or time trends were not statistically significant. Each of the chemicals

390   that were tested inhibited spiggin induction in a concentration-dependent manner,

391   confirming that the AFSS effectively detects androgen receptor antagonists (Fig. 2). It

392   was possible to determine a concentration that elicited full inhibition of spiggin

393 production (relative to the negative control) for three chemicals FL, FN, and VZ and

394 for these, the lowest tested concentration did not evoke effects significantly

395 different from the untreated controls. This allowed the estimation of near-complete

396 concentration response curves without needing to extrapolate to untested effect

397 levels (Fig. 2; Table 1). Based on the measured concentrations, the most potent anti-

398 androgen was VZ (IC50 = 8.57 µg/L), and the least potent was LN (IC50 = 172 µg/L).

399

400 Between-study differences in absolute spiggin concentrations (U/g bw) were

401 relatively small. For example, mean female spiggin values for the DHT control were

402 within one order of magnitude (interquartile range: 34,000 - 63,000 U/g bw). The

403 normalisation approach we adopted for the spiggin data further improved the

404 comparability of concentration-response data from different studies (Fig. 3). Here

405 data obtained from two LN exposure studies are plotted as absolute (Fig. 3a) and

406 transformed (Fig. 3b) spiggin values. Because the means of the DHT controls differed

407 slightly between studies better agreement of the data at low effect concentrations

408 was achieved following normalisation.

409

410 *3.2. Mixture studies*

411 The mixture of anti-androgenic chemicals was tested at both Lancaster and Bergen,

412 and the actual and nominal concentrations for each component of the mixture are

413 given in Table 2. The average variation of the single component measurements

414 between the sampling days was found to be random in nature, i.e. trends across the

415 testing period could not be detected (data not shown). As was the case for the single

416 substance studies, we found no consistent agreement between nominal and

417 measured concentrations of the test compounds, with average recovery in most

418 cases of less than 100%. The estimated concentrations which were derived from the

419 regression method outlined in the Appendix are given in Table 2. The entirety of the

420 curves estimated are shown in Figure 4. The corresponding model parameters can be

421 found in Table S2. There were marked differences in the actual chemical

422 concentration relative to the nominal concentration not only for compounds within

423 the mixture, but also between laboratories. Data for FL were most consistent in

424 terms of both recovery and agreement between laboratories (Fig. 4a), and both FL

425 and LN (Fig. 4c) exhibited a mostly linear relationship between nominal and

426 measured concentrations. However, for FN (Fig. 4b) and VZ (Fig. 4d) more complex

427 relationships between nominal and actual concentrations were evident.

428 Nevertheless, in all cases it was possible to establish a clear functional relationship

429 between nominal and measured concentrations. For most compounds the second-

430 order model parameter θ_3 was not statistically significant (Table S2), and a linear

431 nominal-measured model assumption would have led to nearly identical estimates.

432 However, a significant non-linearity term was estimated for VZ. This approach

433 yielded a significant improvement for VZ where the non-linearity arose mainly

434 because the lower concentrations of VZ showed better recovery rates than the

435 higher concentrations. Overall, the analytically determined chemistry data indicated

436 that the relative composition differed significantly between both mixtures and that

437 therefore separate data analysis for each mixture was appropriate. The chemical

20

438   data also showed that not all mixture concentrations can be described by a common

439   ratio between the component concentrations, which justified our decision to

440   estimate mixture concentrations through variable mixture ratios.

441

442   For studies in both laboratories the mixture of VZ, FL, LN and FN produced a

443   concentration-dependent inhibition of spiggin induction in DHT-primed female

444   sticklebacks (Figure 5). The lowest tested mixture concentration induced no

445   statistically significant changes, and the highest tested mixture concentration

446   produced a maximal spiggin inhibition, equivalent to spiggin levels in non-DHT-

447   exposed controls, in both studies. Data variability was comparable with that from the

448   single component studies (Table 3). The concentration-response data for the single

449   components, pooled from all studies (see Figure 2 and Table 1), were used to

450   compute predicted concentration-additive combination effects covering the entire

451   range of effects (Figure 5a and 5b; solid lines). For both studies, the anticipated

452   combination effects fell within the range of the effects that were observed

453   experimentally. The pooled data sets provided sufficient information for predictions

454   of low statistical uncertainty (Figure 5; dotted lines), and were therefore a good basis

455   for the comparative mixture assessment. The comparison of the observed spiggin

456   induction with the prediction curve yielded a good agreement for most effect levels.

457   No statistical deviation could be detected, with the average spiggin induction lying

458   within or close to the narrow 95% confidence limits along the full length of the curve.

459   The only exceptions to this trend were the responses we observed at 142.8 µg/L

460   (nominal), which were significantly overestimated by CA in the studies conducted at

461 both laboratories. The observed responses were surprising inasmuch as they were

462 similar to those observed at the preceding concentration (nominal 57.1 µg/L), i.e. a

463 concentration-dependent decrease could not be detected between these two

464 successive dilutions. Nevertheless, and notwithstanding this anomaly, these findings

465 provided overall evidence that anti-androgenic chemicals act in an additive manner

466 *in vivo* and that their effects can be predicted accurately using CA.

467

468 **4. Discussion**

469 The results of this study confirm the utility of the female three-spined stickleback as

470 a model organism for evaluating the *in vivo* anti-androgenicity of compounds in an

471 aquatic environment (Katsiadaki et al., 2006, 2012; OECD, 2011). In addition they

472 established that spiggin is an endpoint for anti-androgenic activity with sufficient

473 resolution and sensitivity to permit complex mixture effect analysis.

474

475 *4.1. Mechanistic basis of the spiggin response*

476 Spiggin synthesis in sticklebacks is assumed to be regulated by a renal androgen

477 receptor (Olsson et al., 2005) and therefore an obvious route by which the inhibition

478 of spiggin production can occur is via antagonism of androgen binding to androgen

479 receptors in the kidney by competing ligands. The four anti-androgens used in this

480 study exhibit anti-androgenic activity in mammalian systems via the competitive

481 inhibition of androgen binding to the androgen receptor (Lambright et al., 2000;

22

482 Tamura et al., 2001; Wong et al., 1995) and three of the compounds (VZ and

483 metabolites, LN and FL) have also been demonstrated to displace androgens from a

484 teleost androgen receptor *in vitro* (Wilson et al., 2007). In the current study the

485 degree to which the selected anti-androgens interfered with androgen-stimulated

486 synthesis of renal spiggin in female sticklebacks was broadly consistent with earlier

487 reports of the relative activity of these antagonists *in vitro*. We found LN to be the

488 least potent of the compounds tested and this is consistent with its activity relative

489 to FN and VZ in a human breast cancer reporter cell line (Orton et al., 2011). We also

490 found VZ to be more potent than FL in suppressing androgen-stimulated spiggin

491 production, which conforms to reports of the relative anti-androgenic activities of

492 these compounds in the same cell line (Aït-Aïssa et al., 2010). However, in the

493 present study the apparent potency of VZ may have been inflated due to the

494 degradation of VZ to active derivatives, which were not measured directly. The low

495 recoveries reported for VZ may be due to the fact that via hydrolysis, photolysis

496 and/or microbial metabolism, VZ yields several degradation products. Compounds 2-

497 [[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and 3',5'-

498 dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) ultimately degrade further to the

499 terminal degradation product 3,5-dicholoraniline (M3; Dhananjeyan et al., 2006).

500 Metabolites M1 and M2 are androgen receptor antagonists (Wong *et al*, 1995).

501 Metabolite M3 requires at least 21 days of reaction time to appear (Szeto et al.,

502 1989) in an aqueous medium and may therefore have contributed less to the

503 observed effects. Concentrations of the parent compound VZ only (rather than the

504 metabolites M1 and M2) were determined in this study.

506 *4.2. Sensitivity of spiggin production to anti-androgens*

507 The IC50s identified for VZ and FL in the single agent calibration exposures in the

508 present study fall within or below the lower range of the concentrations used in

509 previous studies with fish. However, previous reports in which immersive exposure

510 to anti-androgens was employed, rather than direct dosing via the food, have not

511 always employed range-finding studies to set exposure levels. Consequently, the

512 range of concentrations of anti-androgens reported to be bioactive in fish is wide.

513 For VZ, effects in fish have been reported for concentrations of 100 μg/L (medaka,

514 *Oryzias latipes*; León et al., 2008), 60 - 450 μg/L (Martinović et al., 2008), 600 μg/L

515 (zebrafish, *Danio rerio*; Martinović-Weigelt et al., 2011) and 2500 μg/L (medaka;

516 Kiparissis et al., 2003). Between-study comparisons are to some degree confounded

517 by variation in exposure conditions, endpoints, species, and developmental stage of

518 the test fish so, for example, exposure to VZ at 90 – 1200 μg/L evoked no significant

519 effects in embryos whereas in the same study adults exposed to VZ at 700 μg/L did

520 exhibit adverse outcomes (fathead minnow, *Pimephales promelas*; Makynen et al.,

521 2000). Flutamide at a concentration of 412 μg/L was found to elicit only minor

522 phenotypic alterations in exposed fish accompanied by more pronounced effects on

523 gene expression (fathead minnow; Filby et al., 2007). At levels of 651 μg/L (fathead

524 minnow; Jensen et al., 2004), 1000 μg/L (medaka; León et al., 2008) and 1700 μg/L

525 (zebrafish; Martinović-Weigelt et al., 2011), significant effects were observed. Fewer

526 data are available describing sub-lethal endocrine disruptive effects on fish exposed

527 to FN and LN but the IC50s identified within the present study for each are

528 consistent with other investigations in which anti-androgenic activity was detected in

529 sticklebacks at concentrations of between 15 and 200 µg/L (FN) and 150 - 250 µg/L

530 (LN) (Hogan et al., 2012; Katsiadaki et al., 2006; Sebire et al., 2009). Overall, the

531 concentration-response data presented here underline the effectiveness of spiggin

532 as an endpoint for detection of anti-androgenicity and suggest that sticklebacks may

533 be more sensitive to environmental anti-androgens than has hitherto been evident.

534

535 *4.3. Specificity of the spiggin response to anti-androgens and compliance with the CA*

536 *model*

537 The model anti-androgen compounds deployed in this study are assumed to

538 interfere in a specific manner with androgen receptor-dependent signaling pathways

539 throughout the animal. Nevertheless, it is possible that collateral effects within the

540 reproductive endocrine system contributed to the magnitude of the reduction in

541 spiggin. In principle, this could have resulted in unpredictable interactions between

542 the effects of each chemical in the mixture. Certainly, anti-androgenic compounds

543 are reported to exert a wide range of phenotypic effects on the teleost reproductive

544 system (Baatrup and Junge, 2001; Bayley et al., 2002, 2003; Jensen et al., 2004;

545 Kinnberg and Toft, 2003; Kiparissis et al., 2003; Makynen et al., 2000; Martinović et

546 al., 2008; Panter et al., 2004). More recent studies have highlighted the extent to

547 which gene expression within the reproductive axis of fish is modulated by anti-

548 androgens in fish (Filby et al., 2007; Garcia-Reyero et al., 2009; León et al., 2008;

549 Martinović-Weigelt et al., 2011; Villeneuve et al., 2007). Given the wide range of

550 genomic and phenotypic responses that are reported to occur in fish exposed to

551 anti-androgens, it was not clear whether the assumption inherent in the CA model,

552 that the components of the mixture do not influence each others uptake,

553 distribution or metabolism, could be met (Backhaus and Faust, 2012). The mixture

554 composition used in the present study was formulated on the basis of the response

555 of sticklebacks to single agent exposures; each component was present in the final

556 mixture at a ratio proportional to their individual potencies. Interactive effects

557 between the anti-androgens which might be evident only during concurrent

558 exposure to several or all the components of the mixture could not be taken into

559 account during the planning stage of the experiment. For example, individual

560 components of a mixture may exhibit different abilities to induce biotransformation

561 enzymes which in turn may impact on the potency of the mixture overall (discussed

562 by Petersen and Tollefsen, 2011). In addition, VZ has been shown to upregulate the

563 expression of the androgen receptor gene in zebrafish and fathead minnow

564 (Martinović et al., 2008; Smolinsky et al., 2010) with uncertain consequences for the

565 interplay between androgen and anti-androgen. In principle, there might have been

566 disparity in the ability of the tested chemicals to displace or compete with other

567 ligands at the androgen receptor site arising from factors affecting access to the

568 receptor, susceptibility to biotransformation, interaction with other elements of the

569 endocrine system such as sex hormone binding globulin, or differences in the

570 breadth of effects, including non-receptor-mediated effects, exerted by each

571 compound. These are issues that hold greater significance in a whole animal

572 exposure system such as that employed here, than in *in vitro* test systems, and might

573 negatively impact upon the usefulness of CA to predict joint effects *in vivo*. However,

574 the fact that substantial deviations from anticipated CA did not become apparent

575 when the individual effects of all mixture components were used as the basis for the

576 predictions, suggests that the importance of these intervening factors was minimal.

577 In this context, it is noteworthy that in contrast with the findings of the current *in*

578 *vivo* study in which VZ was shown to be the most potent anti-androgen tested, an *in*

579 *vitro* stickleback kidney cell assay identified both FN and FL as more potent anti-

580 androgens than VZ (Jolly et al., 2009). This may relate to the degradation of VZ to

581 active metabolites which is more likely to occur in a large scale mixed

582 solvent/aqueous tank-based exposure system than a small-scale *in vitro* system. The

583 discontinuity in the mixture concentration-response curve (between 57.1 and 285.6

584 µg/L nominal), which was observed in the current study at both laboratories

585 independently, and does not reflect the response profiles seen in the single agent

586 exposures, is currently inexplicable. It might be due to a more complex response to

587 the mixture exposure than that assumed by the model however the mechanism by

588 which this might have occurred is unclear and requires further investigation.

