Supporting Information

Can organophosphates and carbamates cause synergisms by inhibiting esterases responsible for biotransformation of pyrethroids?

Yi Cao*, Alberto Ibáñez Navarro, Lucas Perrella, Nina Cedergreen*

Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark

* Corresponding author.

E-mail addresses: y.cao@plen.ku.dk (Yi Cao), cjk223@alumni.ku.dk (Alberto Ibáñez Navarro), jvk471@alumni.ku.dk (Lucas Perrella), ncf@plen.ku.dk (Nina Cedergreen)

Number of pages: 14

Number of Tables: 7

Number of Figures: 4

Table of Contents

Section 1. Verification of exposure concentrations

The exposure concentrations of insecticides were verified at the beginning of the acute toxicity tests. Samples (40 mL each) were taken in triplicates corresponding to the highest treatment, and were mixed with 5 mL of dichloromethane and stored at -20 ºC.

In the sample preparation of alpha-cypermethrin (α-cyp), 20 µL esfenvalerate (4 mg/L) was added to the thawed samples, which were then transferred to 100 mL separation funnels. The sample flasks were washed with 5 mL dichloromethane, which was subsequently transferred to the separation funnels as well. The separation funnels were shaken manually for one minute, the dichloromethane phase was allowed to precipitate for a couple of minutes, and 2 mL of dichloromethane phase was then collected. The extraction procedure was repeated twice. Then 5 mL of the combined dichloromethane solutions was transferred to a beaker, and a teaspoon of dried sodium sulfate was added. The mixture was gently whirled and filtrated into a 10-mL-conical-bottom tube. After the addition of 1.5 mL cyclohexane:acetone (9:1 v/v), the solution was evaporated to a final volume of approximately 1 mL under a nitrogen flow. The concentrated solution was then to a GC-vial, 20 µL deltamethrin of 4 mg/L was added, and the final volume was adjusted to 1.5 mL with acetone.

Samples of α-cyp were analyzed using a GC-µECD (Agilent 7820 A) equipped with a Zebron ZB-5HT column (5% phenyl-arylene, 95% dimethylpolysiloxane, 15 m, 250 µm, and 0.1 µm). The injection mode was chosen to splitless mode, and volume was 3 µL at an inlet temperature of 280 °C. The carrier gas was H_2 at 2.5 mL/min. The oven temperature was initially set to 120 °C, holding for 1.5 minutes, followed by 160, 170, 215, and 260 °C, holding for 4, 4, 3, and 3 minutes, respectively. The total time of the temperature program was 22 minutes. Temperatures were raised at a rate of 40 °C/min. The detector was operated at 300 °C with a makeup flow of N_2 at 60 mL/min.

In the sample preparation of organophosphates and carbamates, 20 μL recovery standards (aldicarb or diazinon) of proper concentrations (10 – 10000 ppm), which is comparable with the concentrations of the analytes, were added to the thawed samples and the samples were shaken. Then, 20 mL of each sample was transferred into a plastic centrifuge tube and 20 mL acetonitrile (1% acetic acid) was added to the tube. The mixture was agitated for 30 s in a vortex shaker at 2500 ppm. Extraction salts (8.4 g $MgSO_4$, 3.5 g $NaOAc \cdot 3H_2O$, 1.4 g NaCl) were added to the mixture which was shaken at 2500 rpm for 20 minutes on a vortex. After shaking, the mixture was centrifuged for 7 min at 4100 rpm at 10 ºC to separate the aqueous phase and the acetonitrile phase. 10 mL of acetonitrile phase was then collected in a 10-mL conical bottom glass tube and was evaporated down to approximately 500 μL under a gentle stream of N_2 at approx. 25 °C. The concentrated liquid was filtered through an acetonitrile pre-washed 0.2 μm RC filter. Finally, the internal standard carbofuran was added, and the volume of the samples was adjusted to 1.5 mL with acetonitrile. For some samples with an extremely high or low concentration of the analyte, the final volume was adjusted to ensure the final concentration of each analyte was within the quantitation ranges.

