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RESEARCH ARTICLE

Reproductive and developmental toxicity of the herbicide Betanal® Expert and corresponding active ingredients to *Daphnia* spp.

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Abstract The commercial herbicide formulation Betanal® Expert and its active ingredients (a.i.s) ethofumesate, phenmedipham and desmedipham were focused in this study. Following questions yielding from a previous study, an in-depth analysis of the reproductive toxicity of the pesticide was made. Long-term exposures of *Daphnia magna* and *Daphnia longispina* to Betanal® Expert, to each a.i. and to a customised mixture matching the a.i.s ratio within the commercial formulation were carried out, and deleterious effects in the offspring were recorded. This intended to clarify whether (1) the tested compounds induce reproductive injury; (2) there is interspecific variation in daphnids tolerance to the compounds; (3) there is an interaction between chemicals in combined treatments; and (4) the so-called inert ingredients added to the commercial formulation contribute to the toxicity of the herbicide. Generally, developmental impair was observed in both species (egg abortion and release of undeveloped embryos or dead offspring) at concentrations of any of the a.i.s below 1 mg L^{-1}. Ethofumesate was invariably the least toxic pesticide, and *D. magna* tended to be of slightly higher sensitivity to the exposures compared to *D. longispina*. Joint exposures indicated that the a.i.s can interact, inducing more than and less than additive effects for Betanal® Expert and the customised a.i. mixture, respectively. This indicates that inert ingredients co-formulating the commercial pesticide (which are absent from the customised a.i. mixture) actually contribute to its overall toxicity. This study constitutes an add-on to the discussion on the ecotoxicological framework required for authorisation of pesticide trade and usage. The results support the need to consider test species, long-term hazardous potential and toxicity of commercial formulations rather than solely that of active ingredients, as relevant variables in pesticide regulation.

Keywords Phenmedipham · Desmedipham · Ethofumesate · Developmental effects · Pesticide formulations

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Introduction

Pesticide application is a common practice worldwide to face increasing demands of agricultural productivity since these chemicals often allow the effective control of pests, pathogens and weeds. Pesticide application is generally regulated by legislation concerning authorisation for its placement on the market (e.g. European Union regulation No. 1107/2009 and former directive 91/414/EC), where ecotoxicological evaluation is generally recommended as an appropriate tool to assess the hazardous potential of pesticides to several environmental matrices, including surface water bodies. Indeed, the accidental contamination of surface water bodies adjacent to agricultural fields by pesticide residues can easily occur during application (e.g. aerial spray drift and spilling) and through soil-driven transport processes such as runoff and leaching (Carlsen et al. 2006; Cerejeira et al. 2003; Tariq et al. 2007; Wilson and Foos 2006).

Three major shortcomings can be identified in the recommended ecotoxicological workflow, particularly in early stages

of risk assessment protocols or effects assessment routines envisaging the establishment of protective benchmarks. First, the active ingredient(s) (a.i.) of pesticides are the assessment targets focused by environmental protection recommendations, with such assessments largely ignoring the potential of surfactants and other so-called inert ingredients within commercial formulations to promote toxic effects (Cedergreen and Streibig 2005; Cox and Surgan 2006; Krogh et al. 2003; Pereira et al. 2000, 2009). Second, since assessments tend to focus on each pesticide component individually, potential interactions between components within multi-way formulations have been overlooked. Finally, little emphasis is generally given to life-history toxicity assessment for regulatory purposes, although regulation supportive guidelines refer to the relevance of addressing such long-term sub-lethal effects (e.g. EC 2002); for instance, ignoring effects on reproduction may underestimate serious population impacts in the wild.

Betanal® Expert is a three-way herbicide co-formulation using an Advanced Micro Droplet approach where a.i.s and formulants are coated with vegetable oil to enhance pesticide delivery and activity (Bayer 2009; Tominack 2000). Betanal® Expert combines three a.i.s: phenmediphan, desmediphan and ethofumesate. Phenmedipham [IUPAC: 3-methoxycarbonylaminophenyl 3′-methylcarbanilate] and desmedipham (ethyl 3-phenylcarbamoyloxyphenylcarbamate) are both systemic herbicides belonging to the bis-carbamates chemical group (Velkoska-Markovska et al. 2008), thus designed to inhibit photosynthetic electron transport at the photosystem II receptor site (PPDB 2009; Tomlin 2001). These compounds are poorly water soluble (1.8 and 7 mg L^{-1}, respectively), slightly mobile in soil (Koc values of 888 and 10,512, respectively) and have high potential for bioaccumulation (log *P* values of 3.59 and 3.39, respectively). Ethofumesate [IUPAC: (±)-2-ethoxy-2,3-dihydro-3,3-dimethylbenzofuran-5-yl methanesulphonate] is a selective benzofuranyl alkanesulphonate herbicide that acts as a growth inhibitor in target plants by retarding cell division, impairing photosynthesis and respiration, as well as affecting the formation of the waxy cuticle and lipid synthesis through ACCase inhibition (EPA 2005a; Roberts 1998; Velkoska-Markovska et al. 2008). It is poorly soluble in water (50 mg L^{-1} at 20 °C) with moderate mobility in soils (Koc = 147) and moderate potential for bioaccumulation (log $P = 2.7$) (Kegley et al. 2009; PPDB 2009; Tomlin 2001).

Residues of phenmedipham, desmedipham and ethofumesate have been found in surface water bodies (Carabias-Martinez et al. 2003; García de Llasera and Bernal-González 2001; Neumann et al. 2003; Saraji and Esteki 2008); e.g. Neumann et al. (2003) registered 51.1 μg L^{-1} ethofumesate from a sample collected in drainage channels acting as input sources into a stream. Despite the hazardous potential of Betanal® Expert and the confirmed presence of its components in surface water bodies, only a few studies addressed its toxicity to non-target aquatic species. Fritz and Braun (2006) tested phenmedipham and found a 48 h EC_{50} of 5.3 μmol L^{-1} (1.6 mg L^{-1}) for *Daphnia magna*, 72 h EC_{50} values of 35 and 0.34 μmol L^{-1} (10 and 0.1 mg L^{-1}) for green microalgae growth and a 30 min EC_{50} of 0.94 μmol L^{-1} (0.3 mg L^{-1}) for *Vibrio fischeri* luminescence. Vidal et al. (2009) tested the effects of Betanal® Expert to green microalgae growth and *Daphnia* survival and recorded 96 h IC_{50} values ranging within 2.22–266 μg L^{-1} and 48 h EC_{50} values ranging within 2.73–4.45 μg L^{-1}, respectively. More recently, Vidal et al. (2012) reported the toxicity of both Betanal® Expert and each of its three a.i.s towards bacteria luminescence, microalgae and macrophyte growth survival and life-history of daphnids.

