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Environmental stress increases synergistic effects of pesticide mixtures on Daphnia magna

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1 **Title**

2 Environmental stress increases synergistic effects of pesticide mixtures on *Daphnia magna*

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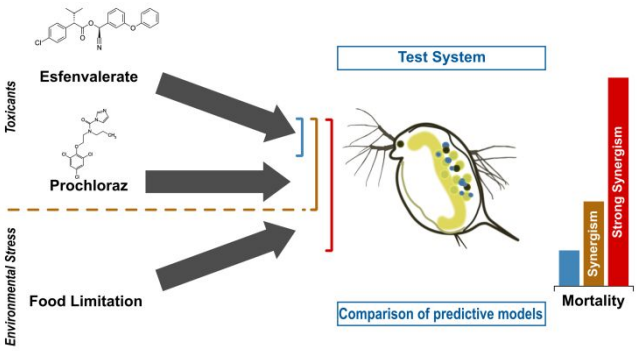
ABSTRACT

Some widely used pesticide mixtures produce more than additive effects according to conventional combined effect models. However, synergistic effects have been so far generally observed at unrealistically high pesticide concentrations. Here, we used *Daphnia magna* as a test organism and investigated how food limitation – a common ecological stressor – affects the mixture toxicity of a pyrethroid insecticide and an azole fungicide. We also compared three models regarding the prediction of mixture effects including concentration addition (CA), effect addition (EA) and stress addition model (SAM). We revealed that especially under low food, the strength of synergism between esfenvalerate and prochloraz increased with an increasing concentration of prochloraz independent of the null model. Under high food conditions and at concentrations of prochloraz ≥ 32 µg/L, we observed a marginal synergistic effect with an MDR = 2.1 at 32 µg/L prochloraz and 2.2 at 100 µg/L prochloraz when using CA as null model. In contrast, the combination of both pesticides and food stress caused synergistic effects shown by an MDR = 10.9 even at 1 µg/L of prochloraz that is frequently detected in the environment. The combined effects of pesticides and food stress could be predicted best with the stress addition model (SAM) that showed the lowest mean deviation between effect observation and prediction (mean deviation SAM = 16 [SD = 28], EA = 1072 [2105], CA = 1345 [2644]). We conclude that common environmental stressors can strongly increase the synergistic effects of toxicants. This knowledge is especially relevant considering current efforts to include the additional risk of pesticide mixtures and environmental stressors into the environmental risk assessment of pesticides.



Abstract Art

INTRODUCTION

Over the last few decades, pesticide contamination originating from intensive agricultural land use has been observed to cause negative impacts on the structure of freshwater communities[1-3] and ecosystem functions.[4-7] Other studies have further discussed the decline in aquatic invertebrate biodiversity[5] or decline in terrestrial biomass[8, 9] due to pesticides.

The frequent occurrence of negative effects of pesticides on non-target organisms in the field shows that the current environmental risk assessments of pesticides fail to determine protective thresholds of risk. This scenario mainly occurs due to (i) an error prone estimation of pesticide exposure[10, 11] and (ii) because pesticides are commonly evaluated as single products without considering realistic environmental stress and exposure conditions.[12] In agricultural practice, pesticides are often applied together as tank mixtures in spray series and hence co-occur in the environment. For example, high loads of pesticide mixtures can be found in streams, especially after run-off events.[2, 3, 13-17]

Especially, azole fungicides have been reported to cause synergistic effects when co-occurring with pyrethroids,[18-22] neonicotinoids,[23] organophosphates,[24] strobilurin fungicides[25, 26] and bipyridylium herbicides.[27] These pesticides are frequently detected in agricultural streams.[3, 28-31]

However, most studies on synergistic effects of pesticide mixtures only report interactions at higher concentrations than those commonly detected in the aquatic environment.[20, 25] Additionally, studies on synergistic mixture effects are generally based on experiments without additional stress.[20, 21, 32] Organisms in the field experience sub-optimal conditions and occasionally have to cope with severe environmental stress.[33] A recent meta-analysis revealed that environmental stress severely enhances the toxicity of individual pesticides.[12] Examples in the meta-analysis include food stress,[34, 35] competition[36] and UVB radiation[37] that can increase the sensitivity of organisms to toxicants up to a factor of 100 depending on the strength of environmental stress.