589

590 *4.4. Deviation of exposure concentrations from nominal and implications for the CA*

591 *model*

592 The flow-through system adopted for these exposures presented technical

593 challenges, including maintenance of the desired concentrations of chemicals, both

594 singly and in the mixture. Failure to maintain steady-state concentrations has

595 implications for the assumptions inherent in the CA model. In flow-through systems,

596 measured chemical concentrations can differ from the intended nominal values for a

597 number of reasons including: uptake by the fish, losses due to degradation,

598 adsorption to surfaces, evaporation, photolysis, hydrolysis, or simply by inaccuracies

599 in the preparation of stock solutions or the dosing of tanks. A sufficiently high flow

600 rate might overcome some of these problems, but there are physical limits to the

601 rate of flow that can be sustained through aquaria of the size employed in the

602 present study (30 L). High flows have practical and cost implications for the volumes

603 of chemical stock solutions that are required and the frequency with which they

604 must be prepared and replenished. Given that the measured concentrations for all

605 the chemicals in the test tanks were below the expected concentrations, the decision

606 to carry out the concentration-response analysis on the basis of measured exposure

607 concentrations was vitally important. Consequently, the originally intended

608 composition of the mixture, in a strict quantitative sense, varied along the

609 concentration profile and therefore affected our mixture assessment which was

610 based on data obtained from fixed-ratio mixture designs. This design is particularly

611 suited for multi-component mixtures as it allows, with a relatively small amount of

612 data, an accurate comparison between observed and predicted effect

613 concentrations. However, outcomes are limited to the relative composition of the

614 tested mixture. If data analysis is based on measured concentrations and these vary,

615 then each mixture concentration can be considered to have its own unique

616 composition and the tested concentrations are no longer sequential dilutions of each

617 other. In cases where the differences between nominal and measured exposures are

618 minimal and do not deviate significantly from the original fixed ratio composition,

619 then the applicability of the traditional data analysis for fixed-ratio mixtures is

620 unaffected (Brian et al, 2005). The same holds true if the concentrations of chemicals

621 comprising the mixture are changed, but across all the tested combinations retain

622 the same proportional relationship with each other (constant ratio). In that case the

623 relative composition of the mixture can be re-calculated on the basis of the

624 measured exposures. For example, Correia et al. (2007) studied the joint effect of

625 estradiol-17β, 17α-ethinylestradiol, and bisphenol A on vitellogenin in sea bass

626 (*Dicentrarchus labrax*) and faced the problem of very low recovery rates for the two

627 steroids. Adjustment of the mixture ratio to the measured exposures allowed these

628 authors to perform a comparative mixture assessment for a broad range of mixture

629 exposures for which measured concentrations were not available. In the current

630 study the components of the mixture were not at a constant ratio for all the tested

631 mixture exposures (see Figure 4) an issue which was particularly evident for FN.

632 Nevertheless, by regression modeling it was possible to smooth the measured

633 concentrations, which manifested varying mixture ratios that allowed predictions for

634 a broader range of mixture concentrations. Where exposure data fail to provide a

635 clear functional pattern between the measurements of the component within the

636 mixture, it is nevertheless desirable to investigate outcomes for exposures outside

637 the tested mixture concentrations. Thus all the single compound and mixture data

638 can then be used to estimate a so-called response surface (Gennings and Carter,

639 1995).

640

641 *4.5. Environmental relevance of the findings*

642 This study has confirmed that the inhibition of spiggin production in androgen-

643 primed female sticklebacks provides a sound basis for evaluating the inhibitory

644 potency of mixtures of similarly-acting anti-androgenic chemicals. The data available

645 that describe the occurrence of anti-androgenic substances in the aquatic

646 environment suggests that this class of chemicals may represent a significant

647 developing wildlife and environmental issue, notwithstanding existing concerns

648 about possible human health issues arising via other routes of exposure (Diamanti-

649 Kandarakis et al., 2009). Anti-androgenic compounds appear to be present in most

650 final effluent discharges from wastewater treatment works (WWTW) in the United

651 Kingdom (Johnson et al., 2007). They are found in both the water column (Grover et

652 al., 2011; Urbatzka et al., 2007; Zhao et al., 2011) and in sediments (Weiss et al.,

653 2009; Zhao et al., 2011) at combined concentrations sufficiently high to raise

654 concerns about effects on exposed biota. For example, total anti-androgenic activity

655 (as FL equivalents; eq.) in effluents from forty one UK WWTW were found to range

656 from 29.5 to 844 µg/L FL eq. (mean of two samples at each site) with a median value

657 of 102 µg/L FL eq. and an overall mean of 201 µg/L FL eq. (Johnson et al., 2007).

658 Given that WWTW discharges can contribute a large proportion of the total flow in

659 many receiving waters, this suggests that the range of concentrations of anti-

660 androgens deployed within the mixture studies described for the present study were

661 environmentally realistic for UK rivers. This supposition is supported by the findings

662 of Grover et al. (2011) who reported within-river measurements of anti-androgen

663 concentrations in the R. Ray (southern England) as 206 to 1070 µg/L FL eq. at 100 m

30

664 downstream of a WWTW and as high as 200-400 µg/L FL eq. up to 10 km

665 downstream of the effluent discharge. Similar levels of anti-androgenicity have been

666 reported for rivers elsewhere, including southern China (935 µg/L FL eq., Pearl River;

667 Zhao et al., 2011) and Italy (460 µg/L FL eq., R. Lambro; Urbatzka et al., 2007). These

668 concentrations lie comfortably within the sensitivity range of the assay system

669 employed in the present study. The *in vivo* androgenised female stickleback screen

670 (AFSS) is thus fit for the purpose of the investigation of mixture issues concerning

671 chemicals with anti-androgenic properties, both with regard to the use of an

672 ecologically relevant test species and the sensitivity of the endpoint to anti-androgen

673 exposure. This is underlined by a recent study in which an increase in spiggin levels in

674 female sticklebacks downstream of a WWTW in southern England was detected after

675 remediation of the WWTW effluent, and removal of much of the anti-androgenic

676 activity (Grover et al., 2011; Katsiadaki et al., 2012).

677

678 The array of chemicals with anti-androgenic properties that enter the aquatic

679 environment via WWTW effluents appears to be extensive (Rostkowski et al., 2011)

680 and is likely to be augmented by anti-androgenic chemicals from both agricultural

681 and industrial sources. The methodological approach described here is likely to be

682 too targeted to play a direct role in assessing the risks to wildlife of specific effluents

683 in which the interactions of numerous, ill-defined components are responsible for

684 final effects. However, in order to inform regulatory decisions regarding complex

685 effluents over which some control can be exerted, or in which some components are

686 clearly quantitatively dominant, it is necessary that the manner in which individual

687 components act together is understood. In this context there is an urgent need for

688 the methodology adopted in the present study.

689

697

698 **References**

699 Aït-Aïssa, S., Laskowski, S., Laville, N., Porcher, J.-M., Brion, F., 2010. Anti-androgenic

700   activities of environmental pesticides in the MDA-kb2 reporter cell line.

701   Toxicol. in Vitro 24, 1979-1985.

702 Baatrup. E., Junge, M., 2001. Antiandrogenic pesticides disrupt sexual characteristics

703   in the adult male guppy (*Poecilia reticulata*). Environ. Health Perspect. 109,

704   1063-1070.

705 Backhaus, T., Faust, M., 2012. Predictive environmental risk assessment of chemical

706   mixtures: a conceptual framework. Environ. Sci. Technol. 46, 2564-2573.

32

707   Bayley, M., Junge, M., Baatrup, E., 2002. Exposure of juvenile guppies to three

708   antiandrogens causes demasculinization and a reduced sperm count in adult

709   males. Aquat. Toxicol. 56, 227-239.

710   Bayley, M., Larsen, P.F., Baekgaard, H., Baatrup, E., 2003. The effects of vinclozolin,

711   an anti-androgenic fungicide, on male guppy secondary sex characters and

712   reproductive success. Biol. Reprod. 69, 1951-1956.

713   Berenbaum, M.C., 1985. The expected effect of a combination of agents: the general

714   solution. J. Theor. Biol. 114, 413-431.

715   Brian, J.V., Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, G.,

716   Jonkers, N., Runnalls, T., Bonfa, A., Marcomini, A., Sumpter, J.P., 2005.

717   Accurate prediction of the response of freshwater fish to a mixture of

718   estrogenic chemicals. Environ. Health. Perspect. 113, 721-728.

719   Correia, A.D., Freitas, S., Scholze, M., Goncalves, J.F., Booij, P., Lamoree, M.H.,

720   Mananos, E., Reis-Henriques, M.A., 2007. Mixtures of estrogenic chemicals

721   enhance vitellogenic response in sea bass. Environ. Health. Perspect. 115

722   (Suppl 1), 115-121.

723   Dhananjeyan, M.R., Erhardt, P.W., Corbitt, C., 2006. Simultaneous determination of

724   vinclozolin and detection of its degradation products in mouse plasma, serum

725   and urine, and from rabbit bile, by high-performance liquid chromatography.

726   J. Chromatog. A 1115, 8-18.

727 Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto,

728   A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: an

729   Endocrine Society scientific statement. Endocr. Rev. 30, 293-342.

730 Drescher, K., Boedeker, W., 1995. Assessment of the combined effects of substances

731   - the relationship between concentration addition and independent action.

732   Biometrics 51, 716-730.

733 Efron, B., Tibshirani, R., 1993. An Introduction to the Bootstrap. London: Chapman &

734   Hall.

735 Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P.,

736   Hamer, V., Scholze, M., Vighi, M., Grimme, L.H., 2001. Predicting the joint

737   algal toxicity of multi-component s-triazine mixtures at low-effect

738   concentrations of individual toxicants. Aquat. Toxicol. 56, 13-32.

739 Filby, A.L., Thorpe, K.L., Maack, G., Tyler, C.R., 2007. Gene expression profiles

740   revealing the mechanisms of anti-androgen-and estrogen-induced

741   feminization in fish. Aquat. Toxicol. 81, 219-231.

742 Garcia-Reyero, N., Villeneuve, D.L., Kroll, K.J., Liu, L., Orlando, E.F., Watanabe, K.H.,

743   Sepúlveda, M.S., Ankley, G.T., Denslow, N.D., 2009. Expression signatures for

744   a model androgen and antiandrogen in the fathead minnow (*Pimephales*

745   *promelas*) ovary. Environ. Sci. Technol. 43, 2614-2619.

746 Gennings, C., Carter, W.H., 1995. Utilizing concentration-response data from

747   individual components to detect statistically significant departures from

748   additivity in chemical mixtures. Biometrics 51, 1264-1277.

749 Grover, D.P., Balaam, J., Pacitto, S., Readman, J.W., White, S., Zhou, J.L., 2011.

750   Endocrine disrupting activities in sewage effluent and river water determined

751   by chemical analysis and *in vitro* assay in the context of granular activated

752   carbon upgrade. Chemosphere 84, 1512-1520.

753 Hill, E.M., Evans, K.L., Horwood, J., Rostkowski, P., Oladapo, F.O., Gibson, R., Shears,

754   J.A., Tyler, C.R., 2010. Profiles and some initial identifications of

755   (anti)androgenic compounds in fish exposed to wastewater treatment works

756   effluents. Environ. Sci. Technol. 44, 1137-1143.

757 Hogan, N.S., Gallant, M.J., van den Heuvel, M.R., 2012. Exposure to the pesticide

758   linuron affects androgen-dependent gene expression in the three-spined

759   stickleback (*Gasterosteus aculeatus*). Environ. Toxicol. Chem. 31, 1391-1395.

760 Jakobsson, S., Borg, B., Haux, C., Hyllner, S.J., 1999. An 11-ketotestosterone induced

761   kidney-secreted protein: the nest building glue from male three-spined

762   stickleback, *Gasterosteus aculeatus*. Fish Physiol. Biochem. 20, 79-85.

763 Jensen, K.M., Kahl, M.D., Makynen, E.A., Korte, J.J., Leino, R.L., Butterworth, B.C.,

764   Ankley, G.T., 2004. Characterization of responses to the antiandrogen

765   flutamide in a short-term reproduction assay with the fathead minnow.

766   Aquat. Toxicol. 70, 99-110.

35

767   Jobling, S., Burn, R.W., Thorpe, K., Williams, R., Tyler, C., 2009. Statistical modeling

768   suggests that antiandrogens in effluents from wastewater treatment works

769   contribute to widespread sexual disruption in fish living in English rivers.

770   Environ. Health Perspect. 117, 797-802.

771   Johnson, I., Hetheridge, M., Tyler, C.R., 2007. Assessment of (anti-) oestrogenic and

772   (anti-) androgenic activities of final effluents from sewage treatment works.

773   Science report-SC020118/SR, Environment Agency, Bristol, UK; pp. 61.

774   http://cdn.environment-agency.gov.uk/scho0207bmax-e-e.pdf

775   Jolly, C., Katsiadaki, I., Morris, S., Le Belle, N., Dufour, S., Mayer, I., Pottinger, T.G.,

776   Scott, A.P., 2009. Detection of the anti-androgenic effect of endocrine

777   disrupting environmental contaminants using *in vivo* and *in vitro* assays in the

778   three-spined stickleback. Aquat. Toxicol. 92, 228-239.