Whilst assessing the long-term toxicity of Betanal® Expert and its three a.i.s to *Daphnia* spp. by recording standard endpoints, Vidal et al. (2012) noticed remarkable abnormal egg development in some of the monitored broods, confirming suspects raised by Kegley et al. (2009) on the ability of all the a.i.s to induce developmental and reproductive injury. Here, we followed-up through an in-depth scrutiny of the reproductive effects of Betanal® Expert and its a.i.s in *Daphnia* sp.. Long-term toxicity tests were carried out with detailed observation of each brood in order to register the frequency of viable and unviable progeny, accounting in particular for egg/embryo development. Based on these records, specific aims were (1) to provide evidence on the deleterious effects of the pesticides in cladoceran's fecundity and fertility and (2) to assess interspecific variation as to the extension of pesticide-driven reproductive injury by testing with *Daphnia magna* (standard model in ecotoxicological assessment) and *Daphnia* cf *longispina* (widespread in European freshwaters). By comparing the outcome of exposure to Betanal® Expert and its a.i.s, this study further intended (3) to determine whether the toxicity of the commercial formulation Betanal® Expert was comparable to that of the three a.i., thus identifying potential interactions between the chemicals; and (4) to indirectly assess the role of the so-called inert ingredients in the overall pesticide toxicity, by comparing reproductive effects in *Daphnia* following exposure to Betanal® Expert and to a customised mixture of its three a.i.s.

Material and methods

Test organisms

Monoclonal bulk cultures of *Daphnia* cf *longispina* (hereinafter referred to as *Daphnia longispina*) and *Daphnia magna* had been reared in the laboratory for several generations, in ASTM hard water (ASTM 1980) enriched with vitamins

(Elendt and Bias 1990) and supplemented with *Ascophyllum nodosum* seaweed extract (Baird et al. 1989), at 20 ± 2 °C under a 16 h^L:8 h^D photoperiod. Culture medium was renewed, and the organisms were fed with *Raphidocelis subcapitata* (1.50 and 3.00×10^5 cells mL^{-1} for *D. longispina* and *D. magna*, respectively; Pereira and Gonçalves 2008) three times per week. Following Stein (1973), *P. subcapitata* was cyclically reared in the laboratory in non-axenic batch cultures in Woods Hole MBL medium, at 20 ± 2 °C and under permanent illumination.

Chemicals and preparation of test solutions

The commercially available herbicide Betanal® Expert (oil-EC; Bayer CropScience, Portugal) combines 10.28 % ethofumesate (112 g L^{-1}), 8.35 % phenmedipham (91 g L^{-1}) and 6.51 % desmedipham (71 g L^{-1}) as a.i.s. All a.i.s were purchased from Sigma-Aldrich as Pestanal® (Fluka) analytical standards. Stock solutions were prepared by directly dissolving (a.i.s) or diluting (commercial formulation) the appropriate amounts of each compound in *Daphnia* culture medium. Concentration ranges used in all toxicity tests are depicted in Figs. 1 and 2. In order to facilitate comparison between different tests (e.g. commercial formulation vs corresponding customised mixture of a.i.s), concentration ranges used were additionally transformed into dimensionless Toxic Units (TU) ranges. The sum of the quotients $Ci/EC_{50}i$ was applied for the purpose, where *i* refers to each a.i. involved in the mixture, C to the concentration of *i* within the mixture and EC_{50} to the median effect concentration found for reproduction (live offspring) in single-chemical exposures to *i* (see e.g. Jonker et al. 2005 for details on the TU approach).

Exposure and assessment endpoints

D. longispina and *D. magna* were exposed for 21 days to geometric concentration ranges of the commercial formulation Betanal® Expert, each a.i. and a mixture of the three a.i. in the same proportions as those present in Betanal; a blank ASTM hard water treatment was used as the control in all tests. OECD (2008) was followed to guide the assays. In brief, a semi-static design was applied, where the test organisms were transferred to new test solutions every other day. Ten individual replicates, each with one newborn daphnid (<24 h; from the 3rd–5th brood of bulk cultures), were assigned to each treatment; each replicate was carried out in a glass vessel filled with 50 mL test solution. Daphnids were fed daily with the corresponding algal ration (see above), and incubation conditions were kept as described for cultures. The organisms were checked daily for mortality and reproductive output. Each brood was observed and monitored on the number of (1) living neonates, (2) dead neonates, (3) undeveloped embryos and (4) undeveloped eggs. Within this study, fecundity was defined as the number of eggs extruded from the ovaries in the brood chamber, whilst fertility refers to the successful yield of living neonates. The body size of the females was estimated by extrapolation from the moult exopodite length (Pereira et al. 2004), immediately after the release of the first brood and at the beginning and end of the test, allowing the calculation of the somatic growth rate (SGR) through the following equation:

$$SGR = [\ln(l_f) - \ln(l_0)]/\Delta t\left(day^{-1}\right)$$

where l_f is the final body length (mm), l_0 is the initial body length (mm) and Δt is the time range (days). These measurements, as well as classification within the progeny, were carried out under a stereoscope (Olympus SZX9).