A Water Acquity UPLC I-class module was used for chromatographic separation equipped with a 2.1 mm × 50 mm waters Acquity UPLC BEH C18 column, particle size 1.7 μM. The injection volume was 10 μL at an inlet temperature of 60 ºC. To separate the different components of the samples, gradient elution was used at a flow rate of 0.3 mL/min. Eluent A was 0.1% formic acid water and eluent B was 0.1% formic acid in methanol. The elution started with 80% A and 20% B over 11 minutes, followed by 15% A and 85% B over three minutes, and then 80% A and 20% B in the last one minute. The MS was operated on a Waters Xevo TQD triple quadrupole mass spectrometer with electrospray ionization in positive ion mode. The retention time, parent ions, product ions, cone voltage, and collision energy and limit of detection (LOD) and quantification (LOQ) is shown in Table S1.

Table S1. UPLC-MS/MS parameters for organophosphates and carbamates analysis

Compound	Retention time (min)	Parent ion	Cone voltage (V)	Product ion	Collision energy (V)	LOD/LOQ (μg/L)
Aldicarb	3.67	116	24	89	24	1.18/3.93
Diazinon	8.94	305.1	42	169	18	1.66/5.54
Chlorpyrifos	10.39	349.9	32	96.9	30	3.32/11.1
Parathion	8.46	292.01	30	235.9	16	7.13/23.8
Chlorfenvinphos	9.09	359	38	98.9	30	0.43/1.44
Iso-OMPA	6.66	343.2	38	284.15	16	0.85/2.82

Section 2. 96 h acute toxicity test of alpha-cypermethrin with the addition of iso-OMPA

In the first mixture toxicity tests, seven concentration-response setups for α-cyp were tested together with seven different constant concentrations of iso-OMPA (Table S1), controls (iso-OMPA in the M7 medium) and solvent controls (0.01% v/v acetone and iso-OMPA in the M7 medium). The α-cyp concentrations were adjusted to capture a full concentration-response curve, also when iso-OMPA induced synergy. There was no mortality of iso-OMPA alone for the concentrations used in the test. The mixture tests of α-cyp and iso-OMPA showed no significant mortality during the first two days (Figure S1a and S1b). Fraction surviving *D. magna* after 72 h and 96 h exposed to α-cyp and iso-OMPA mixture is described as a function of the concentration of α-cyp (Figure S1c and S1d), and the fitted parameters are given in table 1 in the manuscript.

Table S2. Compositions of iso-OMPA and α-cyp mixture tests towards *D. magna*. Seven (or eight) nominal concentrations of iso-OMPA were combined with different nominal ranges of α-cyp.

Iso-OMPA (mg/L)					α-cyp (μg/L)			
20	0.5	0.25	0.05		0.01	0.002	0.0004	0.00008
10	0.5	0.25	0.1	0.05	0.01	0.002	0.0004	0.00008
5	1	0.5	0.25	0.1	0.05	0.01	0.002	0.0004
1	1	0.5	0.25		0.1	0.05	0.01	0.002
0.04	2	1	0.5		0.25	0.05	0.01	0.002
0.008	2	1	0.5		0.25	0.05	0.01	0.002
0	2	1	0.5		0.25	0.05	0.01	0.002