779   Jones, I., Lindberg, C., Jakobsson, S., Hellqvist, A., Hellman, U., Borg, B., Olsson, P.E.,

780   2001. Molecular cloning and characterization of spiggin - An androgen-

781   regulated extraorganismal adhesive with structural similarities to von

782   Willebrand Factor-related proteins. J. Biol. Chem. 276, 17857-17863.

783   Jukosky, J.A., Watzin, M.C., Leiter, J.C., 2008. The effects of environmentally relevant

784   mixtures of estrogens on Japanese medaka (*Oryzias latipes*) reproduction.

785   Aquat. Toxicol. 86, 323-331

786   Kang, I.H., Kim, H.S., Shin, J.-H., Kim, T.S., Moon, H.J., Kim, I.Y., Choi, K.S., Kil, K.S.,

787   Park, Y.I., Dong, M.S., Han, S.Y., 2004. Comparison of anti-androgenic activity

788   of flutamide, vinclozolin, procymidone, linuron, and p,p-DDE in rodent 10-day

789   Hershberger assay. Toxicology 199, 145-159.

790   Katsiadaki, I., Scott, A.P., Hurst, M.R., Matthiessen, P., Mayer, I., 2002. Detection of

791   environmental androgens: A novel method based on enzyme-linked

792   immunosorbent assay of spiggin, the stickleback (*Gasterosteus aculeatus*)

793   glue protein. Environ. Toxicol. Chem. 21, 1946-1954.

794   Katsiadaki, I., Morris, S., Squires, C., Hurst, M.R., James, J.D., Scott, A.P., 2006. Use of

795   the three-spined stickleback (*Gasterosteus aculeatus*) as a sensitive in vivo

796   test for detection of environmental antiandrogens. Environ. Health. Perspect.

797   114 Suppl 1, 115-121.

798   Katsiadaki, I., Sanders, M., Sebire, M., Nagae, M., Soyano, K., Scott, A.P., 2007. Three-

799   spined stickleback: an emerging model in environmental endocrine

800   disruption. Environ. Sci. 14, 263-283.

801   Katsiadaki, I., Sanders, M., Henrys, P.A., Scott, A.P., Matthiessen, P., Pottinger, T.G.,

802   2012. Field surveys reveal the presence of anti-androgens in an effluent-

803   receiving river using stickleback-specific biomarkers. Aquat. Toxicol. 122-123,

804   75-85.

805   Kinnberg, K., Toft, G., 2003. Effects of estrogenic and antiandrogenic compounds on

806   the testis structure of the adult guppy (*Poecilia reticulata*). Ecotoxicol.

807   Environ. Safety 54, 16-24.

808   Kiparissis, Y., Metcalfe, T.L., Balch, G.C., Metcalf, C.D., 2003. Effects of the

809   antiandrogens, vinclozolin and cyproterone acetate on gonadal development

810   in the Japanese medaka (*Oryzias latipes*). Aquat. Toxicol. 63, 391-403.

811   Kortenkamp, A., 2007. Ten years of mixing cocktails: a review of combination effects

812   of endocrine-disrupting chemicals. Environ. Health Perspect. 115, 98-105.

813   Lambright, C., Ostby, J., Bobseine, K., Wilson, V., Hotchkiss, A.K., Mann, P.C., Gray,

814   L.E., 2000. Cellular and molecular mechanisms of action of linuron: An

815   antiandrogenic herbicide that produces reproductive malformations in male

816   rats. Toxicol. Sci. 56, 389-399.

817   León, A., Wu, P.S., Hall, L.C., Johnson, M.L., The, S.J., 2008. Global gene expression

818   profiling of androgen disruption in Qurt strain medaka. Environ. Sci. Technol.

819   42, 962-969.

820   Makynen, E.A., Kahl, M.D., Jensen, K.M., Tietge, J.E., Wells, K.L., van der Kraak, G.,

821   Ankley, G.T., 2000. Effects of the mammalian antiandrogen vinclozolin on

822   development and reproduction of the fathead minnow (*Pimephales*

823   *promelas*). Aquat. Toxicol. 48, 461-475.

824   Martinović, D., Blake, L.S., Durhan, E.J., Greene, K.J., Kahl, M.D., Jensen, K.M.,

825   Makynen, E.A., Villeneuve, D.L., Ankley, G.T., 2008. Reproductive toxicity of

826   vinclozolin in the fathead minnow: Confirming an anti-androgenic mode of

827   action. Environ. Toxicol. Chem. 27, 478-488.

828 Martinović-Weigelt, D., Wang, R.L., Villeneuve, D.L., Bencic, D.C., Lazorchak, J.,

829   Ankley, G.T., 2011. Gene expression profiling of the androgen receptor

830   antagonists flutamide and vinclozolin in zebrafish (*Danio rerio*) gonads.

831   Aquat. Toxicol. 101, 447-458.

832 OECD, 2011. Guidance document on the androgenised female stickleback screen.

833   Series on Testing and Assessment. No. 148. pp. 47. Environment Directorate,

834   Organisation for Economic Co-Operation and Development, Paris.

835   http://www.oecd.org/chemicalsafety/testingofchemicals/48532191.pdf

836 Olsson, P.E., Berg A.H., von Hofsten, J., Grahn, B., Hellqvist, A., Larsson, A., Karlsson,

837   J., Modig, C., Borg, B., Thomas, P., 2005. Molecular cloning and

838   characterization of a nuclear androgen receptor activated by 11-

839   ketotestosterone. Reprod. Biol. Endocrinol. 3, 37.

840 Orton, F., Rosivatz, E., Scholze, M., Kortenkamp, A., 2011. Widely used pesticides

841   with previously unknown endocrine activity revealed as *in vitro*

842   antiandrogens. Environ. Health. Perspect. 119, 794-800.

843 Panter, G.H., Hutchinson, T.H., Hurd, K.S., Sherren, A., Stanley, R.D., Tyler, C.R., 2004.

844   Successful detection of (anti-) androgenic and aromatase inhibitors in pre-

845   spawning adult fathead minnows (*Pimephales promelas*) using easily

846   measured endpoints of sexual development. Aquat. Toxicol. 70, 11-21.

847 Petersen, K., Tollefsen, K.E., 2011. Assessing combined toxicity of estrogen receptor

848   agonists in a primary culture of rainbow trout (*Oncorhynchus mykiss*)

849   hepatocytes. Aquat. Toxicol. 101, 186-195.

850   Pottinger, T.G., Carrick, T.R., Yeomans, W.E., 2002. The three-spined stickleback as

851   an environmental sentinel: effects of stressors on whole-body physiological

852   indices. J. Fish Biol. 61, 207-229.

853   Pottinger, T.G., Cook, A., Jurgens, M.D., Rhodes, G., Katsiadaki, I., Balaam, J.L., Smith,

854   A.J., Matthiessen, P., 2011. Effects of sewage effluent remediation on body

855   size, somatic RNA: DNA ratio, and markers of chemical exposure in three-

856   spined sticklebacks. Environ. Int. 37, 158-169.

857   Pottinger, T.G., Henrys, P.A., Williams, R.J., Matthiessen, P., 2013. The stress

858   response of three-spined sticklebacks is modified in proportion to effluent

859   exposure downstream of wastewater treatment works. Aquat. Toxicol. 126,

860   382-392.

861   Rostkowski, P., Horwood, J., Shears, J.A., Lange, A., Oladapo, F.O., Besselink, H.T.,

862   Tyler, C.R., Hill, E.M., 2011. Bioassay-directed identification of novel

863   antiandrogenic compounds in bile of fish exposed to wastewater effluents.

864   Environ. Sci. Technol. 45, 10660-10667.

865   Sanchez, W., Katsiadaki, I., Piccini, B., Ditche, J.M., Porcher, J.M., 2008. Biomarker

866   responses in wild three-spined stickleback (*Gasterosteus aculeatus* L.) as a

867   useful tool for freshwater biomonitoring: A multiparametric approach.

868   Environ. Int. 34, 490-498.

869   Scholze, M., Boedeker, W., Faust, M., Backhaus, T., Altenburger, R., Grimme, L.H.,

870   2001. A general best-fit method for concentration-response curves and the

871   estimation of low-effect concentrations. Environ. Toxicol. Chem. 20, 448-457.

872   Scott, A.P., Mackenzie, D.S., Stacey, N.E., 1984. Endocrine changes during natural

873   spawning of the white sucker, *Catostomus commersoni* . II. Steroid hormones.

874   Gen. Comp. Endocrinol. 56, 349-359.

875   Sebire, M., Scott, A.P., Tyler, C.R., Cresswell, J., Hodgson, D.J., Morris, S., Sanders,

876   M.B., Stebbing, P.D., Katsiadaki, I., 2009. The organophosphorous pesticide,

877   fenitrothion, acts as an anti-androgen and alters reproductive behavior of the

878   male three-spined stickleback, *Gasterosteus aculeatus*. Ecotoxicology 18, 122-

879   133.

880   Smolinsky, A.N., Doughman, J.M., Kratzke, L.T.C., Lassiter, C.S., 2010. Zebrafish

881   (*Danio rerio*) androgen receptor: sequence homology and up-regulation by

882   the fungicide vinclozolin. Comp. Biochem. Physiol. C – Toxicol. Pharmacol.

883   151, 161-166.

884   Sumpter, J.P., Johnson, A.C., 2008. 10th Anniversary Perspective: Reflections on

885   endocrine disruption in the aquatic environment: from known knowns to

886   unknown unknowns (and many things in between). J. Environ. Monit. 10,

887   1476-1485.

888   Szeto, S.Y., Burlinson, N.E., Rahe, J.E., Oloffs, P.C., 1989. Kinetics of hydrolysis of the

889   dicarboximide fungicide vinclozolin. J. Agric. Food Chem. 37, 523-529.

41

890   Tamura, H., Maness, S.C., Reischmann, K., Dorman, D.C., Gray, L.E., Gaido, K.W.,

891   2001. Androgen receptor antagonism by the organophosphate insecticide

892   fenitrothion. Toxicol. Sci. 60, 56-62.

893   Thorpe, K.L., Hutchinson, T.H., Hetheridge, M.J., Scholze, M., Sumpter, J.P., Tyler,

894   C.R., 2001. Assessing the biological potency of binary mixtures of

895   environmental estrogens using vitellogenin induction in juvenile rainbow

896   trout (*Oncorhynchus mykiss*). Environ. Sci. Technol. 35, 2476-2481.

897   Urbatzka, R., van Cauwenberge, A., Maggioni, S., Viganò, L., Mandich, A., Benfenati,

898   E., Lutz, I., Kloas, W., 2007. Androgenic and antiandrogenic activities in water

899   and sediment samples from the river Lambro, Italy, detected by yeast

900   androgen screen and chemical analyses. Chemosphere 67, 1080-1087.

901   Villeneuve, D.L., Blake, L.S., Brodin, J.D., Greene, K.J., Knoebl, I., Miracle, A.L.,

902   Martinovic, D., Ankley, G.T., 2007. Transcription of key genes regulating

903   gonadal steroidogenesis in control and ketoconazole- or vinclozolin-exposed

904   fathead minnows. Toxicol. Sci. 98, 395-407.

905   Vonesh, E., Chinchilli, V.M., 1996. Linear and Nonlinear Models for the Analysis of

906   Repeated Measurements. New York: Marcel Dekker.

907   Weiss, J.M., Hamers, T., Thomas, K.V., van der Linden, S., Leonards, P.E.G., Lamoree,

908   M.H., 2009. Masking effect of anti-androgens on androgenic activity in

909   European river sediment unveiled by effect-directed analysis. Anal. Bioanal.

910   Chem. 394, 1385-1397.

911 Wilson, V.S., Cardon, M.C., Gray, L.E., Hartig, P.C., 2007. Competitive binding

912   comparison of endocrine-disrupting compounds to recombinant androgen

913   receptor from fathead minnow, rainbow trout, and human. Environ. Toxicol.

914   Chem. 26, 1793-1802.

915 Wong, C.I., Kelce, W.R., Sar, M., Wilson, E.M., 1995. Androgen receptor antagonist

916   versus agonist activities of the fungicide vinclozolin relative to

917   hydroxyflutamide. J. Biol. Chem. 270, 19998-20003.

918 Zhang, H., Kong, F.-X., Yu, Y., Shi, X.-L., Zhang, M., Tian, H.-E., 2010. Assessing the

919   combination effects of environmental estrogens in fish. Ecotoxicology 19,

920   1476-1486.

921 Zhao, J.-L., Ying, G.-G., Yang, B., Liu, S., Zhou, L.-J., Chen, Z.-F., Lai, H.-J., 2011.

922   Screening of multiple hormonal activities in surface water and sediment from

923   the pearl river system, South China, using effect-directed *in vitro* bioassays.

924   Environ. Toxicol. Chem. 30, 2208-2215.

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Figure legends

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Figure 1: Graphical representation of a fixed- and variable-ratio for a mixture of two

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931 compounds. The line A refers to the planned mixture composition based on nominal

932 concentrations, and the lines B and C represent scenarios in which both compounds

933 were measured with different recovery ratios: line B assumes constant ratios

934 between nominal and measured concentrations, and line C occurs when for one

935 compound at least the ratio varies independently of the tested mixture

936 concentration.