Data analysis

One-way ANOVA followed by Dunnett's post hoc test was applied to the growth endpoints (size of primipara and SGR) and each reproductive endpoint (viable and unviable progeny), in order to assess differences between control and pesticide treatments within each *Daphnia* species. The dataset was graphically inspected for normality and homocedasticity prior to running ANOVA, and data transformation was applied when necessary to better meet the assumptions. A multiple regression approach was employed to test the fit of the predictor variables (living neonates, dead neonates, undeveloped embryos and undeveloped eggs) on fecundity, allowing the extraction of the relative individual contribution of each predictor to the overall fit through the relative increase in the adjusted coefficient of determination (Quinn and Keough 2002). A fixed significance level (α) of 0.05 was used in all analyses.

As an indirect measure of the role of inert ingredients in the toxicity of Betanal® Expert, we compared the toxicity of the mixture of the three a.i. with that of Betanal® Expert using the Toxic Unit (TU) approach based on the sum of the ratios between EC_{50} values obtained following single and joint exposures (e.g. Woods et al. 2002):

$$TUmix = [EC_{50}P(mix)/EC_{50}P(single)] + [EC_{50}D(mix)/EC_{50}D(single)] + [EC_{50}E(mix)/EC_{50}E(single)]$$

where P, D and E stand for phenmedipham, desmedipham and ethofumesate, and EC_{50}(mix) and EC_{50}(single) represent the toxicity of each chemical within the mixture or single exposure, respectively. If the model outcome equals 1, the toxicity of the mixture would be additive, whilst values below or above 1 denote interaction between chemicals within the mixture i.e. a more than or less than additive behaviour of the mixture, respectively. TUmix was calculated for fertility using the corresponding EC_{50} values, which were estimated

following the fitting of a non-linear regression model (general logistic equation) to the data through the least squares statistical method.

Results

Whilst no clear pattern could be found for fecundity following exposure to either Betanal® Expert or the corresponding ternary mixture of the a.i.s (Fig. 1), the endpoint was significantly impaired at the highest concentrations of desmedipham and ethofumesate (Fig. 2). As to this endpoint, desmedipham was clearly more toxic than ethofumesate since it induced significant decrease of total fecundity at 0.300 mg L^{-1} compared to similar effects of the latter only at 11.11 mg L^{-1} (Fig. 2; Table 1). In both daphnid species, a great release of unviable progeny was observed following exposure to both the three-way formulation Betanal® Expert and its a.i.s in single and combined exposures (Figs. 1 and 2). Whenever unviable progeny was released, undeveloped eggs were generally released, as well as undeveloped embryos, but dead neonates were found only occasionally. As to the release of unviable progeny and comparing solely amongst single exposures to a.i.s, there was a marked difference in toxicity expressed by changes in LOEC values of about one order of magnitude (Fig. 1; Table 1). Ethofumesate was the least toxic pesticide, showing LOEC values of 1.905 and 4.630 mg L^{-1} for egg release in *D. magna* and *D. longispina*, respectively, followed by phenmedipham with LOEC values one order of magnitude lower; desmedipham was the pesticide showing higher toxicity by significantly inducing release of