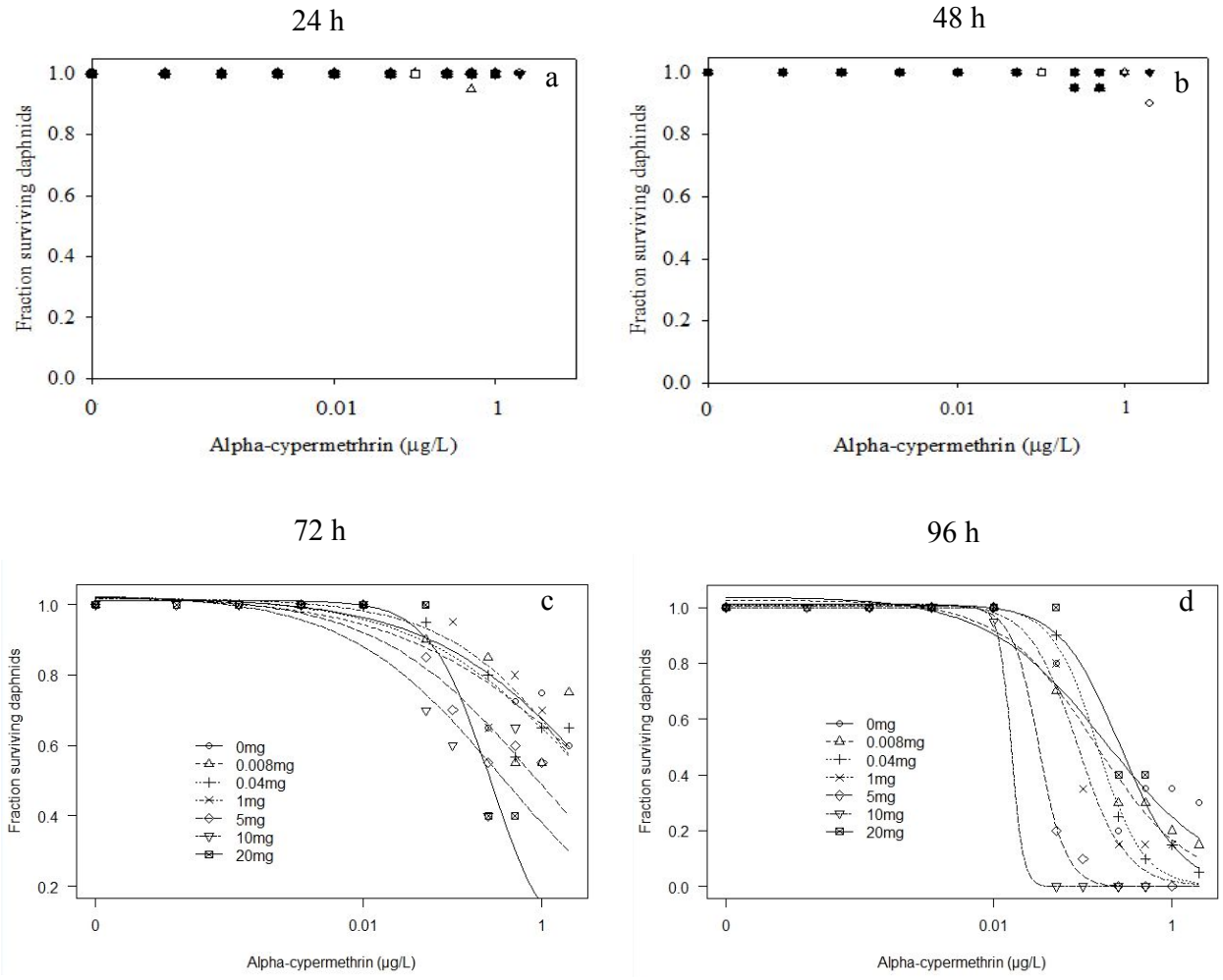

Figure S1. Fraction surviving *D. magna* exposed to α-cyp for 4 h (a), 48 h (b), 72 h (c), and 96 h (d) with addition of increasing nominal concentrations of iso-OMPA (0, 0.008, 0.04, 1, 5, 10, and 20 mg/L) as a function of α-cyp nominal concentrations. There is no significant mortality after 24 h and 48 h exposure. The Data of 72 h and 96 h are fitted with three-parameter log-logistic models by R. For clarity, all data are given as mean (n = 20). Fit parameters are given in Table S3 for 72h and Table 1 in the manuscript for 96h.

Table S3. Model parameters for the concentration-response curves fitted to the mixture tests of α-cyp and iso-OMPA data after 72h exposure. The concentrations of α-cyp of each treatment are given in Table S2, and the EC_{50} values are given as α-cyp concentration, while the treatments are given as the iso-OMPA concentration added. MDR was expressed by the fold-decrease in EC_{50} of α-cyp in the mixture relative to EC_{50} of α-cyp alone. All model parameters are given as mean ± SE.