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938 **Figure 2.** Concentration-response data and fitted curves for (a) flutamide, FL; (b)

939 fenitrothion, FN; (c) linuron, LN; (d) vinclozolin, VZ. Data are pooled from

940 independent studies carried out in two laboratories (Lancaster and Bergen; see

941 section 2.4.1 for details). Each point is the mean ± standard error. The best-fitting

942 regression models (solid lines; see Table 1) are shown together with the

943 corresponding 95% confidence intervals (dotted lines).

944

945 **Figure 3.** Between-study variability in the spiggin response and normalisation of the

946 absolute effect scale. Concentration-effect data for linuron (LN) from two

947 independent studies (denoted by circles and stars) conducted at Bergen are shown

948 based on (a) raw spiggin values, and (b) spiggin values normalized to solvent and

949 positive DHT controls. Each point represents the median value with the 25th and 75th

950 sample percentiles indicated. The horizontal lines correspond to the control means.

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Figure 4. Measured concentrations and estimated measured concentrations for (a)

flutamide, FL; (b) fenitrothion, FN; (c) linuron, LN; (d) vinclozolin, VZ. Measured data

are from at least three sample points during each of two independent mixture

studies conducted at Lancaster and Bergen. Estimated measured concentrations are

shown as second-order regression curves (see Table S.2). The dotted black lines

indicate parity between measured and nominal concentrations.

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Figure 5: Concentration-response data for a mixture of four anti-androgens, tested in

two different laboratories (A: Lancaster; B: Bergen). Open circles denote individual

data, solid circles show the median effect, and vertical lines delineate the inter-

quartile range (i.e. distance between the 25th and the 75th sample percentiles).

Mixture effects were predicted according to the Concentration Addition method and

are shown as an unbroken line with the approximate 95% confidence intervals as

dotted lines.

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972 **Table 1:** Anti-androgenicity of individual compounds

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Substance (by order of IC$_{50}$)	Concentration response function						IC10[1]	IC50[1]
	RM[2]	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_3$	$\hat{\theta}_{min}$	θ_{max}	µg/L [CI]	µg/L [CI]
vinclozolin	Logit	6.73	-7.21	-	-0.01	1	4.25E+0 [1.51E+0 - 6.45E+1][3]	8.57E+0 [6.64E+0 - 1.03E+1][3]
fenitrothion	Logit	3.57	-2.33	-	-0.33	1	2.86E+0 [1.52E+0 - 5.01E+1]	2.07E+1 [1.34E+1 - 3.50E+1]
flutamide	G.Logit I	15.93	-2.67	55241	-0.44	1	7.81E+0 [3.91E+0 - 1.33E+1]	3.63E+1 [3.08E+1 - 4.70E+1]
linuron	Weibull	3.39	-1.68	-	0.01	1	3.32E+1 [2.19E+1 - 5.07E+1]	1.72E+2 [1.21E+2 - 2.58E+2]

974 [1]IC50, IC10: measured concentration provoking 50% and 10% inhibition of the effect produced by nominal 5 µg/L DHT, respectively.

975 [2]The column "RM" indicates the mathematical regression function as defined by Scholze *et al.* (2001).

976 $\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_3, \hat{\theta}_{min}$: estimated model parameters, given for concentrations expressed in µg/l (rounded values), θ_{max} were not estimated, but set to 1

977 relating to the mean value of the DHT controls.

978 [3]Values in brackets denote the upper and lower limits of the approximate 95% confidence interval.

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980 **Table 2.** Nominal and measured exposure concentrations for both mixture studies.

| | | Nominal mixture concentrations [µg/L] | | | | | | | | | | |
| | | 5.71 | | 19.04 | | 57.12 | | 142.8 | | 285.6 | | 571.21 | |
Components	Laboratory:	Lancaster	Bergen	Lancaster	Bergen	Lancaster	Bergen	Lancaster	Bergen	Lancaster	Bergen	Lancaster	Bergen
vinclozolin	nominal	0.41		1.36		4.08		10.19		20.38		40.75	
	measured[1]	0.36	0.35	0.67	0.33	1.61	1.16	4.45	2.75	7.20	6.85	16.22	12.92
	recovery [%]	87.3	85.0	49.0	23.9	39.5	28.5	43.7	26.9	35.3	33.6	39.8	31.7
	estimated[2]	0.34	0.30	0.72	0.47	1.67	1.02	3.84	2.52	7.73	5.80	16.60	15.29
fenitrothion	nominal	0.37		1.24		3.72		9.29		18.58		37.16	
	measured	1.30	0.15	1.55	0.45	2.14	1.57	4.46	3.08	6.68	13.43	16.60	23.34
	recovery [%]	349.5	41.0	125.1	36.0	57.6	42.3	48.0	33.2	35.9	72.3	44.7	62.8
	estimated	1.33	0.15	1.47	0.45	2.25	1.42	4.11	4.14	7.53	10.0	15.70	25.6
flutamide	nominal	0.72		2.40		7.21		18.01		36.03		72.06	
	measured	0.86	1.03	2.05	1.78	6.55	5.80	15.60	17.70	23.76	28.65	45.77	45.85
	recovery [%]	120.1	143.2	85.4	74.3	90.9	80.4	86.6	98.3	65.9	79.5	63.5	63.6
	estimated	0.82	0.90	2.37	2.34	6.24	8.00	13.90	13.80	25.39	26.70	46.27	53.02
linuron	nominal	4.21		14.04		42.12		105.31		210.62		421.24	
	measured	4.21	2.31	10.04	5.38	34.27	15.04	95.52	52.05	150.94	103.96	274.20	166.52
	recovery [%]	100.0	54.7	71.5	38.3	81.3	35.7	90.7	49.4	71.7	49.4	65.1	39.5
	estimated	3.87	2.13	12.10	6.08	34.05	17.18	80.31	43.29	153.28	90.19	291.86	193.66

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983   (Footnote for Table 2)

984   [1]Measured values given for the individual compounds represent their geometric mean concentrations in the mixture determined during a minimum of

985   three independent occasions of the mixture studies.

986   [2]Estimated concentrations were derived by a second-order regression modeling the relationship between nominal and measured values (see Table S2 for

987   more details)

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992 **Table 3.** Observed and predicted spiggin induction (normalized to the means of solvent and DHT controls; see Section 2.7) for a mixture of four
993 anti-androgenic compounds.

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	Nominal mixture concentrations [µg/L]					
	5.71	19.04	57.12	142.8	285.6	571.21
observed (mean ± SEM)						
Lancaster	1.02 ± 0.036 (n=15)	0.76 ± 0.047 (n=15)	0.64 ± 0.062 (n=15)	0.59 ± 0.086 (n=11)	0.17 ± 0.035 (n=14)	0.04 ± 0.024 (n=15)
Bergen	0.97 ± 0.023 (n=13)	0.91 ± 0.030 (n=12)	0.69 ± 0.099 (n=13)	0.78 ± 0.035 (n=14)	0.33 ± 0.086 (n=12)	0.02 ± 0.021 (n=15)
predicted by CA (mean with 95% confidence interval)						
Lancaster	0.93 [0.88-0.96]	0.87 [0.82-0.90]	0.65 [0.59-0.70]	0.29 [0.23-0.34]	0.18 [0.13-0.24]	<0.01 [0.0-0.02]
Bergen	0.98 [0.95-.99]	0.95 [0.92-0.97]	0.77 [0.73-0.80]	0.41 [0.35-0.45]	0.10 [0.05-0.14]	<0.01 [0.0-0.02]

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Appendix

The mathematical relationship f between nominal ($c_{nominal}$) and measured concentrations ($c_{measured}$) were modeled for each compound independently by a second order regression after a $\log_{10}$-transformation of the concentration scale, i.e. for $\log_{10}(c_{measured})= f(\log10(c_{nominal})) + error$ we set

$$f\left(\log_{10}(c_{nominal})\right)=\theta_1+\theta_2*\log_{10}(c_{nominal})+\theta_3*\left(\log_{10}(c_{nominal})\right)^2. \qquad (A.1)$$

Here the statistical error term is assumed to be normal distributed with zero mean, and θ_1, θ_2 and θ_3 are the model parameters which have to be estimated. Occasionally we estimated a functional minimum at very low concentrations, although always below the lowest test concentration. To ensure that decreasing nominal concentrations lead always to decreasing and coherent estimates, we restricted estimates only to the strict monotonic ranges of Equation (A.1), and concentrations below this minimum were estimated by a linear function between the corresponding estimate and the zero origin. If $C_{Minimum}$ is the concentration at this global functional minimum, then function f in Equation (A.1) can be expanded to

$$f^*\left(\log_{10}(c_{nominal})\right)=\begin{cases} f\left(\log_{10}(c_{nominal})\right) & \text{for} \quad c_{nominal}\geq C_{Minimum} \\ \dfrac{f\left(\log_{10}(C_{Minimum})\right)}{C_{Minimum}}*c_{nominal} & \text{else} \end{cases}. \qquad (A.2)$$