Despite numerous studies on the influence of environmental stress on the effect of single toxicants, only little attention was paid to the combined effect of environmental stress and pesticide mixtures. For example, Bjergager et al.[19] investigated mixtures of esfenvalerate and prochloraz on *Daphnia magna* under semi-field conditions and detected similar and even higher synergism in the outdoor microcosms compared to those in laboratory studies. Also Delnat et al.[38] reported that the daily temperature variation can increase the toxicity of a pesticide mixture of an organophosphate chlorpyrifos and a biopesticide *Bacillus thuringiensis* var. To our knowledge, apart from these studies, there is no information on pesticide mixtures under relevant field conditions, including environmental stressors.

To determine protective concentration levels of individual pesticides for regulatory purposes, we need to understand and quantify to what extent pesticide toxicity is increased by synergistic interactions and additional environmental stressors. Until now, approaches are lacking to predict the effects of mixtures that act synergistically. Traditional approaches such as concentration addition (CA) for similar acting compounds and effect addition (EA, also known as “independent

action") for dissimilar acting compounds assume additive effects. Among these two approaches, CA is usually considered the most conservative approach.[32, 39, 40] In comparison, Liess et al.[12] recently developed a new model, the 'stress addition model' (SAM), to specifically predict the synergy between environmental stressors and individual toxicants. However, SAM has not been tested yet for pesticide mixtures alone or in combination with environmental stress.

The aim of the present study is to identify the synergistic interactions of a frequently applied pesticide mixture, esfenvalerate and prochloraz[41] in combination with a common stressor, food limitation.[34, 35, 42] For this, we performed experiments with *D. magna* for 28 days that included mixtures of environmentally realistic concentrations of both pesticides and the additional environmental stress. Furthermore, we analysed the prediction of the combined effects using traditional approaches for toxicant mixtures (i.e., CA, Loewe and Muischnek[43] and EA, Bliss[44]). We further tested the SAM to predict combined effects of environmental and toxicant stressors.

MATERIALS AND METHODS

We studied the combined effect of the insecticide esfenvalerate and the fungicide prochloraz under high and low food conditions. For pesticide exposure, we set up a fully crossed factorial design with eight esfenvalerate treatments (0, 0.0001, 0.001, 0.01, 0.1, 0.316, 1, 3.16 µg/L) × four prochloraz concentrations (0, 1, 32, 100 µg/L) × two food levels (high, low) (Table S1). The experiment was repeated three times for all treatments apart from 0.0001, 0.001 and 0.01 µg/L of esfenvalerate using <24 h old neonates. These low concentrations were additionally included later (in second or third repetition) to better understand the effects of prochloraz under low food and low esfenvalerate conditions. Before pesticide exposure, organisms were acclimatized to the corresponding food conditions for 7 days. Organisms were exposed to pesticides for 24 h, and survival was monitored for 3 weeks. For each treatment, we tested 15 daphnids with one

individual per vessel containing 80 mL of the test solution (see also Table S1). The mortality of the daphnids was checked daily and dead individuals were removed from the experiment. Neonates from each vessel were removed daily. The total duration of the experiment was 4 weeks including the period of 1 week for acclimation to the respective food levels.