Treatment mg/L iso-OMPA	Slope *b*	EC_{50} of α-cyp (μg/L)	MDR
0	0.50 ± 0.14	4.33 ± 2.76	1.00
0.008	0.43 ± 0.10	4.62 ± 2.74	0.94
0.04	0.50 ± 0.10	3.47 ± 1.52	1.25
1	0.63 ± 0.17	3.08 ± 1.88	1.41
5	0.53 ± 0.09	0.88 ± 0.28	4.92
10	0.54 ± 0.09	0.34 ± 0.11	12.7
20	1.33 ± 0.15	0.26 ± 0.02	16.7

Section 3. The relative activities of general esterases and cytochrome P450 exposed to iso-OMPA

D. magna were exposed to different concentration of iso-OMPA for 96 h. In this process, the *in vivo* GE activity of *D. magna* was measured every 24 h.[1] The relative activities were calculated based on the controls average for each exposure period (Table S4). Iso-OMPA knocked down GE activity after 24 h, but at 48 h GE activity was stimulated, just to decrease again after 72 h and 96 h (Figure S2a). Potential inhibition of iso-OMPA on cytochrome P450 monooxygenase was tested *in vitro* on

rat liver microsomes, as those resemble P450 enzymes of *D. magna* in terms of chemical inhibition reasonably well (Figure S2b).[2]

Table S4. Activities of *in vivo* GE after 24, 48, 72, and 96 h exposed to increasing nominal concentrations of iso-OMPA. Data are shown as mean ± SD (n = 3).

Iso-OMPA (mg/L)	Activity of GE after 24 h (nmol h^{-1} $organism^{-1}$)	Activity of GE after 48 h (nmol h^{-1} $organism^{-1}$)	Activity of GE after 72 h (nmol h^{-1} $organism^{-1}$)	Activity of GE after 96 h (nmol h^{-1} $organism^{-1}$)
0	0.34 ± 0.25	0.48 ± 0.09	0.48 ± 0.17	1.14 ± 0.49
0.008	0.23 ± 0.01	0.39 ± 0.08	0.54 ± 0.16	0.86 ± 0.10
0.04	0.21 ± 0.06	0.63 ± 0.24	0.50 ± 0.29	0.82 ± 0.19
1	0.35 ± 0.03	0.74 ± 0.02	0.57 ± 0.13	1.02 ± 0.25
5	0.29 ± 0.02	0.44 ± 0.14	0.33 ± 0.06	0.67 ± 0.27
10	0.22 ± 0.04	0.44 ± 0.06	0.23 ± 0.15	0.27 ± 0.24
20	0.14 ± 0.02	0.37 ± 0.13	0.24 ± 0.08	0.46 ± 0.06

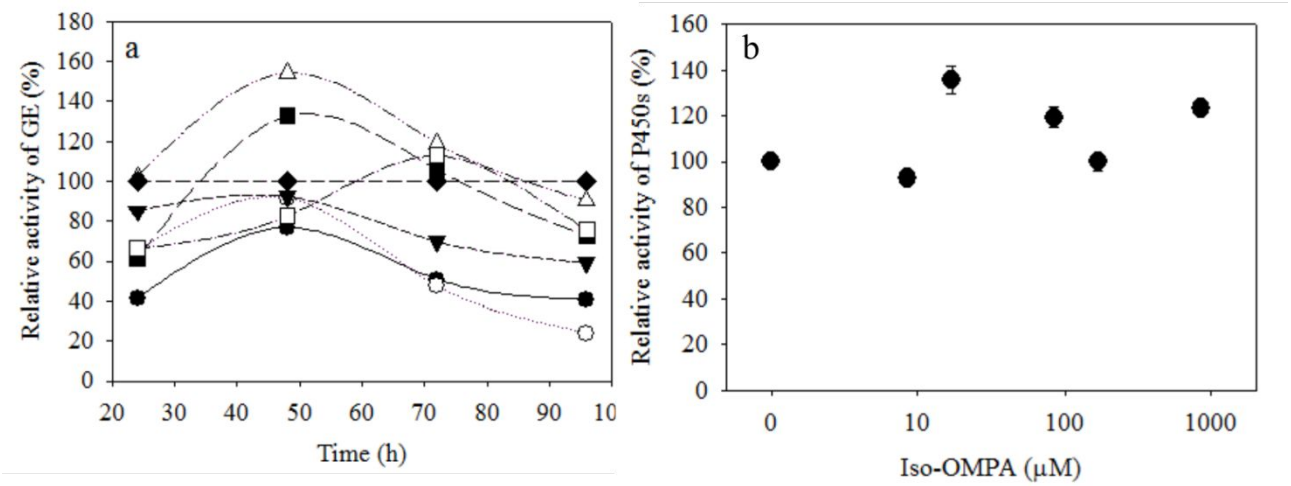

Figure S2. (a) *In vivo* GE relative activities of each iso-OMPA treatment (black circles: 20 mg/L; white circles: 10 mg/L; black triangles: 5 mg/L; white triangles: 1 mg/L; black squares: 0.04 mg/L; white squares: 0.008 mg/L; black diamonds: 0 mg/L) fluctuating over time. For clarity, standard deviations are omitted. (b) *In vitro* cytochrome P450 relative activity of rat liver microsomes exposed