According to calculus, a global minimum is given only when the term $2*\theta_3$ is positive, and then $C_{Minimum}$ can be calculated as $\log_{10}(C_{Minimum})= - \theta_2/(2* \theta_3)$.
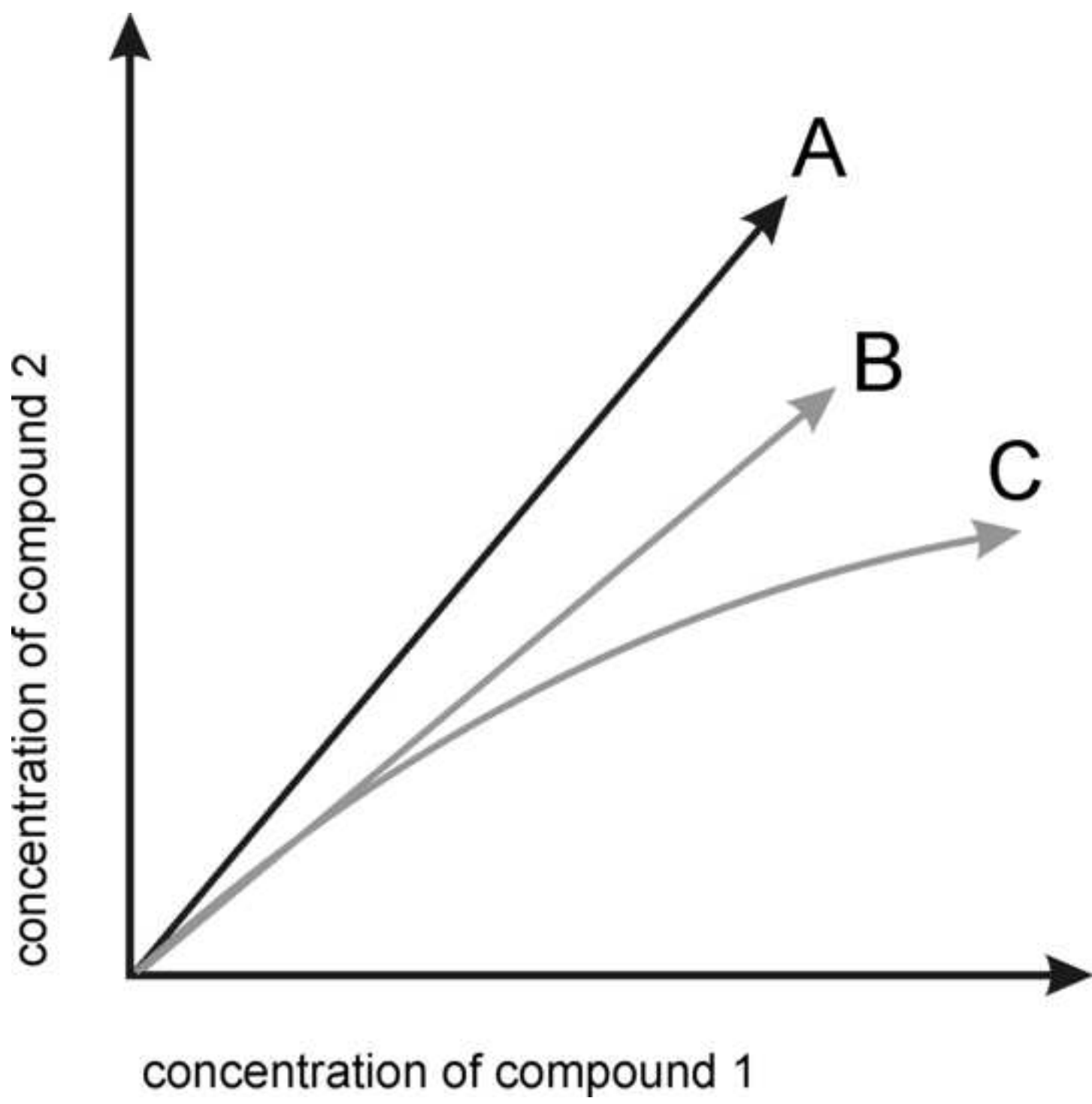
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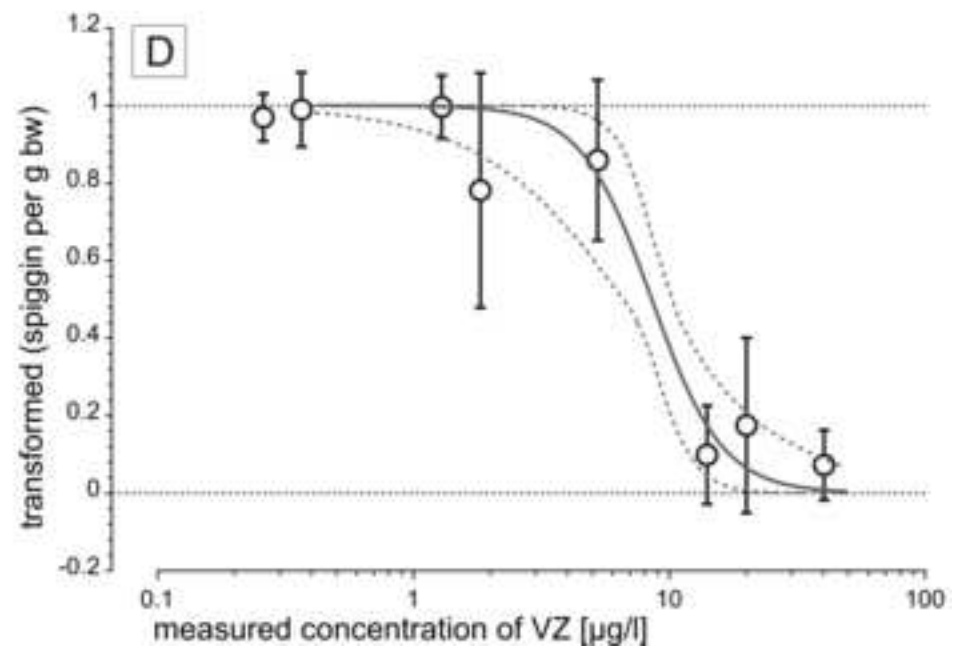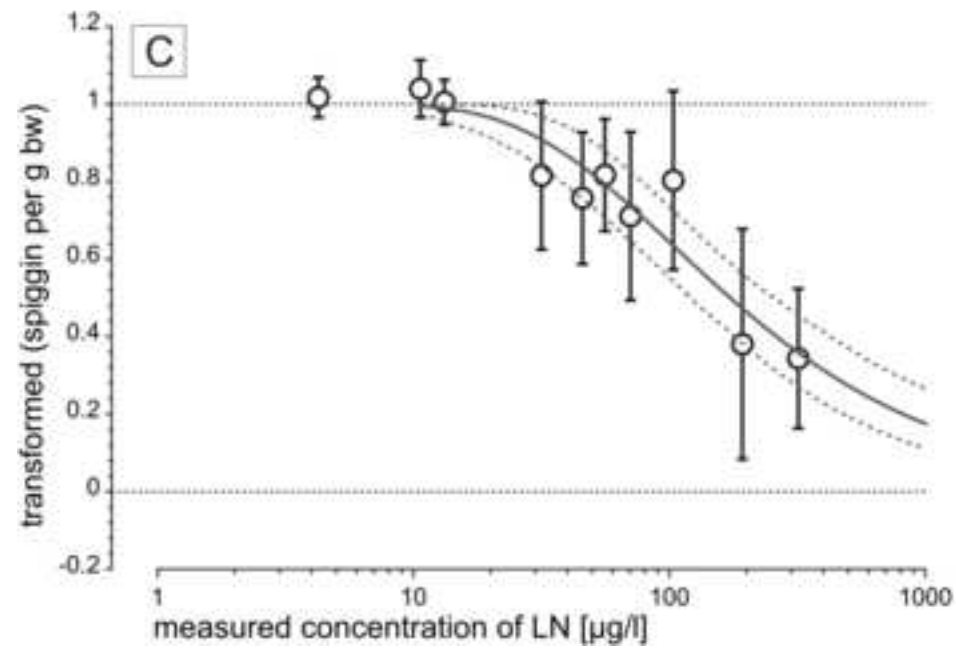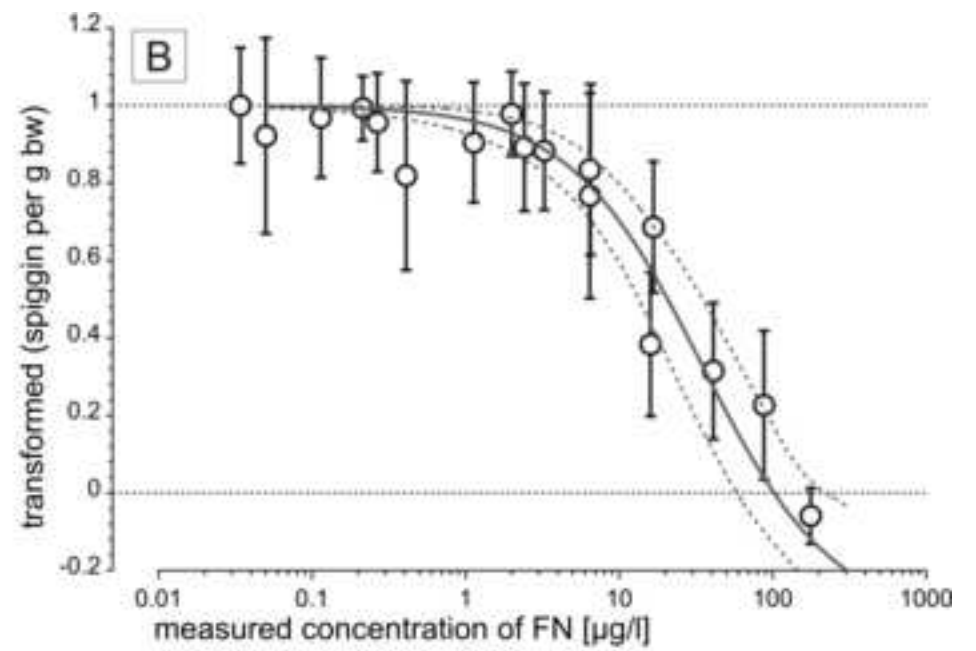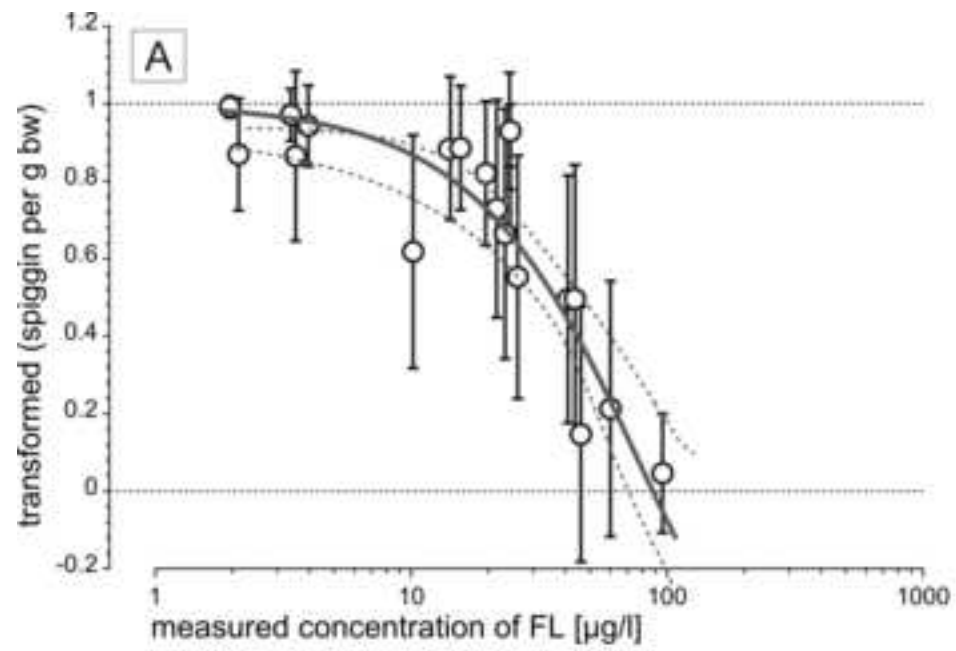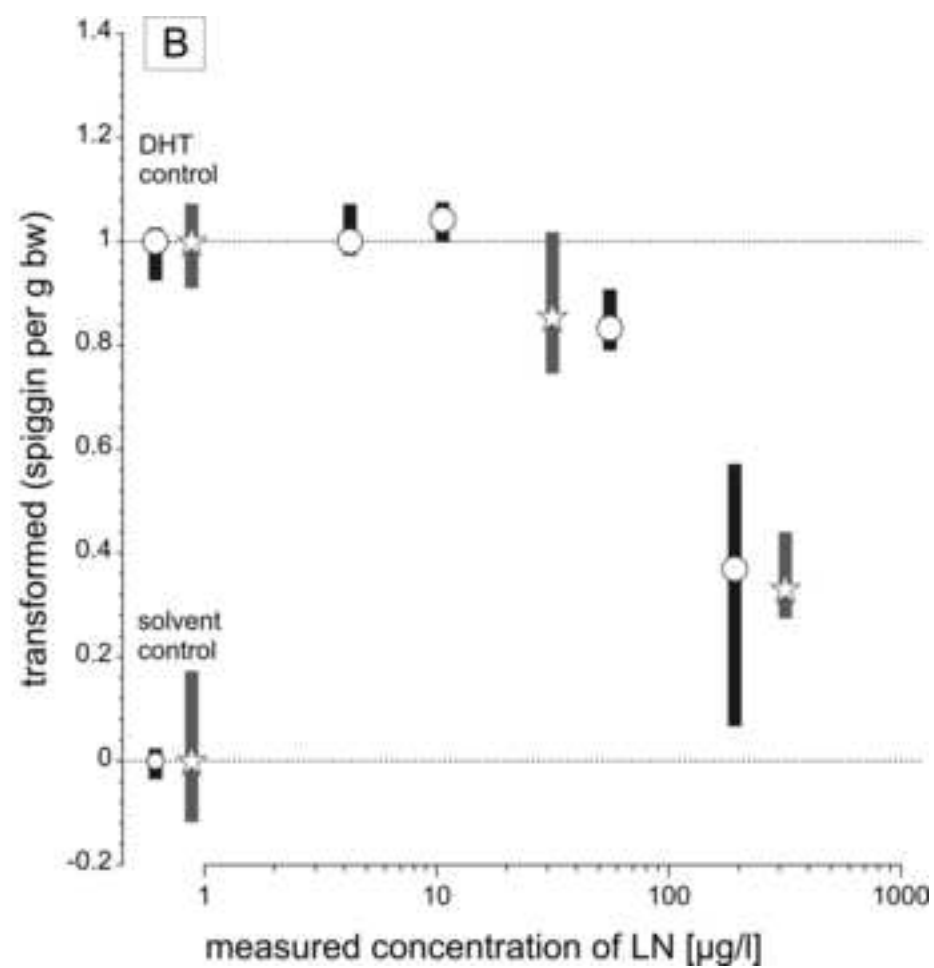