Test organisms

In all experiments, we used *D. magna* individuals obtained from a clone "Aachen V" cultured at the Department System-Ecotoxicology, Helmholtz Centre for Environmental Research − UFZ, Leipzig, Germany. Daphnids were cultured in beakers (20 individuals/beaker) with 1800 mL of artificial *Daphnia* medium (ADaM).[45] The temperature of the culture medium was maintained at 20.0 ± 1 °C under a photoperiod of a 16/8 h light/dark cycle that facilitated continuous amictic reproduction.[46] Individuals were fed with a suspension of green algae *Desmodesmus subspicatus* at 0.5×10^9 cells ind^{-1} day^{-1} in the first week and 0.75×10^9 cells ind^{-1} day^{-1} in the second week. On weekends daphnids were additionally fed with yeast (0.6 mg/L). In the culture and during the experiments, the medium was changed every second day, and neonates were removed within 24 h. The microalgae *D. subspicatus* was cultured in a mixture of distilled water and algae medium (ratio 9:1)[47] at 20.0 ± 1.0 °C under continuous light and shaken through a mixture of CO_2 and compressed air (air: 300 bar, CO_2: 3 bar). The algae were harvested in the exponential growth phase and centrifuged, and the pellets were re-suspended in ADaM to obtain the required dilutions. During the test, the organisms used in the high food treatment were fed with 0.5×10^9 cells ind^{-1} day^{-1} the first week, 1.15×10^9 cells ind^{-1} day^{-1} the second week, and 1.35×10^9 cells ind^{-1} day^{-1} the third and fourth weeks. In contrast, organisms in the low food treatment were fed with 0.5×10^7 cells ind^{-1} day^{-1} the first week, 1.15×10^7 cells ind^{-1} day^{-1} the second week, and 1.35×10^7 cells ind^{-1} day^{-1} in the third and fourth weeks. The food dosage for low food conditions was

established according to preliminary range finding tests that showed a minor effect on the survival of individuals (around 15% as compared to high food conditions) until the end of experiment (i.e., 4 weeks). Fecundity rates at the low food condition were decreased (number of eggs per female over 21 days = 0.18) as compared to high food conditions, but comparable to temporary conditions in the field. In the field, cladoceran populations have been studied to experience severe food limitation that causes a reduction in egg production close to zero[48] and a crash of the population under observation.[49]

Exposure to contaminants

We selected the pyrethroid esfenvalerate (Chemical Abstracts Service (CAS) 66230-04-4, purity: 99.8%) and the azole fungicide prochloraz (CAS 67747-09-5, purity: 98.6%) for the pesticide mixtures. We selected these pesticides because (i) azole fungicides and pyrethroid insecticides are known to cause synergistic effects and (ii) are frequently applied in agriculture in the form of mixtures.[41]

We tested concentrations of esfenvalerate, except the highest concentrations (1 and 3.16 µg/L esfenvalerate), that are in the range of those detected frequently in the field ranging from trace concentrations to 0.166 µg/L[28, 50] or even 0.76 µg/L.[51] The lowest tested concentration was even below the regulatory acceptable concentration (RAC) of esfenvalerate (EU RAC, 0.0005 µg/L; European Food Safety Authority (EFSA [52]). In comparison, prochloraz concentrations are in the range of low to environmentally unrealistic concentrations of 100 µg/L. Frequently detected concentrations of prochloraz in European surface waters range from trace concentrations to 2.9 µg/L.[28, 53, 54] We applied prochloraz and esfenvalerate at analytical grades (Sigma-Aldrich, Germany). We used dimethyl sulfoxide (DMSO) as a solvent for the preparation of the stock solution of esfenvalerate and prochloraz. The DMSO concentration was always kept below

158 0.02% [vol/vol] that is two orders of magnitude lower than the LOEC (Lowest observed-effect
159 concentration; 2%)[55] and under the solvent limit suggested by Organisation of Economic
160 Cooperation and Development (OECD) guidelines.[56]

161 **Chemical analysis of the test media**

162 Exposure concentrations of esfenvalerate and prochloraz were analysed for all treatments per
163 experimental repetition. Samples were analysed by Wessling GmbH, Landsberg OT, Oppin,
164 Germany, using a Thermo Fisher Scientific TSQ™ 8000 Evo Triple Quadrupole GC-MS/MS.
165 The detection limit of the instrument was 5.7 ng/L. The analytical column used was a TG-5HT
166 guard column with a 0.53 mm id and a 0.15 μm film thickness (Thermo Fisher Scientific,
167 Hennigsdorf, Germany). The software Trace Finder 3.2 (Thermo Fisher Scientific) was applied
168 for data processing. The measured concentrations of esfenvalerate and prochloraz in the
169 experimental repetitions are given in the Supporting Information (Table S2). The median
170 measured concentration of each nominal concentration ranged in acceptable boundaries (± 20%).
171 The concentrations below the detection limit (i.e., 0.0001 and 0.001 μg/L) were confirmed by
172 higher concentrations serving as stock solutions for serial dilutions. Results in subsequent
173 sections are displayed and analysed using nominal concentrations.