to increasing nominal concentration of iso-OMPA (8.5, 17, 85,170, and 850 μM, corresponding to 2.9, 5.8, 29, 58 and 291 mg/L). Data are given as mean ± SD, n = 3.

Section 4. Acute toxicity tests of single chemicals

96 h acute toxicity tests toward *D. magna* were implemented to identify the toxicities of single insecticide (α-cyp, aldicarb, diazinon, chlorpyrifos, parathion, and chlorfenvinphos). The fractions of surviving *D. magna* were recorded every 24 h. The concentration-response curves were fitted by R with a three-parameter log-logistic model, and parameters are given in Table S5.

Table S5. Model parameters for the concentration-response curves fitted to the 96h tests of single insecticides.

		24 h	**48 h**	**72 h**	**96 h**
α-cyp	**Slope *b***	1.30 ± 0.22	1.50 ± 0.23	1.33 ± 0.17	2.58 ± 0.39
	Upper limit *d*	1.00 ± 0.02	0.98 ± 0.02	1.00 ± 0.02	1.00 ± 0.02
	EC_{50} *e* (μg/L)	1.53 ± 0.17	1.10 ± 0.09	0.03 ± 0.004	0.02 ± 0.003
Aldicarb	**Slope *b***	0.54 ± 0.26	1.57 ± 0.24	10.6 ± 43.4	10.6 ± 65.1
	Upper limit *d*	1.00 ± 0.02	0.98 ± 0.02	1.00 ± 0.02	0.99 ± 0.02
	EC_{50} *e* (μg/L)	921 ± 89.8	574 ± 54.8	411 ± 98.3	396 ± 53.7
Diazinon	**Slope *b***	0.50 ± 0.10	1.23 ± 0.16	3.58 ± 1.61	6.99 ± N/A
	Upper limit *d*	1.00 ± 0.023	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.02
	EC_{50} *e* (μg/L)	23.1 ± 10.4	0.98 ± 0.11	0.59 ± 0.13	0.29 ± N/A
Chlorpyrifos	**Slope *b***	0.83 ± 0.24	0.67 ± 0.13	1.68 ± 0.34	5.98 ± 18.32
	Upper limit *d*	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.02
	EC_{50} *e* (μg/L)	0.88 ± 0.34	0.41 ± 0.11	0.07 ± 0.15	0.04 ± 0.06
Parathion	**Slope *b***	0.89 ± 0.60	0.52 ± 0.10	0.70 ± 0.09	6.38 ± 33.4
	Upper limit *d*	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.02
	EC_{50} *e* (μg/L)	39.8 ± 58.7	10.1 ± 3.95	1.57 ± 0.29	0.19 ± 0.46
Chlorfenvinphos	**Slope *b***	0.38 ± 0.12	1.21 ± 0.23	7.14 ± 91.4	5.54 ± 29.6
	Upper limit *d*	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.03
	EC_{50} *e* (μg/L)	277 ± 273	2.20 ± 0.32	0.70 ± 2.18	0.64 ± 0.53