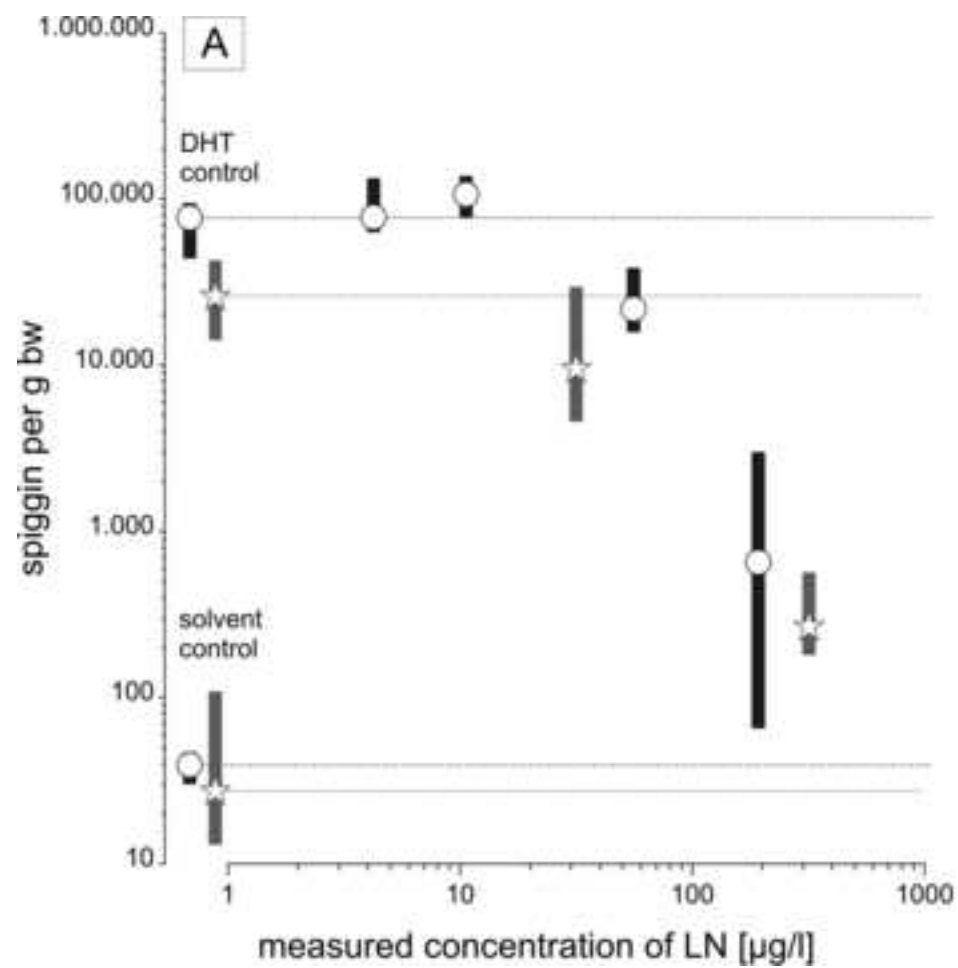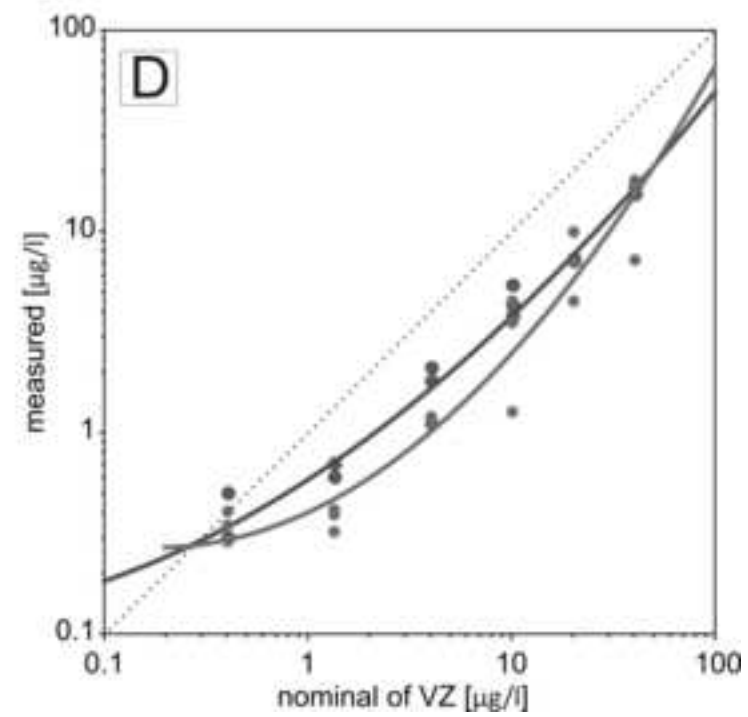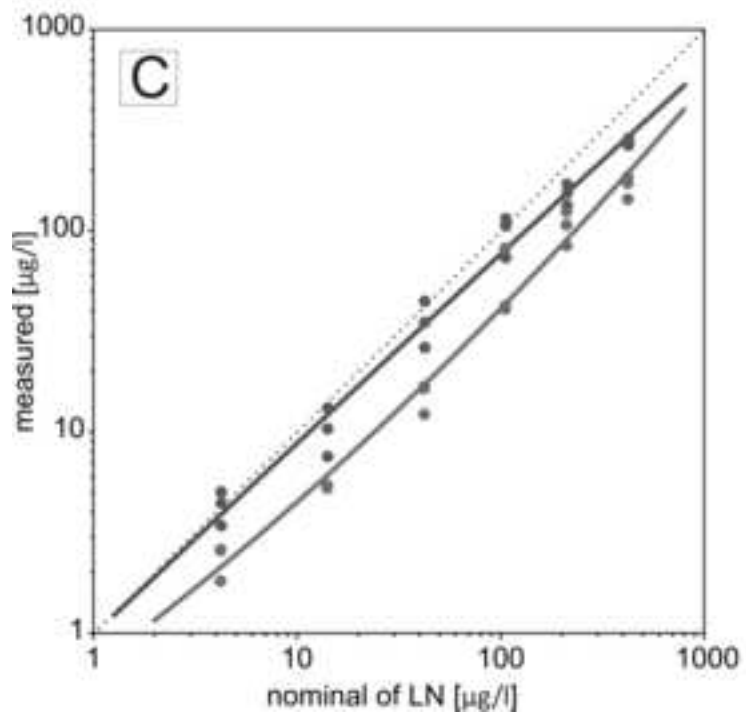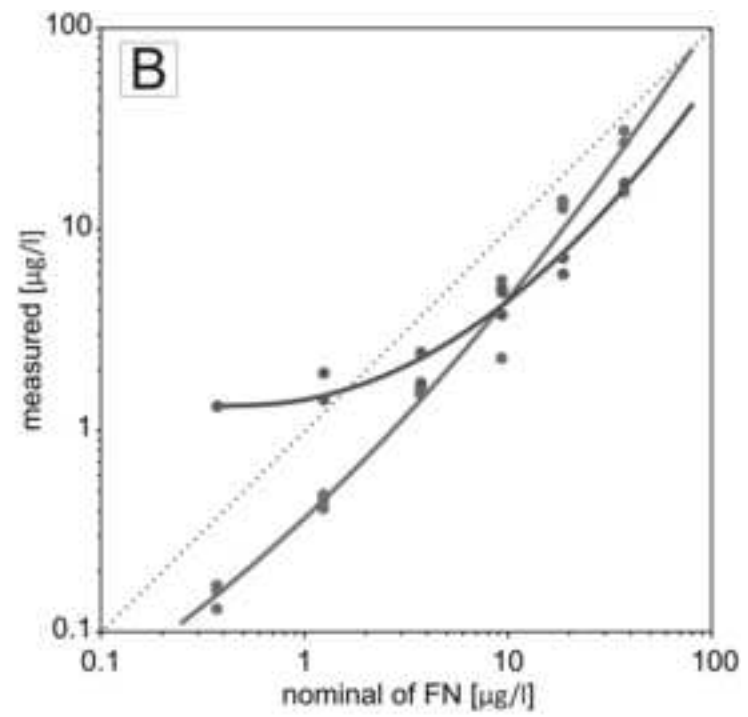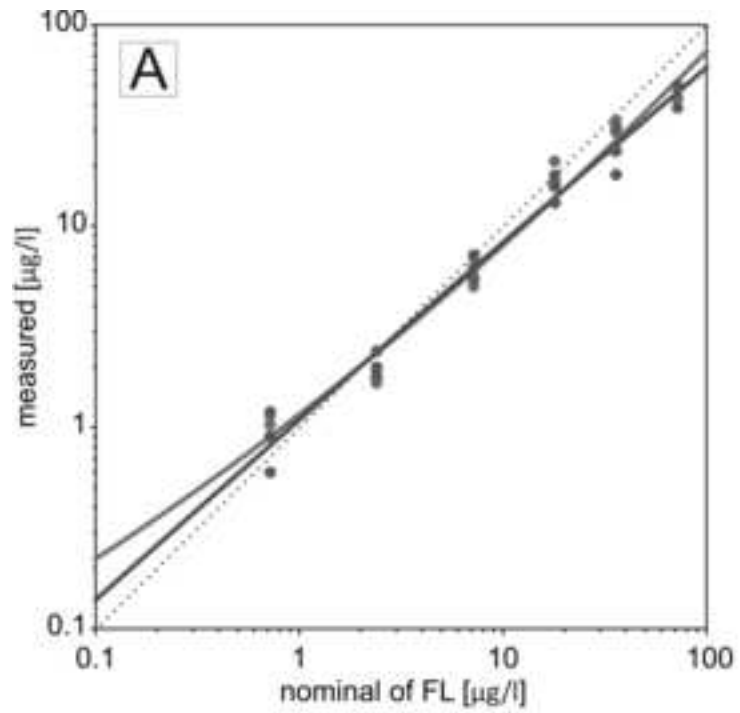
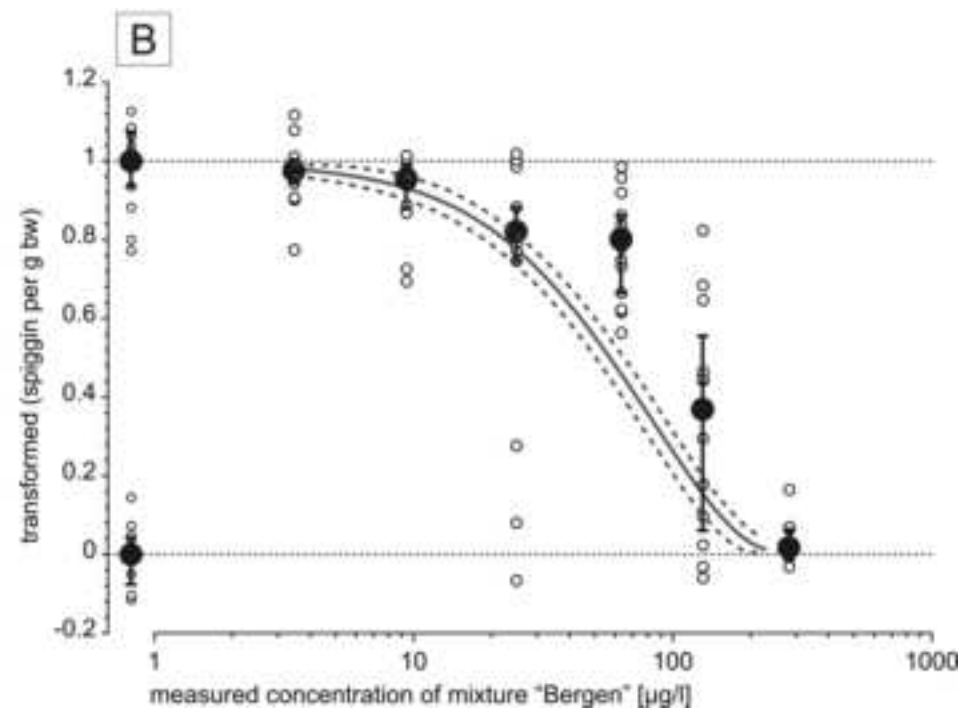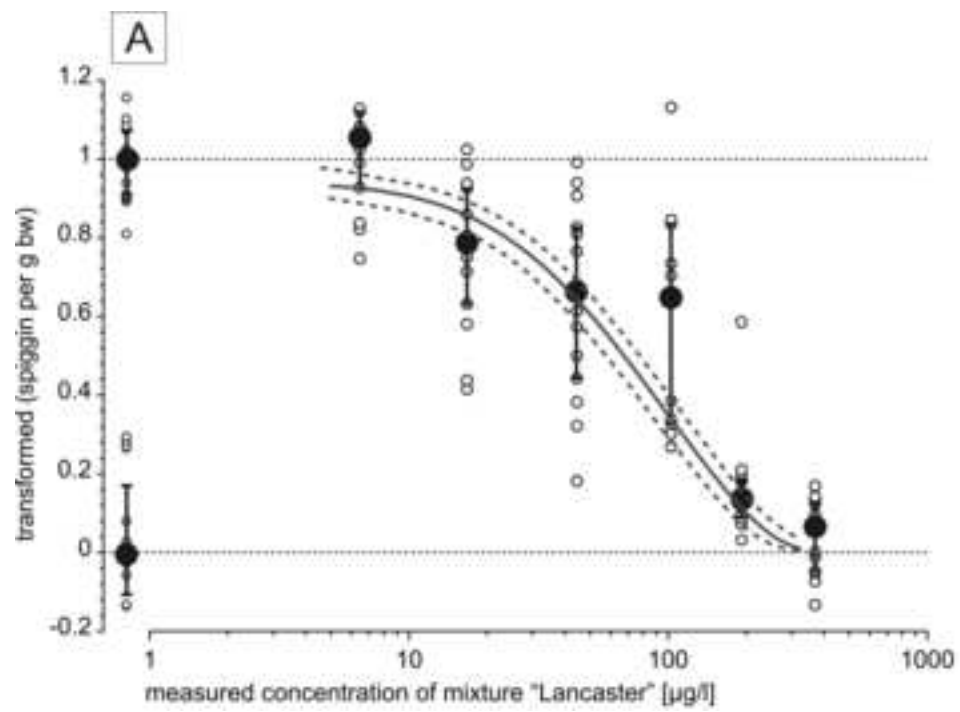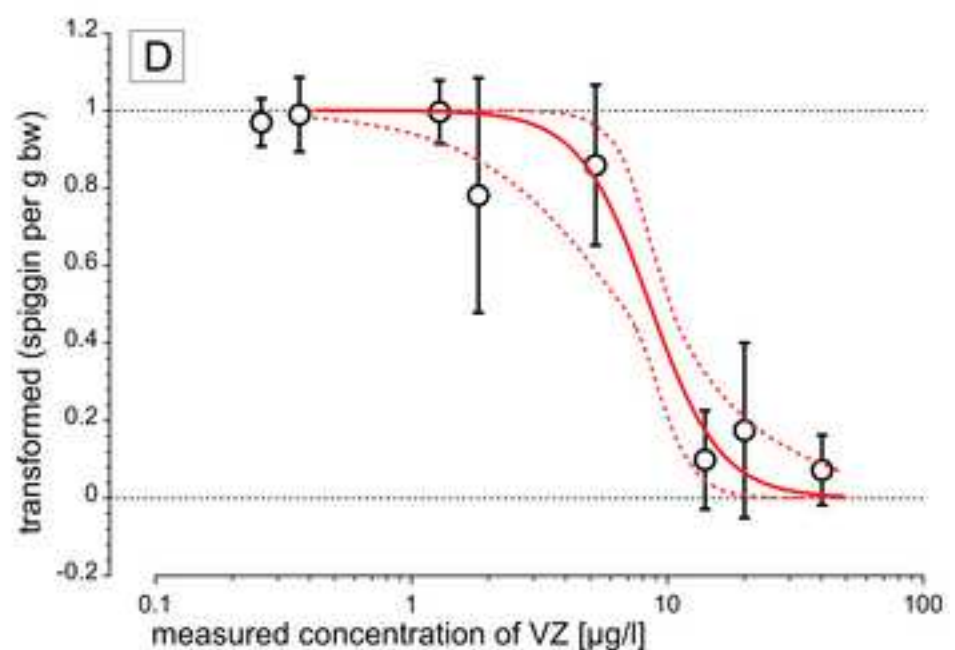**Figure 2 COL**
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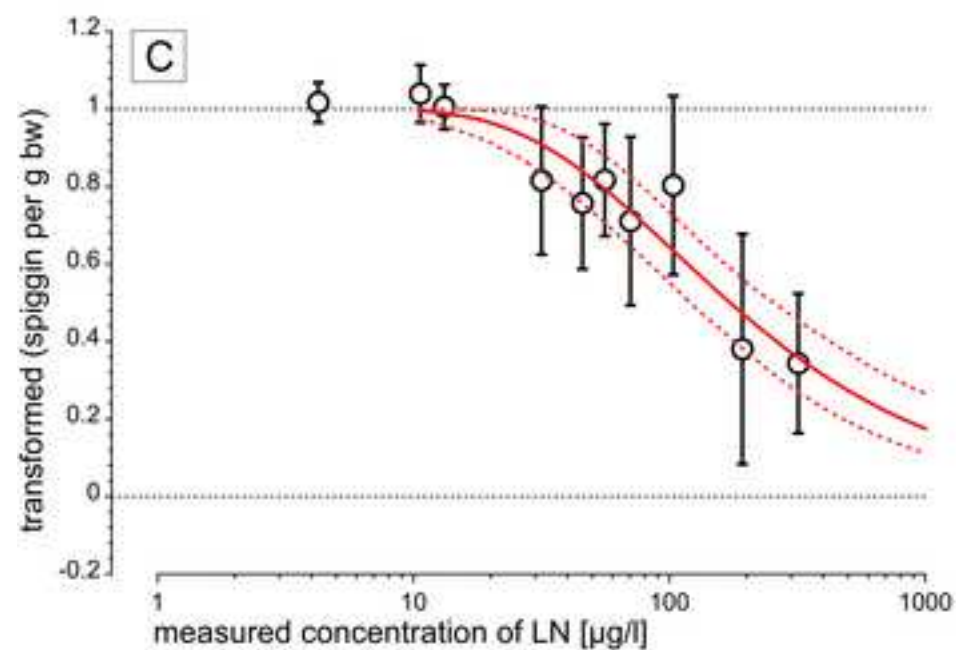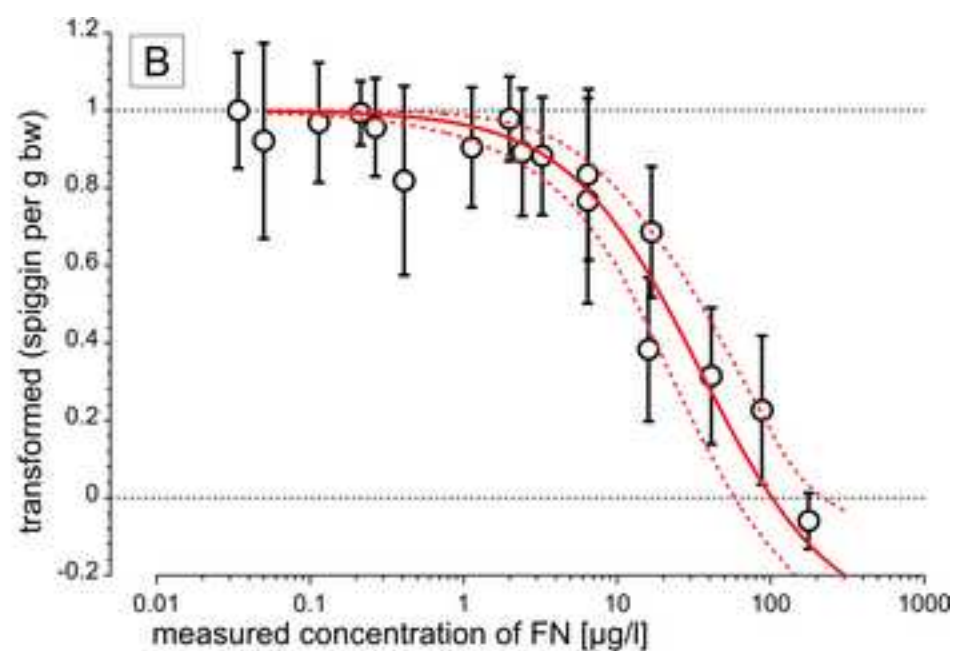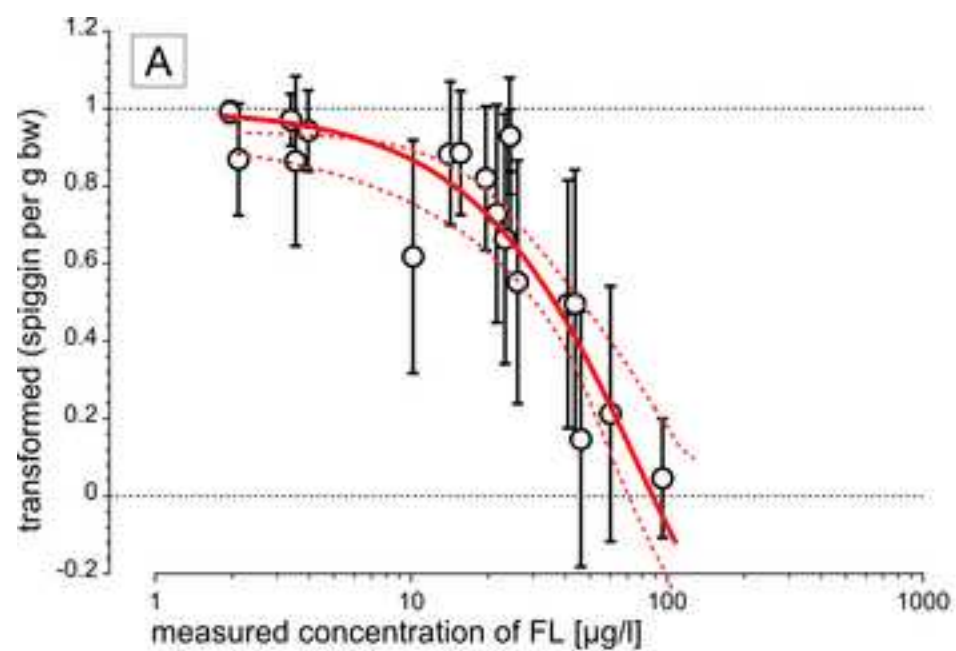