174 **Statistics and comparison of predictive models**

175 To compare the LC_{50} concentrations of esfenvalerate between the different levels of food stress
176 and prochloraz, we calculated LC_{50} and the 95% confidence intervals using a five-parameter log-
177 logistic model for concentration-response relationships.[57] The LC_{50} values of esfenvalerate were
178 derived by fitting a five-parameter log-logistic model to the survival per treatment. The survival
179 per treatment was averaged over the three repetitions before fitting. Single LC_{50} for each
180 repetition were also determined to calculate the confidence intervals. As the survival of *D.*

magna did not significantly differ from 7 days to 21 days after exposure (paired sample *t*-test; *p*-value > 0.05), we used the data for day 7 for further analysis.

In the present study, we first investigated the toxicity of the pesticide mixture under high and low food conditions. For this purpose, we compared the LC_{50} of esfenvalerate for different prochloraz treatments under high and low food conditions in relation to the respective control groups (i.e., high and low food conditions at 0 µg/L prochloraz). Secondly, we investigated the combined effect of pesticide and environmental stressors. For this, we compared different prochloraz treatments under low food conditions in relation to the high food control at 0 µg/L prochloraz as the optimal laboratory condition.

We evaluated the predicted combined effects for the first and second analysis by applying different additive approaches (CA and EA) and one approach designed for synergistic interactions (SAM). Both the EA[44] and CA[43] models are commonly applied to predict mixture effects and assume the additivity of effects.

For the EA approach, the effect was predicted using the following equation (Eq. 1):

$$E(c_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(c_i)) \qquad (1)$$

where *E(cmix)* is the total effect of all stressors *E(ci)*. For the CA approach, the prediction was based on the following equation (Eq. 2):

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i}\right)^{-1} \qquad (2)$$

where ECx_{mix} is the total concentration of the mixture including environmental stress, p_i indicates the proportion of component *i* in the mixture, and ECx_i is the concentration of component *i* producing a ×% effect. Environmental stress was converted into a concentration level via

mortality based on the concentration-response relationship of the toxicant (for details see Liess et al.[12]).

In comparison to the additive approaches CA and EA, the SAM was developed to predict synergistic effects of independent stressors, such as a toxicant and an environmental stressor.[12] According to Liess et al.[12] the prediction of the SAM model are based on three principal assumptions: (i) each individual has a certain stress capacity to tolerate all types of stress without showing an effect; (ii) every stressor can be transferred into a general stress level that ranges from 0 to 1 using stress-level related mortality as the common link (0 = no mortality, 1 = 100 % mortality); and (iii) the joint effect can be estimated by adding up general stress levels exerted by independent stressors. The details and formulas are given in Liess et al.[12] and the software INDICATE.

We applied CA, EA and the SAM to predict LC_{50} using the software INDICATE (Version 1.0.0; http://www.systemecology.eu/indicate/). To quantify the predictive accuracy of the models, a model deviation ratio (MDR) was calculated for the CA, EA and SAM models by dividing the predicted LC_{50} values by the observed LC_{50} values. Belden et al.[39] suggested the model deviation ratio as a simple measure of model accuracy. The authors further suggested the range of $0.5 < MDR < 2$ as an arbitrary benchmark for the accuracy of CA or EA models. For an $MDR > 2$, interactions between stressors are interpreted as synergistic.[58] In the present study, we used the term "high synergism" or "strong synergism" when the MDR values were > 10 using concentration addition (CA) as the null model. Additionally, we calculated the mean deviation factor of all MDRs for different treatments of prochloraz and food using the three prediction models. In cases with MDR values < 1, we determined the deviation factor by dividing the predicted LC_{50} and the observed LC_{50}. Combined effects were considered to be significantly

synergistic if the MDR values were > 2 and, if the 95% confidence intervals of observed and predicted LC_{50} values of the three single repetitions did not overlap.[59, 60]