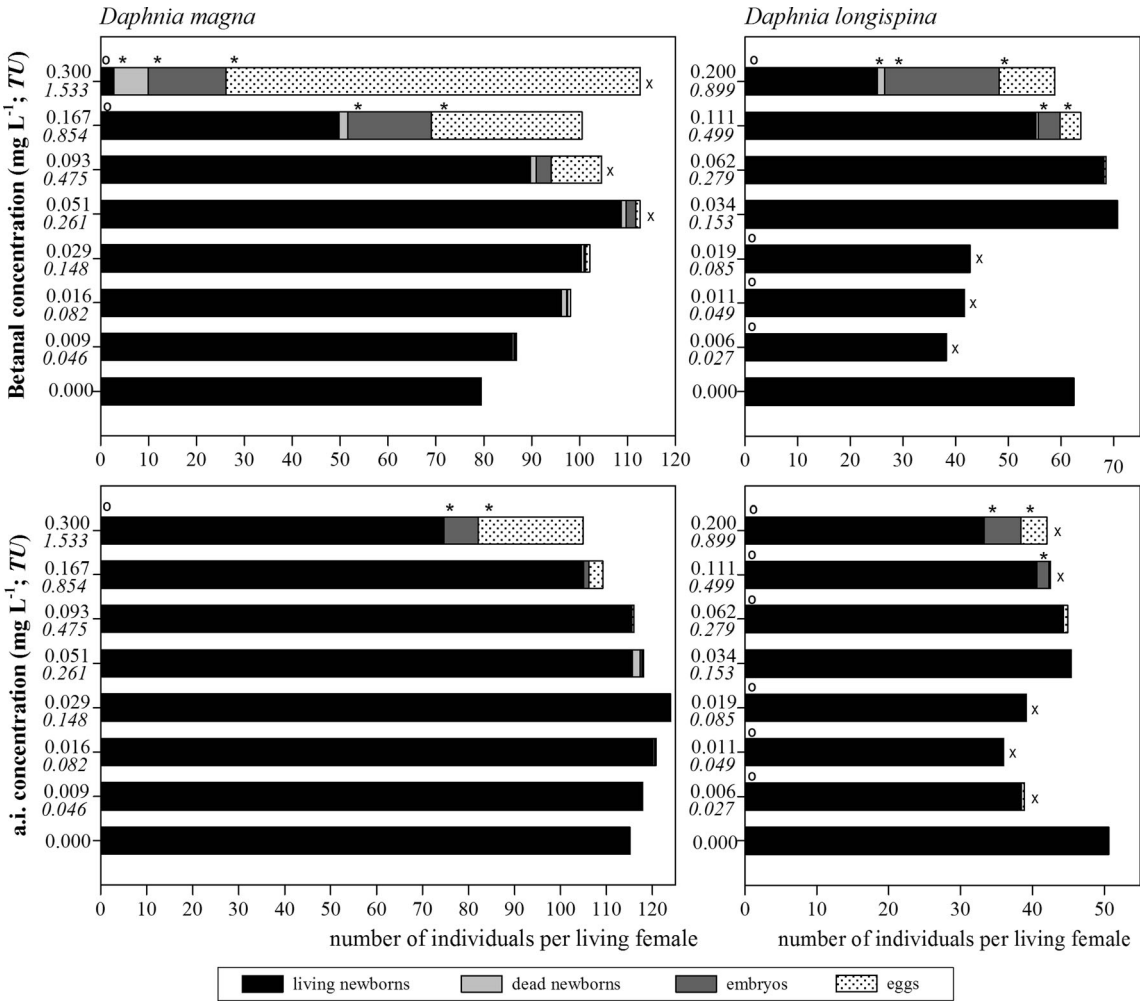

Fig. 1 Fecundity output of *Daphnia magna* (*left-hand panel*) and *Daphnia longispina* (*right-hand panel*) following a 21-day exposure to Betanal® Expert and to a corresponding mixture of its active ingredients (a.i.s) phenmedipham, desmedipham and ethofumesate. Concentration in each treatment is expressed both as milligrams per Litre a.i. and toxic units (TU). *Stacked bars* contain information on viable and unviable progeny as dead newborns, undeveloped embryos and undeveloped eggs, per test treatment; as a whole, *bars* show total fecundity i.e. viable plus unviable progeny. '*x*', '*o*' and '*' indicate significant differences between pesticide treatments and control as to total, viable and unviable fecundity, respectively (one-way ANOVA followed by Dunnett test, $\alpha = 0.05$)

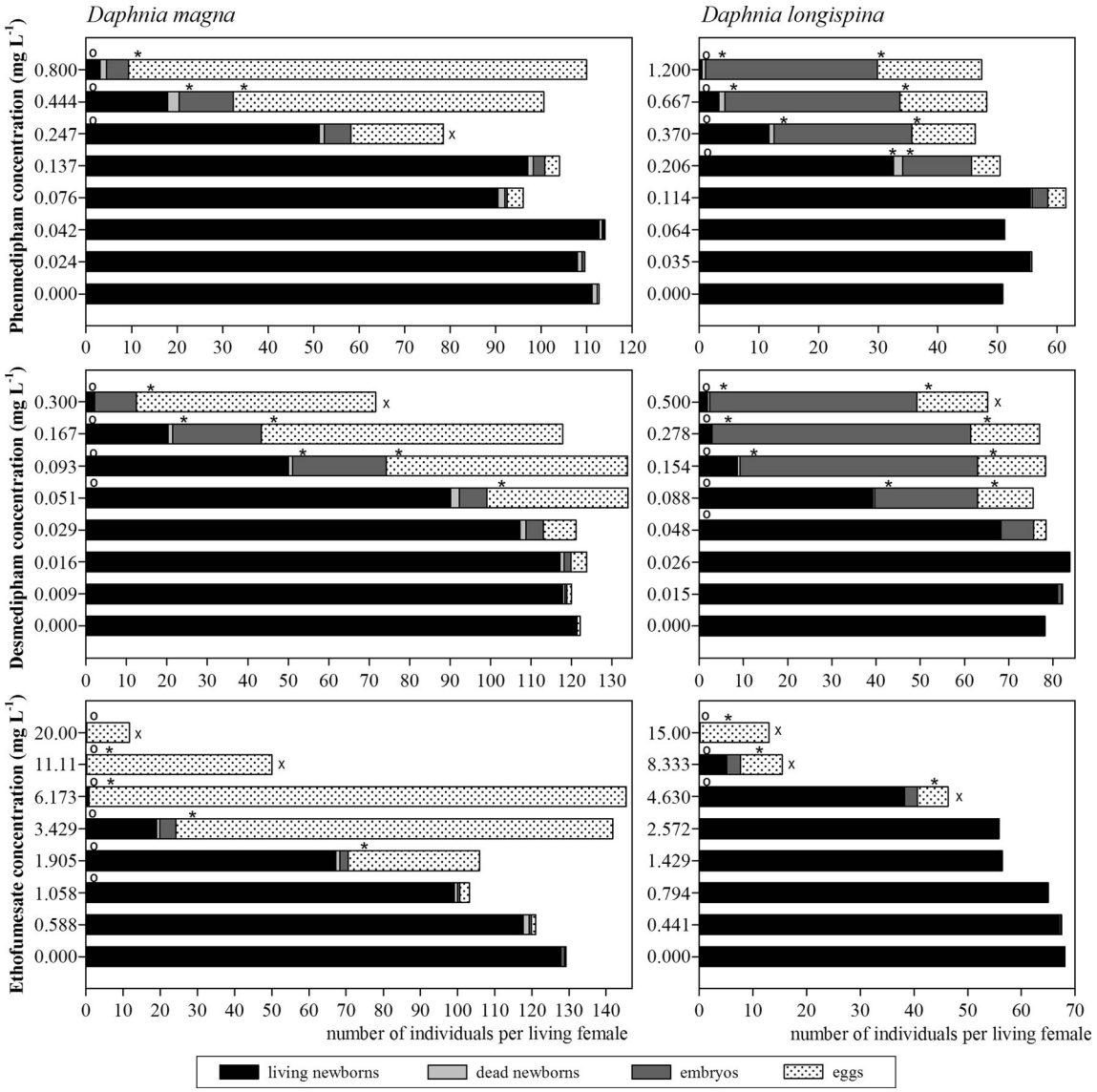

Fig. 2 Fecundity output of *Daphnia magna* (*left-hand panel*) and *Daphnia longispina* (*right-hand panel*) following a 21-day exposure to phenmedipham, desmedipham and ethofumesate. *Stacked bars* contain information on viable and unviable progeny as dead newborns, undeveloped embryos and undeveloped eggs, per test treatment; as a whole, *bars* show total fecundity i.e. viable plus unviable progeny. '*x*', '*o*' and '*' indicate significant differences between pesticide treatments and control as to total, viable and unviable fecundity, respectively (one-way ANOVA followed by Dunnett test, $\alpha = 0.05$)

undeveloped eggs from 0.051 mg L^{-1} onwards in *D. magna* and undeveloped embryos and eggs from 0.088 mg L^{-1} onwards in *D. longispina*.