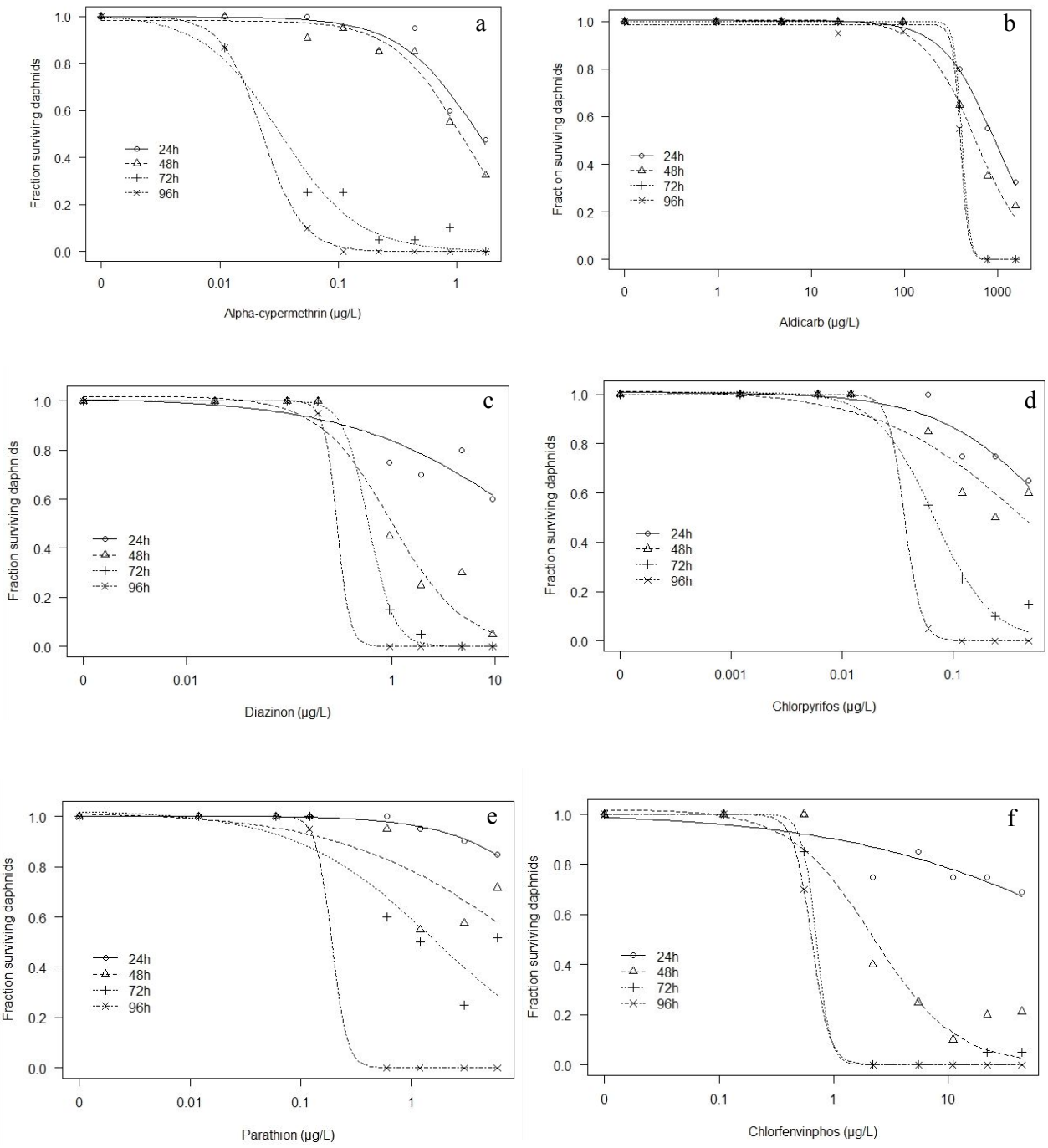

Figure S3. Fraction surviving *D. magna* exposed to α-cyp (a), aldicarb (b), diazinon (c), chlorpyrifos (e), parathion (f), and chlorfenvinphos (d) for different periods (24, 48, 72 and 96 h) as a function of insecticide nominal concentrations. For clarity, all data are given as mean (n = 20).

Section 5. Mixture toxicity tests of alpha-cypermethrin and organophosphates and carbamates

Based on the results of 96 h acute toxicity tests of single insecticides (Figure S3), 96 h acute toxicity mixture tests of α-cyp and organophosphates (OPs) or carbamates (CBs) were set up in a 50:50% effect ratio ray-design. The compositions of concentrations in these tests are shown in table S6. A toxic unit was set based on the 96h EC_{50} values of the individual insecticides (Table S5), and the sum toxic units (TU) of the mixture tests ranged from 0.1 to 10. The fractions of surviving *D. magna* were recorded every 24 h. The concentration-response curves were fitted by R with a three-parameter log-logistic model. There were no mortality of *D. magna* observed in the mixture test of α-cyp and chlorpyrifos (Figure S4d) and the mixture test of α-cyp and parathion (Figure S4e) for the first 24 h exposure. Thionate OPs (diazinon, chlorpyrifos, and parathion), which need bio-activation before exerting toxicity, induced antagonisms. Apart from thionate OPs, the carbamate aldicarb also had an antagonistic effect (Figure S4). Fit parameters are given in Table S7.