Except the determination of observed and predicted LC_{50} values, we generated all figures and statistical analyses using the software R studio (version 1.0.44)[61] and R (version 3.0.3).[62]

RESULTS

Synergistic potential of azole fungicide prochloraz at high and low food conditions

To reveal general differences between the toxicity of the pesticide mixture under different food levels, we compared the toxicity of esfenvalerate at different concentrations of prochloraz under high and low food conditions in relation to respective control groups (i.e., high and low food controls). Under high food conditions, prochloraz alone did not show any significant effect on the survival, even at the highest concentration. However, under low food conditions, the survival was significantly affected by higher concentrations of prochloraz (≥32 µg/L prochloraz, Wilcoxon's rank sum test, p-value < 0.05; Figure 1B). Further, we observed that under both food conditions, the strength of synergism between esfenvalerate and prochloraz increased with increasing concentration of prochloraz. Under high food conditions, synergistic effects between both pesticides could only be observed at higher concentrations of prochloraz (≥32 µg/L prochloraz; Figure 1 A; Table 1). However, these synergistic effects in relation to CA were only moderate under high food conditions, as shown by an MDR of 0.82 to 2.18 but not significant (Table 1). In comparison, the threshold for the synergistic effects of prochloraz under low food conditions was lower than that under high food conditions (≥ 1 µg/L prochloraz; Figure 1 B, Table 1) using CA as the reference model. With increasing concentrations of prochloraz, the MDR for LC_{50} increased to 2.6, 13 and 1925 for 1 µg/L, 32 µg/L and 100 µg/L prochloraz,

247 respectively. However, synergistic effects were only significant at 32 and 100 μg/L prochloraz
248 (Table 1).

249 Regarding the prediction of the mixture effects of esfenvalerate and prochloraz, we observed that
250 under high food conditions, the mean deviation of the predicted combined effect from the
251 observed effect was similar for all three approaches (Figure S1, Table 1). However, under low
252 food conditions, EA and to a lesser extent CA provided the most accurate predictions at lower
253 concentrations of prochloraz (1 and 32 μg/L prochloraz), while the SAM highly overestimated
254 the combined effect. In contrast, at the highest concentration of prochloraz (100 μg/L), the SAM
255 predictions were the most precise (Figure S2, Table 1). Additionally, when we took the average
256 of all treatments (i.e., 1, 32 and 100 μg/L of prochloraz), the SAM predictions deviated two and
257 six times less from the observed effect compared to the predictions of EA and CA, respectively
258 (Figure S2, Table 1). The results indicate that the SAM provides the best predictions of mixture
259 toxicity if strong synergistic interactions are expected.