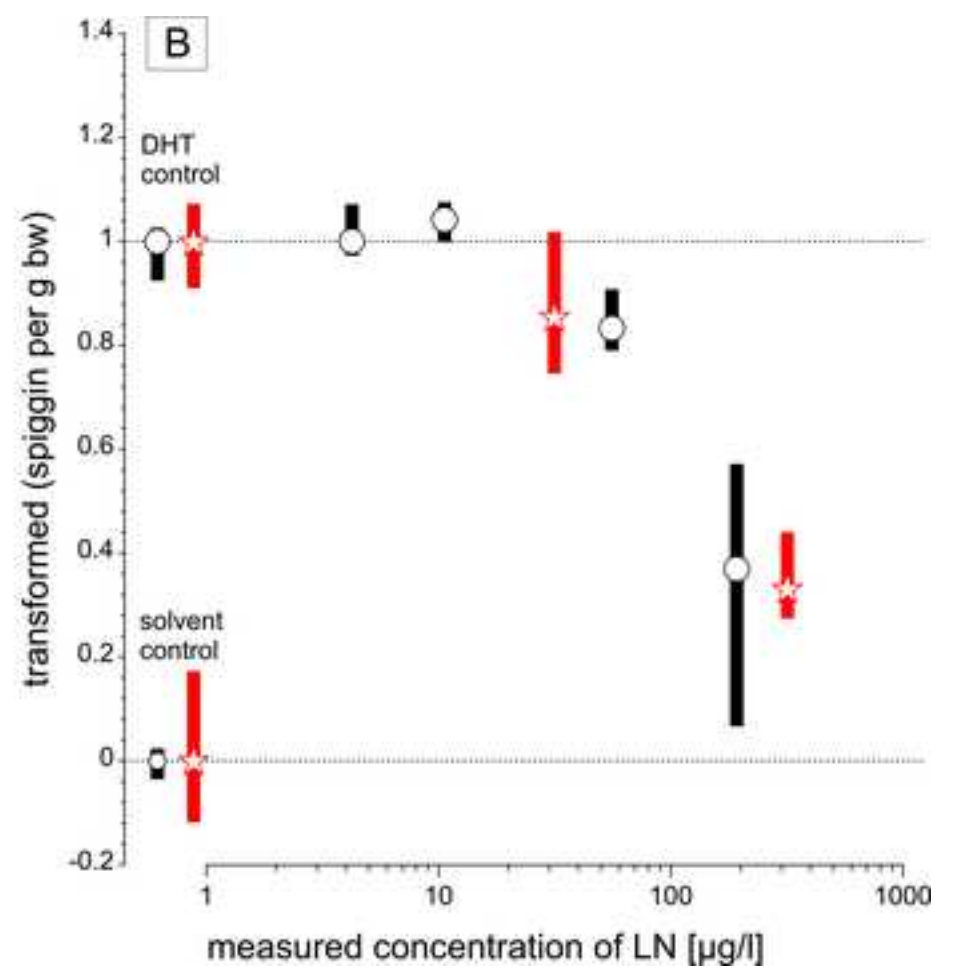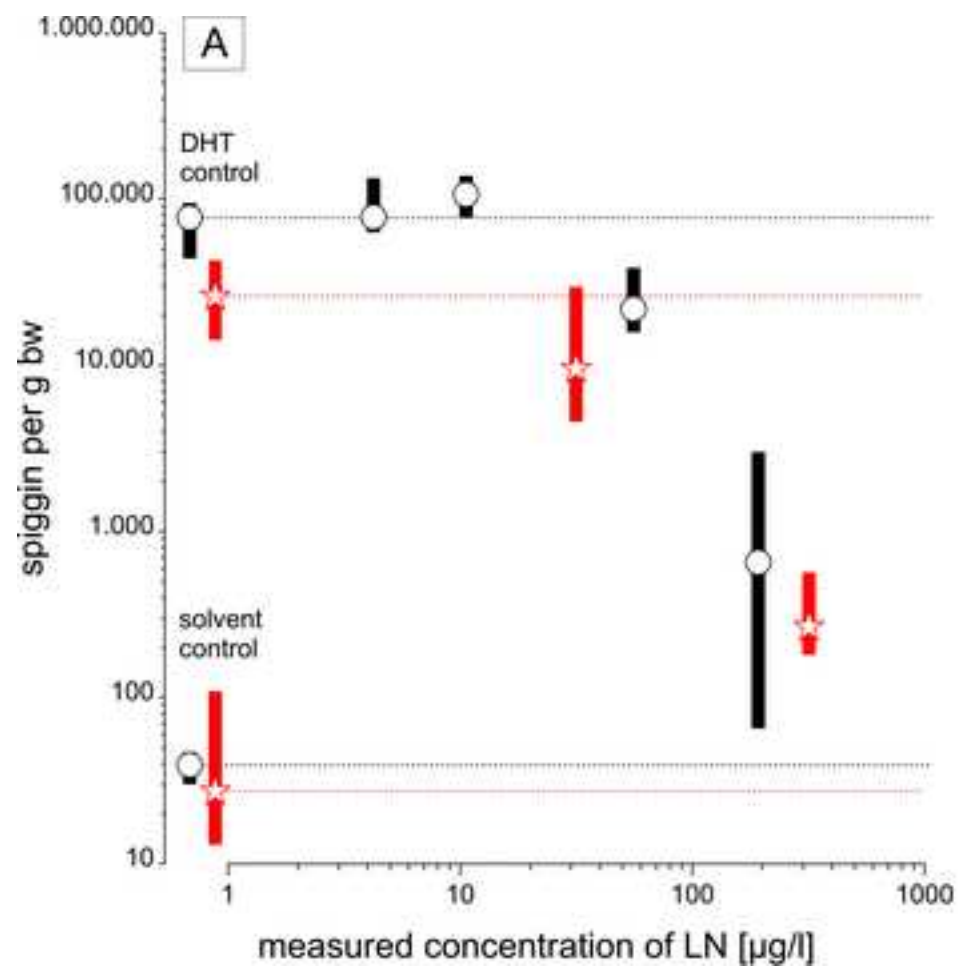**Figure 3 COL**
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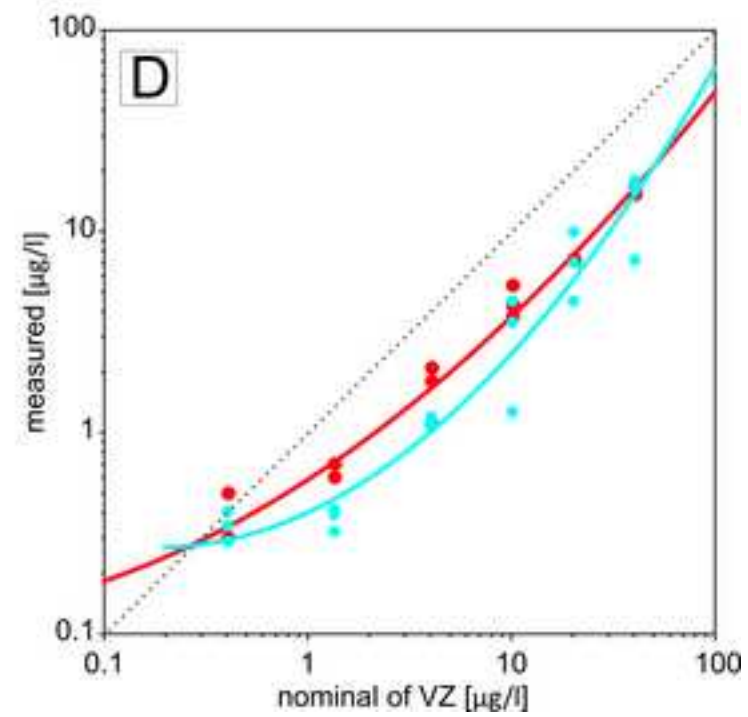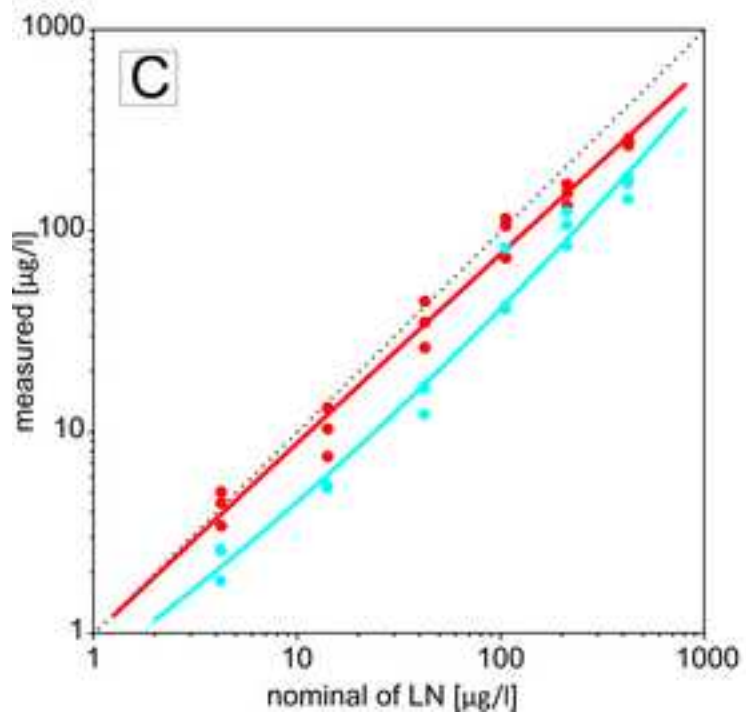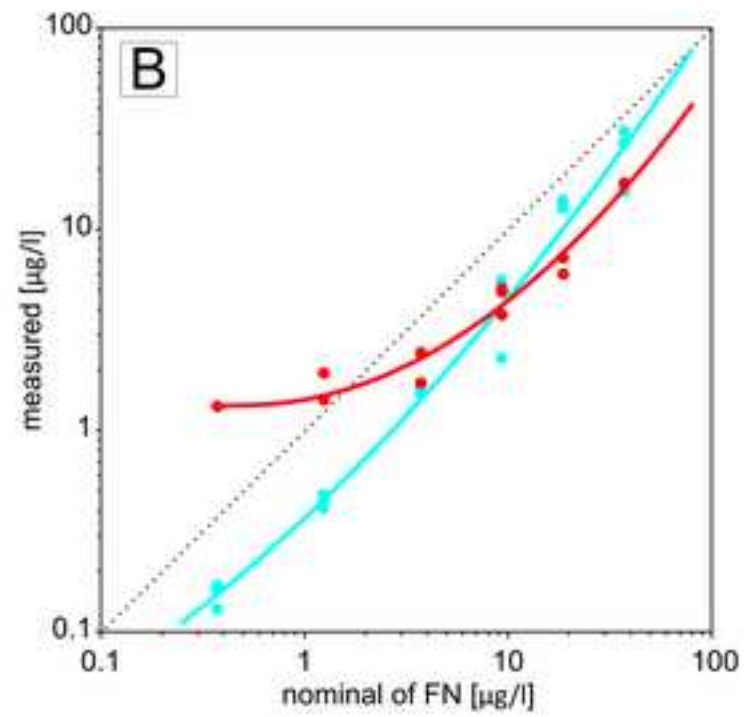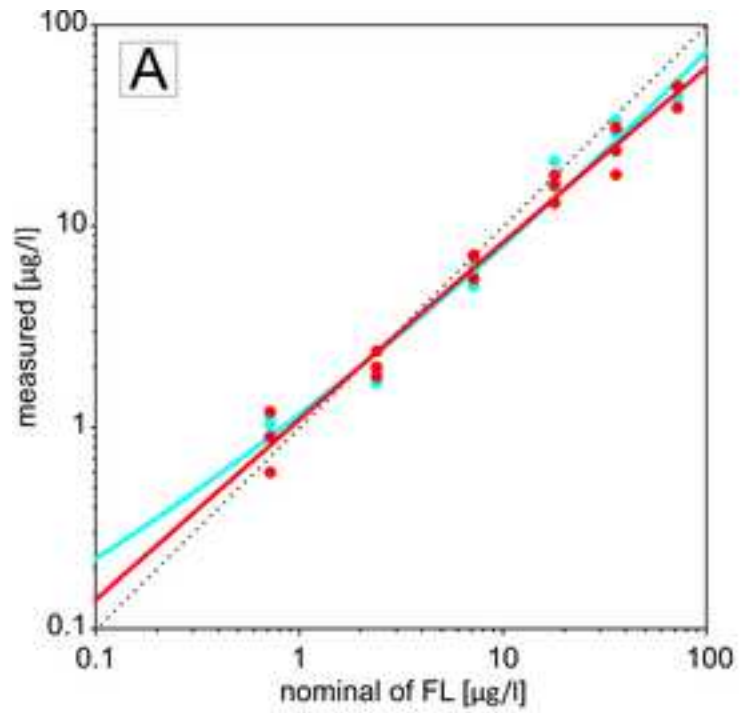
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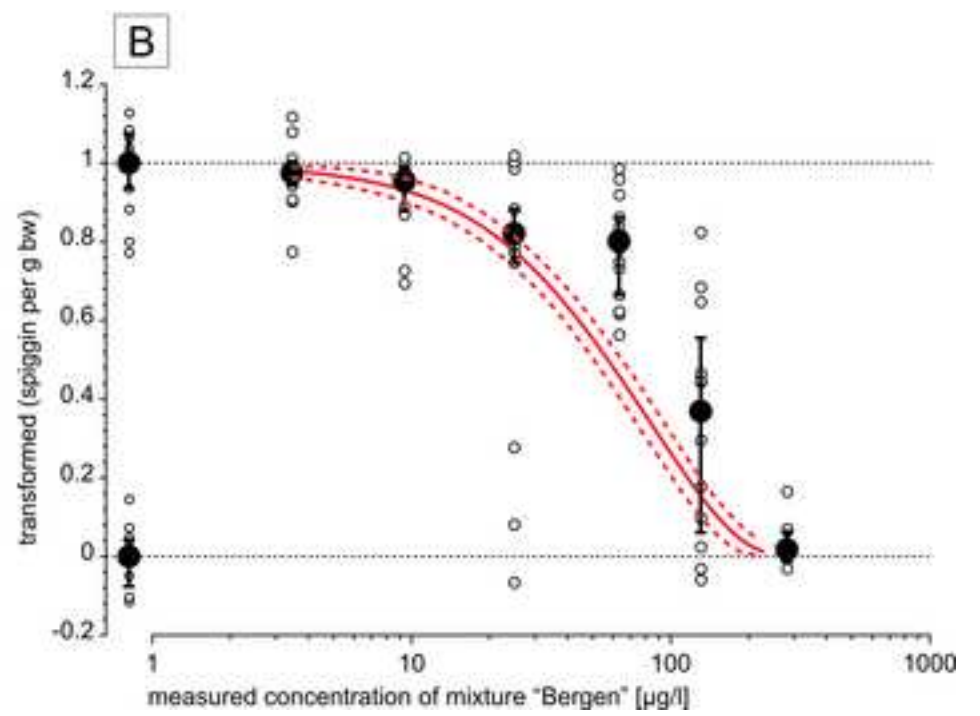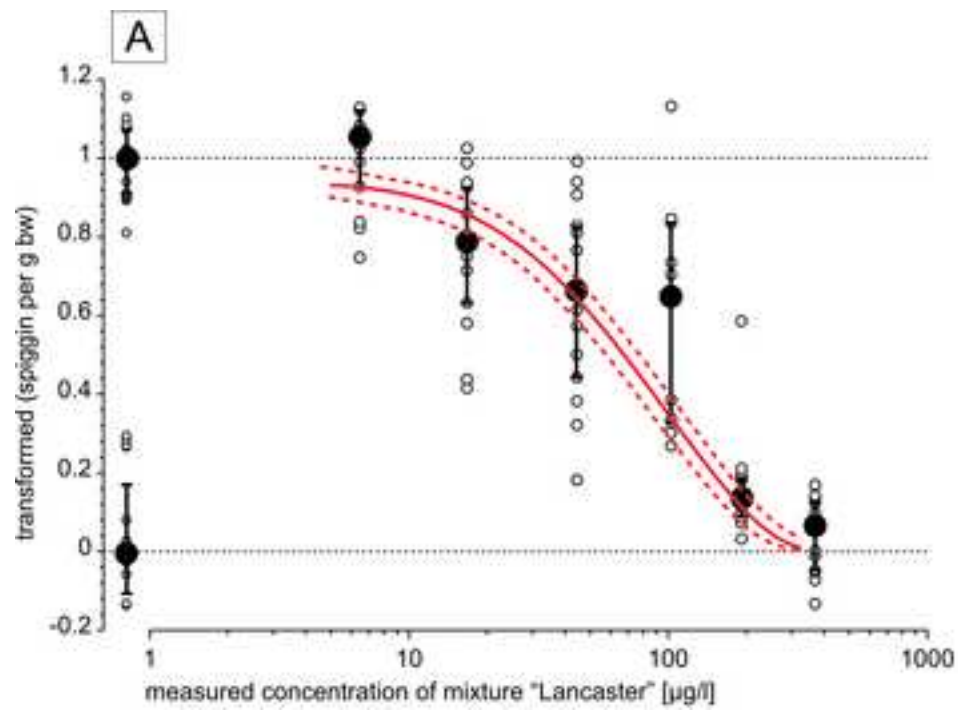
Supplementary Material