Similarly to fecundity, the growth endpoints showed little responsiveness to the tested concentrations (Fig. 3). A slight stimulatory effect can be noticed in the size of primipara and somatic growth rate, but such effect was rarely significant (Fig. 3; Table 1). Ethofumesate was the only toxicant consistently inducing negative effects in these endpoints. In fact, significantly smaller *D. magna* primipara were recorded, and somatic growth rates of both cladocerans decelerated significantly following exposure to the highest ethofumesate concentrations (LOEC values of 11.11 and 8.33 mg L^{-1} for *D. magna* and *D. longispina*, respectively; Table 1).

The general patterns referred above were common to both daphnid species. However, assuming that more extensive damage corresponds to earlier reproductive impacts to offspring development, the progeny of *D. magna* was more sensitive than that of *D. longispina*. At similar toxicant concentrations, the progeny of *D. longispina* included fewer undeveloped eggs than undeveloped embryos, whilst the progeny of *D. magna* included more undeveloped eggs than embryos; this clearly indicates that injury in *D. magna* progeny occurs at an earlier developmental stage than that in *D. longispina*. Such a trend is particularly shown in the fecundity profiles retrieved

Table 1 One-way ANOVA summary (df, degrees of freedom) regarding the fecundity of *Daphnia magna* and *Daphnia longispina* exposed to the herbicide Betanal® Expert and its active ingredients (P+D+E stands for the mixture of active ingredients respecting their ratios in the commercial formulation Betanal® Expert). LOEC values are presented for each endpoint in milligrams per Litre, which can be found converted into Toxic Units for Betanal® Expert and the corresponding mixture of a.i. in Fig. 1. Fecundity endpoints tested included total fecundity (viable plus unviable progeny), viable fecundity and unviable fecundity (dead newborns, undeveloped embryos and eggs yield by the females)

Factor	Endpoint	*Daphnia magna*					*Daphnia longispina*				
		df	$MS_{residual}$	*F*	*P*	LOEC	df	$MS_{residual}$	*F*	*P*	LOEC
Betanal® Expert	Total	7, 65	343.6	3.54	0.003	n.d.	7, 70	79.9	20.12	<0.001	n.d.
	Viable		250.0	42.70	<0.001	0.167		71.0	36.08	<0.001	n.d.
	Dead		7.073	6.560	<0.001	0.300		0.3843	6.500	<0.001	0.200
	Embryos		23.44	20.22	<0.001	0.167		8.64	66.51	<0.001	0.111
	Eggs		103.8	79.84	<0.001	0.167		5.97	23.77	<0.001	0.111
	Prim. size		0.0215	0.58	0.774	>0.300		0.0134	1.450	0.198	>0.200
	SGR		0.000012	0.42	0.886	>0.300		0.0000142	0.53	0.811	>0.200
P+D+E	Total	7, 66	155.0	2.260	0.040	n.d.	7, 69	27.00	7.580	<0.001	n.d.
	Viable		170.0	14.04	<0.001	0.300		24.00	12.96	<0.001	0.006
	Dead		2.370	1.230	0.300	n.d.		0.0493	1.140	0.351	n.d.
	Embryos		3.010	21.46	<0.001	0.300		0.681	48.02	<0.001	0.111
	Eggs		8.450	73.20	<0.001	0.300		0.879	17.15	<0.001	0.200
	Prim. size		0.0341	3.22	0.005	0.300		0.00731	3.60	0.002	0.200
	SGR		0.0000118	221.9	<0.001	n.d.		0.0000089	2.09	0.057	>0.200
Phenmedipham	Total	7, 63	488.7	2.680	0.017	n.d.	7, 64	73.33	2.57	0.021	n.d.
	Viable		330	53.31	<0.001	0.247		38.8	137.2	<0.001	0.206
	Dead		2.150	1.400	0.223	n.d.		0.6293	3.77	0.002	n.d.
	Embryos		25.23	6.370	<0.001	0.444		34.7	48.78	<0.001	0.206
	Eggs		335	40.02	<0.001	0.444		40.10	11.49	<0.001	0.370
	Prim. size		0.0411	0.65	0.712	>0.800		0.00944	0.92	0.496	>1.200
	SGR		0.0000076	1.12	0.359	>0.800		0.0000103	0.99	0.445	>1.200
Desmedipham	Total	7, 67	504.4	5.870	<0.001	0.300	7, 69	0.002223	4.79	<0.001	0.500
	Viable		345	58.70	<0.001	0.051		37	338.2	<0.001	0.048
	Dead		2.263	1.540	0.170	n.d.		0.6221	1.00	0.440	n.d.
	Embryos		92.16	8.950	<0.001	0.093		48.5	126.1	<0.001	0.088
	Eggs		155.9	57.38	<0.001	0.051		42.62	13.21	<0.001	0.088
	Prim. size		0.0324	1.86	0.091	>0.300		0.0166	0.79	0.595	>0.500
	SGR		0.0000157	2.66	0.017	0.300		0.0000286	1.81	0.100	>0.500
Ethofumesate	Total	7, 65	739	27.16	<0.001	11.11	7, 52	102.2	28.15	<0.001	4.630
	Viable		269	105.4	<0.001	1.058		60.3	71.42	<0.001	4.630
	Dead		1.055	2.850	0.012	n.d.		0.2541	0.55	0.795	n.d.
	Embryos		6.528	3.270	0.005	n.d		8.203	1.31	0.266	n.d.
	Eggs		724	42.27	<0.001	1.905		6.973	14.05	<0.001	4.630
	Prim. size		0.0702	3.63	0.002	20.00		0.00966	1.79	0.108	>15.00
	SGR		0.0000159	46.06	<0.001	11.11		0.0000213	4.09	<0.001	8.333

n.d. not determined

following exposure to phenmedipham and desmedipham (Fig. 1). The multiple regression approach using viable and unviable fecundity over total fecundity clearly supports this pattern (Table 2): egg yield was consistently first fitted in the model followed by embryo yield in *D. magna,* whilst the opposite occurred for the *D. longispina* dataset; there was a single exception for *D. longispina* exposed to ethofumesate, where eggs were first fitted to the model followed by embryos. In both species, ethofumesate was the a.i. inducing earlier reproductive injury (littler release of undeveloped embryos or dead neonates) and egg deposition in the brood chamber (fecundity significantly affected) (Fig. 1).