Table S6. Compositions (shown as nominal concentrations) of OPs or CBs and α-cyp mixture tests towards *D. magna*. α-cyp was mixed with OPs or CBs at 50:50 effect ratios, ranging from 10 to 0.1 toxic units (TU).

α-cyp (μg/L)	Aldicarb (μg/L)	Diazinon (μg/L)	Chlorpyrifos (μg/L)	Parathion (μg/L)	Chlorfenvinphos (μg/L)
0.1	1980	1.45	0.2	0.95	3.2
0.05	990	0.725	0.1	0.475	1.6
0.025	495	0.3625	0.05	0.2375	0.8
0.01	198	0.145	0.02	0.095	0.32
0.005	99	0.0725	0.01	0.0475	0.16
0.0025	49.5	0.03625	0.005	0.02375	0.08
0.001	19.8	0.0145	0.002	0.0095	0.032

Table S7. Model parameters for the concentration-response curves fitted to the 96 h mixture tests of mixture tests of α-cyp and organophosphates (OPs) or carbamates (CBs).

Insecticide	Parameters	24 h	48 h	72 h	96 h
α-cyp + Aldicarb	**Slope *b***	4.33 ± 0.50	3.63 ± 0.39	10.4 ± 20.7	9.94 ± 19.1
	Upper limit *d*	1.00 ± 0.01	0.98 ± 0.01	0.99 ± 0.01	1.00 ± 0.01
	EC_{50} *e* (TU)	4.40 ± 0.1	2.53 ± 0.06	2.25 ± 0.47	2.00 ± 0.85
α-cyp + Diazinon	**Slope *b***	4.22 ± 2.98	1.13 ± 0.20	2.94 ± 0.39	4.98 ± N/A
	Upper limit *d*	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.02
	EC_{50} *e* (TU)	13.9 ± 3.34	14.0 ± 2.42	3.11 ± 0.16	2.02 ± 0.13
α-cyp + Chlorpyrifos	**Slope *b***	N/A	0.98 ± 0.60	3.21 ± 0.44	5.84 ± 1.24
	Upper limit *d*	N/A	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.01
	EC_{50} *e* (TU)	N/A	79.9 ± 113	5.72 ± 0.27	3.02 ± 0.14
α-cyp + Parathion	**Slope *b***	N/A	3.77 ± 1.52	2.24 ± 0.42	7.65 ± 5.75
	Upper limit *d*	N/A	1.00 ± 0.01	1.00 ± 0.02	0.99 ± 0.01
	EC_{50} *e* (TU)	N/A	12.6 ± 1.42	9.96 ± 0.77	5.01 ± 0.12
α-cyp + Chlorfenvinphos	**Slope *b***	2.67 ± 0.45	5.01 ± 0.43	4.32 ± 0.71	8.71 ± 12.9
	Upper limit *d*	1.00 ± 0.01	1.00 ± 0.01	1.00 ± 0.01	1.00 ± 0.01
	EC_{50} *e* (µg/L)	11.6 ± 0.62	3.53 ± 0.11	2.05 ± 0.09	1.17 ± 0.28