Highlights

- Spiggin synthesis was stimulated in female sticklebacks by exposure to androgen

- The inhibition of spiggin production by four anti-androgens (AAs) was assessed

- An equipotent mixture of the AAs was formulated using the single agent data

- Concentration addition was used to predict the response of fish to the mixture

- Good agreement between the actual and the predicted outcomes was obtained

Supplementary material

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Table S1. Study design of the four component mixture study. FL - flutamide; FN – fenitrothion; LN –

linuron; VZ – vinclozolin; DHT - dihydrotestosterone

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			Nominal concentrations (µg/L)					
	Treatment	Tank	FL	FN	LN	VZ	Mixture	DHT
	Water	A	-	-	-	-	-	-
Controls	Methanol	B	-	-	-	-	-	-
	DHT	C	-	-	-	-	-	5
	FL	D	72.06	-	-	-	-	5
Single chemical	FN	E	-	37.20	-		-	5
(concentration = IC50)	LN	F	-	-	421.24	-		5
	VZ	G	-	-	-	40.75	-	5
	FL	H	7.20	-	-	-	-	5
Single chemical	FN	I	-	3.70	-	-	-	5
(concentration = IC50/10)	LN	J	-	-	42.12	-	-	5
	VZ	K				4.08	-	5
	Mix (1.0)	L	72.06	37.16	421.24	40.75	571.21	5
	Mix (0.5)	M	36.03	18.58	210.62	20.38	285.60	5
Mixture	Mix (0.25)	N	18.01	9.29	105.31	10.19	142.80	5
(dilutions of stock containing all chemicals at respective IC50)	Mix (0.1)	O	7.21	3.72	42.12	4.08	57.12	5
	Mix (0.33)	P	2.40	1.24	14.04	1.36	19.04	5
	Mix (0.01)	Q	0.72	0.37	4.21	0.41	5.71	5

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9   **Table S2.** Mathematical relationship between measured and nominal concentration of

10   components within test mixtures

Components	Laboratory	Concentration response function[1]		
		$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_3$
vinclozolin	Lancaster	-0.233*	0.657*	0.153*
	Bergen	-0.395*	0.468*	0.318*
fenitrothion	Lancaster	0.146*	0.185*	0.308*
	Bergen	-0.436*	0.948*	0.145
flutamide	Lancaster	0.038	0.888*	-0.006
	Bergen	0.065	0.778*	0.062
linuron	Lancaster	-0.008	0.957*	-0.006
	Bergen	-0.165?	0.744*	0.072

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12   [1] second-order regression, i.e. $\log_{10}(c_{measured}) = \theta_1 + \theta_2 * \log_{10}(c_{nominal}) + \theta_3 * \log_{10}(c_{nominal})^2$, given

13   for concentrations expressed in µg/l.

14     $\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_3$ estimated model parameters (rounded values)

15   * indicates statistical significance

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