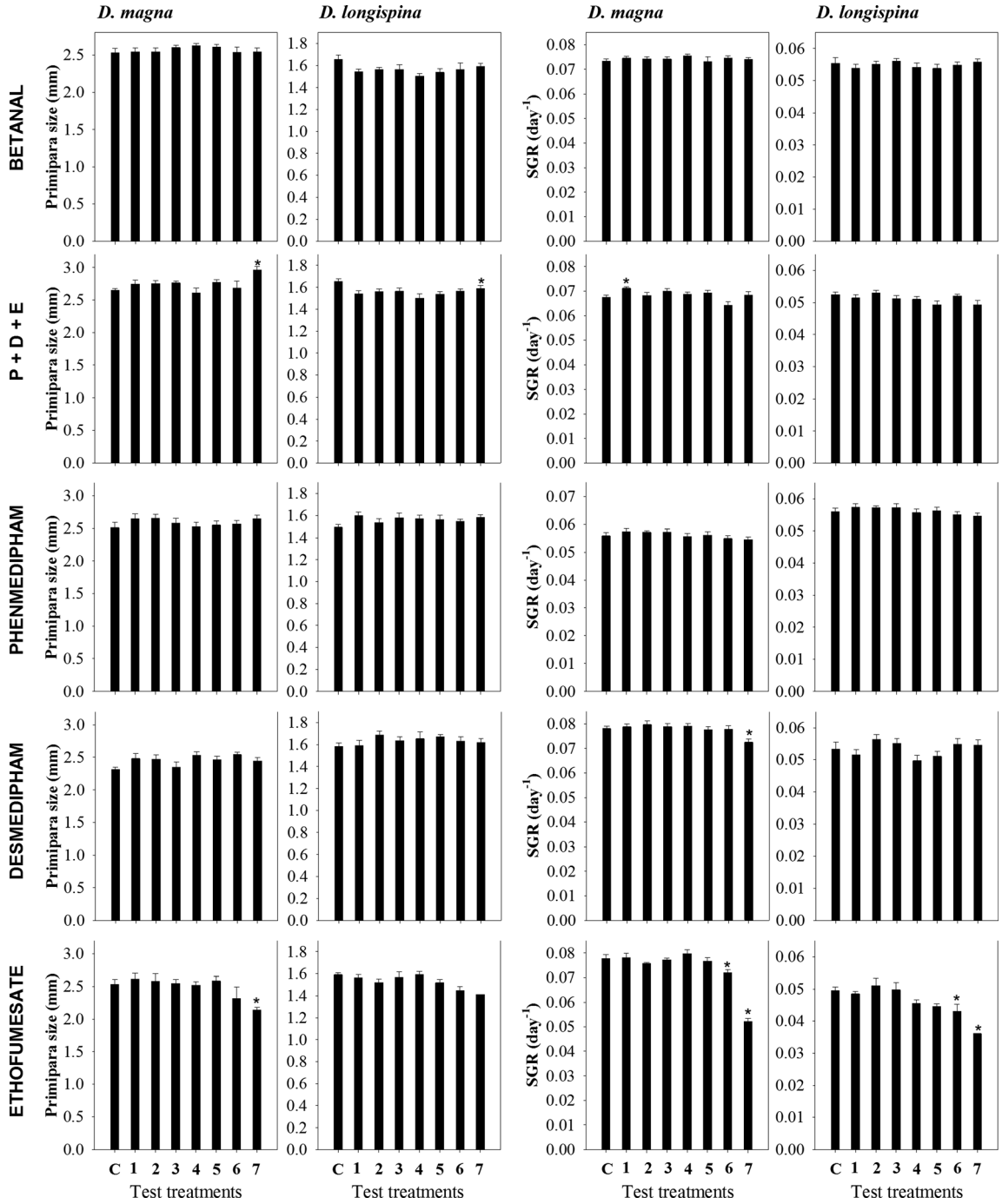

Fig. 3 Primipara size and somatic growth rate (SGR) of *Daphnia magna* and *Daphnia longispina* following a 21-day exposure to Betanal® Expert and to a corresponding mixture of its active ingredients (a.i.s) phenmedipham, desmedipham and ethofumesate, as well as following single exposure to these a.i.s. For graphic convenience, test treatments are presented as C (control) and 1–7, corresponding to increasing concentrations of the toxicants as presented in former figures. '*' indicates significant differences between pesticide treatments and control (one-way ANOVA followed by Dunnett test, $\alpha = 0.05$)

Betanal® Expert may be assumed as a fixed-ratio mixture of the a.i.s phenmedipham, desmedipham and ethofumesate, to which adjuvant ingredients are added. The response patterns found for Betanal® Expert were similar to those above for the a.i.s in single exposures. Along with a significant decrease in fertility, severe lethal effects on the progeny were noticed at the highest concentrations, with significant release of undeveloped eggs and embryos. At comparable toxic strengths, Betanal® Expert seems able to act earlier in *D. magna* broods by eliciting the release of more undeveloped eggs compared to *D. longispina* (Fig. 1; e.g. 0.161 and 0.475 TU in *D. magna* compared to 0.276 and 0.499 TU in

Table 2 Summary of a stepwise multiple regression of the viable and unviable (undeveloped eggs, undeveloped embryos and dead newborns) fecundity on the total fecundity of *Daphnia magna* and *Daphnia longispina* following exposure to the three-way formulation Betanal® Expert and its active ingredients phenmedipham, desmedipham and ethofumesate (P+D+E stands for the mixture of active ingredients respecting their ratios in the commercial formulation Betanal® Expert). The entering order in each regression is indicated by the numbers given to each predictor. Adj r^2 represents the adjusted coefficient of determination for each significant regression and shows the proportion of variation explained by the model when added a significant predictor