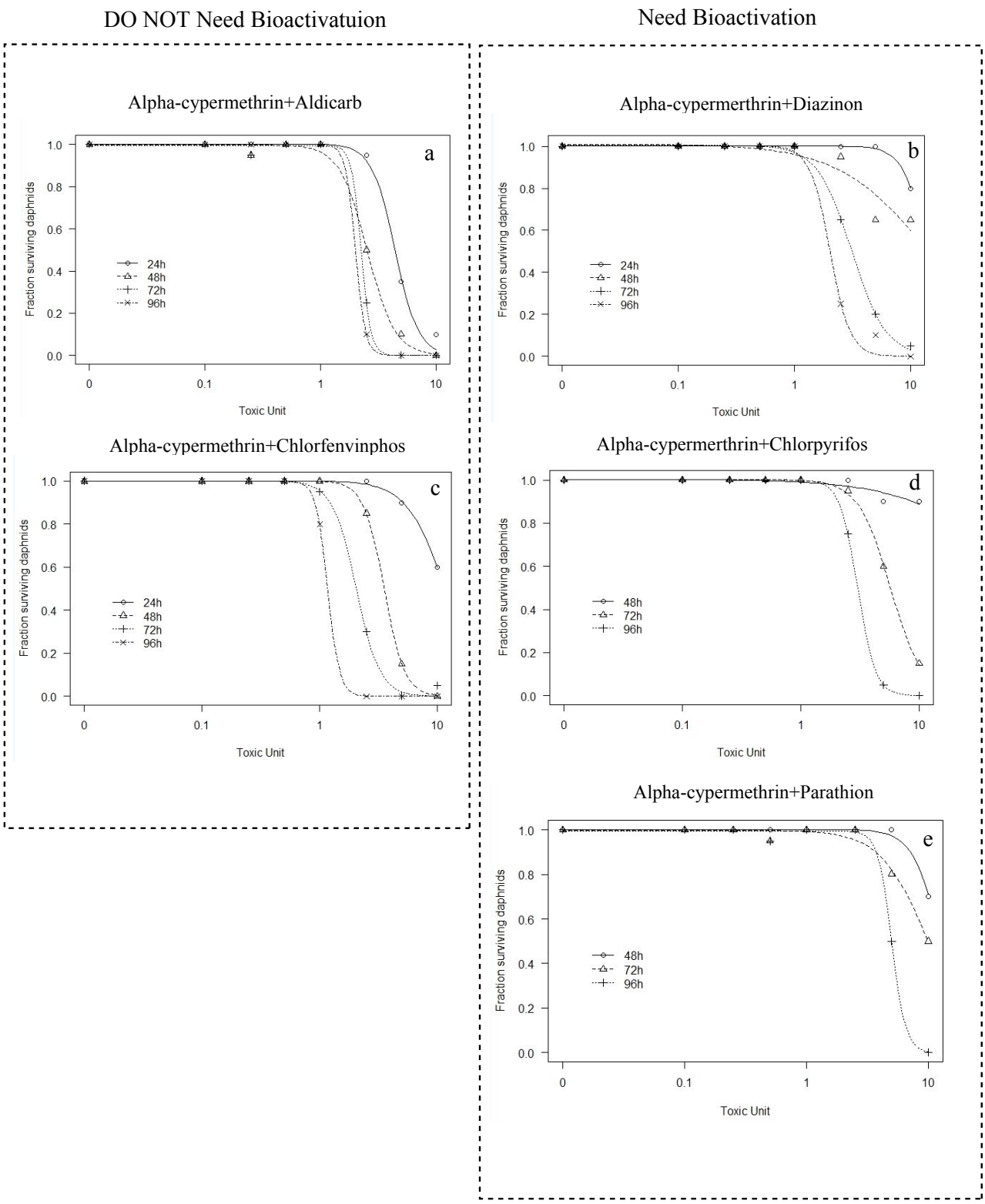

Figure S4. Fraction surviving *D. magna* exposed to the binary combination of α-cyp and aldicarb (a), diazinon (b), chlorfenvinphos (c), chlorpyrifos (d) and parathion (e) for different periods as a function as toxic unit basing EC_{50} values of each insecticide. There was no dead *D. magna* observed in the

mixture test of α-cyp and chlorpyrifos (d) and the mixture test of α-cyp and parathion (e) for the first 24 h exposure. For clarity, all data are given as mean (n = 20).

References

(1) Cao, Y.; Herrero-Nogareda, L.; Cedergreen, N. A comparative study of acetylcholinesterase and general-esterase activity assays using different substrates, *in vitro* and *in vivo* exposures and model organisms. *Ecotoxicol. Environ. Saf.* **2020**, *189*, 109954. doi:10.1016/j.ecoenv.2019.109954

(2) Gottardi, M.; Tyzack, J. D.; Bender, A.; Cedergreen, N. Can the inhibition of cytochrome P450 in aquatic invertebrates due to azole fungicides be estimated with in silico and in vitro models and extrapolated between species? *Aquat. Toxicol.* **2018**, *201*, 11–20. doi:10.1016/j.aquatox.2018.05.017