	Daphnia magna		*Daphnia longispina*	
	Predictors	Adj r^2	Predictors	Adj r^2
Betanal® Expert	1 eggs	11.30	1 viable	53.72
	2 viable	87.05	2 embryos	95.55
	3 embryos	98.32	3 eggs	99.87
P+D+E	1 viable	73.47	1 viable	80.52
	2 eggs	96.19	2 embryos	96.86
	3 embryos	98.76	3 eggs	99.89
	4 dead	99.99		
Phenmedipham	1 viable	11.79	1 viable	24.84
	2 eggs	93.27	2 embryos	52.63
	3 embryos	99.68	3 eggs	99.12
Desmedipham	1 viable	28.81	1 viable	22.59
	2 eggs	83.89	2 embryos	56.52
	3 embryos	99.68	3 eggs	99.21
Ethofumesate	1 eggs	25.50	1 viable	92.64
	2 viable	99.57	2 eggs	98.88
	3 embryos	99.96	3 embryos	99.96

D. longispina). This was not statistically confirmed since slightly higher LOEC values were depicted for the responses of *D. magna*, including when testing undeveloped egg production (Table 1), but the multiple regression approach confirmed that Betanal® Expert acts earlier in *D. magna* brood development (Table 2). The mixture seems to be more toxic than expected through CA as to fertility of both *D. magna* and *D. longispina*, with calculated TUmix values of 0.859 and 0.809, respectively. Whilst the mixture of the active ingredients (P+D+E) resulted in similar response patterns, a different outcome was noticed when addressing the potential interactions between chemicals within the mixture of a.i.s. In fact, TUmix values higher than 1 were found for the combined effect of this mixture in the fertility of both species (1.885 for *D. magna* and 1.772 for *D. longispina*), suggesting a less than additive behaviour of the mixture.

Discussion

This study was devoted to the reproductive toxicity of the herbicide Betanal® Expert and its a.i.s phenmedipham, desmedipham and ethofumesate to *Daphnia* spp.. The experiments were designed so that an indirect measure of the hazardous potential of adjuvant ingredients (i.e. other than a.i.s) could be provided, considering that their exact concentrations are not disclosed in the product safety datasheet (Bayer 2009). Betanal® Expert, its a.i.s and their customised mixture severely impaired *Daphnia* spp. reproduction differentially, and the reported findings constitute meaningful evidences adding to the environmental risk assessment of the pesticides.

Fertility was found severely impaired following all exposures. Decreased fertility was already reported as a reproductive effect elicited by different xenobiotics, including pesticides, e.g. 3,4-dichloroaniline, propanil, propiconazole, endosulfan and chlorpyrifos (Baird et al. 1991; Barata and Baird 2000; Beyerle-Pfnür et al. 1991; Guilhermino et al. 1999; Kast-Hutcheson et al. 2001; Lampert 2006; Palma et al. 2009a, 2009b; Pereira et al. 2007; Trubetskova and Lampert 2002). Following the evidences provided by Baird et al. (1991), and because fecundity was in fact mostly unaffected, it is reasonable to hypothesise that egg production in the ovaries was not severely affected by the pesticides in the present study. This links the observed reproductive impairment to the ability of the chemicals to directly affect eggs after extrusion into the brood pouch, which directly communicates with the external medium for facilitated gas exchanges.

Body size and somatic growth records, along with unaffected fecundity, apparently support the view above on a major toxicity route via direct contact in developing eggs rather than through impairment of its production. The Dynamic Energy Budget theory, as well as its downstream models interpreting toxic effects (DEBtox; e.g. Jager and Zimmer 2012), has been defining resource allocation in animal models such as *Daphnia* challenged by different stressors. In general, assimilated reserves should primarily support structure (somatic maintenance and then growth), and the remainder is invested in reproduction, i.e. maturation in juveniles and egg production in adults (Kooijman and Bedaux 1996; Jager and Zimmer 2012). Our results generally showed that primipara body size does not differ between pesticide and control treatments, as well as that somatic growth rates were not significantly impaired by the pesticides. Taking into account that under unlimited resources (such as in our study), daphnids preferably invest in reproduction often at the expense of somatic growth (Pereira and Gonçalves 2007; Pereira et al. 2007; Polishchuk and Vijverberg 2005; Smolders et al. 2005), a reduced reproductive output would require an upstream scenario of reduced growth as a compensatory change to keep the daphnids' primary ecological strategy. Based on this rationale, the physiological mode of action, as defined by Kooijman and Bedaux (1996), of desmedipham and phenmediphan should relate mostly to direct hazard posed to the embryo.

However, the amount of reserves available to allocate per egg is a key determinant of the success of the first egg divisions (Kooijman 2009), which in turn constrains the success of embryogenesis and the fitness of the released progeny. On this basis, our results can also be suggesting that there was assimilation impairment, achieved via decreased feeding rate or decreased efficiency in food assimilation into reserves, with increasing chemical concentrations. The results obtained following exposure to high concentrations of ethofumesate constitute an important support for this alternative interpretation since significant fertility reduction occurred along with decreased primipara size and somatic growth rate. Ethofumesate acts in target plant pests as an inhibitor of lipid synthesis and constraining fatty acid elongation (EPA 2005a; Roberts 1998). This elongation process also occurs in mammals, and long-chain fatty acids are key constituents of membrane phospholipids and have an important role in neural growth and myelinisation (Shaner 2004). Thus, assuming that there is some parallel between mammals and crustaceans as to this metabolic pathway, the impairment of reserve allocation to egg production and development by ethofumesate in addition to direct poisoning of eggs in the brood pouch is likely to occur. The study by Alda Álvarez et al. (2006) on the toxicity of carbendazim to the parthenogenetic nematode *Acrobeloides nanus* supports our interpretation above. Their data yielded patterns similar to those obtained here and fitted better to the DEBtox model assuming decrease in assimilation as the physiological mode of action.

In order to clarify whether the main toxicity target is the egg or the mother, direct toxicity testing over eggs could be a suitable follow-up to the present study. Baird et al. (1991) designed an experiment accounting to the three main stages of embryonic development in *Daphnia* (oocyte production in the ovary, oocyte vitellogenesis in the ovary and egg development in the brood pouch; Zaffagnini 1987), allowing to assess at which stage 3,4-dichloroaniline would preferably enact developmental abnormalities in the progeny, and Sobral et al. (2001) established an in vitro toxicity test with *D. magna* parthenogenetic eggs, which has been successfully applied by others (e.g. Palma et al. 2009a, 2009b). Regardless the assessment platform (e.g. sensitive and cost-effective in vitro egg testing versus demanding reproduction testing), including developmental effects, and more importantly considering process-based approaches that consistently address the physiological mode of action of toxicants (Jager et al. 2006) in environmental risk assessment, is of critical importance because of their high impact as a constraint of the population fitness in the wild.

The *Daphnia* species used is another constraint that may interfere with the ecotoxicological evaluation of xenobiotics. Indeed, the present study shows that *D. longispina*, which is widespread in European freshwaters, is more tolerant than the standard *D. magna*, supporting the use of the latter as a more conservative indicator of the environmental toxicity of these particular pesticides. Previous studies comparing these two species generally showed the opposite trend (e.g. Abrantes et al. 2009; Antunes et al. 2004; Marques et al. 2004a, 2004b; Pereira et al. 2007, 2009; Pereira and Gonçalves 2007), although the higher sensitivity of *D. magna* was also reported, e.g. for the acute toxicity of the herbicide propanil (Pereira et al. 2007) and for the acute toxicity of phenmedipham and desmedipham (Vidal et al. 2012). Body size is a critical constraint of toxicokinetics (Kooijman and Bedaux 1996) and the smaller size of *D. longispina* compared to *D. magna,* with its consequently greater surface-to-volume ratio, would theoretically increase its exposure to waterborne chemicals (Lilius et al. 1995). The same principle should apply to the toxicity of xenobiotics towards developing eggs, i.e. the larger *D. magna* eggs (0.3–0.4-mm diameter; Trubetskova and Lampert 2002) would be less affected than the smaller eggs of *D. longispina* (0.2–0.3-mm diameter; Guisande and Gliwicz 1992).

There are some points deserving further discussion as eventual sources of inconsistency in differential species sensitivity. First, the effect of organic load in long-term testing media cannot be disregarded. It is likely that the microalgae and even the organic matter particles (organic seaweed extract) load of test medium had an important role featuring species sensitivity in long-term bioassays, which often depended on the endpoint. All a.i.s tested here showed a moderate potential to adsorb onto organic matter (see Koc values in the Introduction section). Adsorption of the contaminants to both microalgae and extract particles would make them less available inside the brood pouch for both species and even less available to *D. magna* since this species was fed with twice the microalgae concentration. Conversely and assuming that the females can also be negatively affected by the pesticides in parallel to their eggs developing in the brood pouch, *D. magna* is a more powerful filter feeder than *D. longispina,* and hence it is likely that *D. magna* was more exposed to the pesticides via ingested contaminated food. Secondly, the multiple regression approach used to explore fecundity versus fertility observations faced technical limitations that could have affected the final outcome. Some predictors necessarily are highly correlated, thus collinearity is likely to occur; in addition, high frequencies of zero values in some predictors resulted in positively skewed distributions of limited adjustment via data transformation. These features can bias the regression outcome (e.g. Quinn and Keough 2002), thus results from such analysis should be regarded carefully.

The commercially available herbicide Betanal® Expert contains a fixed-ratio ternary mixture of the active ingredients phenmedipham, desmedipham and ethofumesate. Results suggest that despite similar response patterns depicted for Betanal® Expert and the chemicals tested alone, they interact when jointly provided to the tested species (TUmix values

different from 1; as interpreted by Woods et al. 2002). Such an interaction was not unexpected. Indeed, phenmedipham and desmedipham act similarly in target species as photosynthetic inhibitors (EPA 1996, 2005b; PPDB 2009; Roberts 1998; Tomlin 2001), but ethofumesate has a completely distinct mode of action (EPA 2005a; Roberts 1998; Velkoska-Markovska et al. 2008). It is then reasonable to hypothesise that the a.i.s also act via different mechanisms of toxicity in non-target organisms, such as *Daphnia*, which is reinforced by the similarities in response patterns after exposure to phenmedipham and desmedipham but not so much for etofumesate as discussed above (see the discussion by Jager et al. (2010) as to the interpretation of physiological modes of action). In this context, phenmedipham and desmedipham are expected to act as dilutions of each other (e.g. Cedergreen et al. 2008; Deneer 2000) since they have similar modes-of-action, whilst ethofumesate may promote deviations from simple additivity. Such interpretation may be biassed by the application of the TU approach in the present study, which is limited by the assumption of concentration addition (CA) for the joint action of similarly acting chemicals (Jonker et al. 2005). Still, CA has been described as more conservative in environmental assessment than the alternative model of independent action (Cedergreen et al. 2008), which improves the level of confidence in the conclusions.

A distinct outcome of the exposures to Betanal® Expert and to the equivalent mixture of its active ingredients was recorded considering the mixture toxicity metrics used. Indeed, the active ingredients apparently interacted, showing a more than additive behaviour when the commercial formulation Betanal® Expert was dosed (TUmix below 1), but the opposite occurred when the a.i.s were jointly tested (TUmix above 1). This clearly indicates that undisclosed adjuvant chemicals added to the commercial formulation Betanal® Expert play a relevant role in enhancing the biological efficacy of the mixture of its a.i.s. Commercial formulations of pesticides contain the active ingredient(s) and a number of other chemical adjuvants (the so-called inert ingredients) which are added to assist proper mixing, dilution, application and stability (Cox and Surgan 2006), i.e. to increase the general efficiency of the product. The inert ingredients are not supposed to be toxic and their identification, as well as percentages within the formulation, generally constitutes confidential information. Despite the lack of toxicity of inert ingredients possibly being true, several authors (Cedergreen and Streibig 2005; Krogh et al. 2003; Pereira et al. 2000, 2009; Solomon and Thompson 2003) and the present study have shown that such a statement does not necessary apply when considering non-target aquatic organisms. Further support was hence given to the argument on the validity of integrating commercial formulations rather than solely the corresponding active ingredients in the ecotoxicological evaluation frameworks required before pesticide placement on the market.

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