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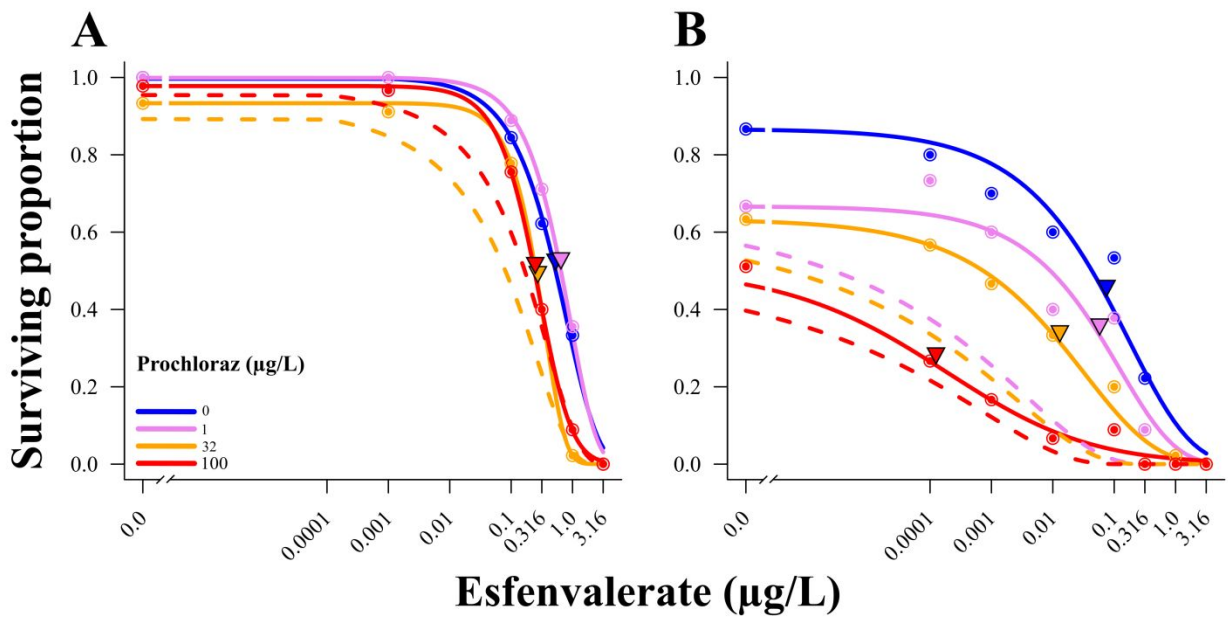

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262 **Figure 1.** Survival of *Daphnia magna* at day 7 after an exposure of 24 h to the mixture of
263 esfenvalerate and prochloraz under (A) high food and (B) low food conditions. Data points
264 represent an average survival based on three experimental repetitions that was calculated relative
265 to the initial number of individuals. The solid lines show the fitted observed concentration-
266 response relationships, and the dashed lines represent the modelled concentration-response
267 relationship under additional stress using the SAM. Under high food conditions (A), the
268 predicted concentration-response relationship at 1 µg/L of prochloraz is not shown; because SAM
269 requires an effect > 0% at control conditions (0 µg/L esfenvalerate). At 1 µg/L prochloraz alone
270 there was no measurable effect on the survival of *D. magna* under high or low food conditions.
271 Triangles display LC_{50} values of different concentration-response curves.

272 **Interaction of three stressors including both pesticides and food limitation**

273 For the combined effect of both pesticides and food stress, we performed similar analysis as in
274 the previous chapter *Synergistic potential of azole fungicide prochloraz at high and low food*
275 *conditions*. In comparison, we here compare all treatments of low food and prochloraz to the

276 control with high food and without prochloraz as the optimal laboratory condition (best case).
277 Our results show that in comparison to prochloraz and esfenvalerate under high food conditions
278 (Figure 1A, Table 1), the combination of food stress and prochloraz notably increased the
279 sensitivity of daphnids to esfenvalerate (Figure 2, Table 1). The MDR values determined for the
280 LC_{50} of esfenvalerate using CA were 7.7, 10.9, 50.2 and 5312 for the low food conditions with 0,
281 1 µg/L, 32 µg/L and 100 µg/L prochloraz, respectively and all treatments showed significant
282 synergistic effects (Table 1).

283 When comparing the predictions of CA, EA and the SAM for the effect of all three stressors, we
284 found that the SAM performed best in terms of the modelled curve (Figure 2, Figure S3) and
285 lowest MDRs (Table 1). The models of CA and EA substantially underestimated the combined
286 effect of all three stressors by up to three orders of magnitude at the highest concentration of
287 prochloraz (Table 1, Figure S3). On average, the underestimation by CA and EA of the observed
288 effect was 1345 and 1072 times, respectively. In comparison, the SAM predicted best at 0, 1
289 µg/L, and 32 µg/L prochloraz (Figure S3; Table 1). Nevertheless, in the case of the highest
290 concentration of prochloraz (100 µg/L), the SAM also underestimated the total effect by a factor
291 of 58, which was still 92 and 73 times greater than those estimated by CA (i.e., 5312 times) and
292 EA (i.e., 4229 times), respectively (Figure S3, Table 1).

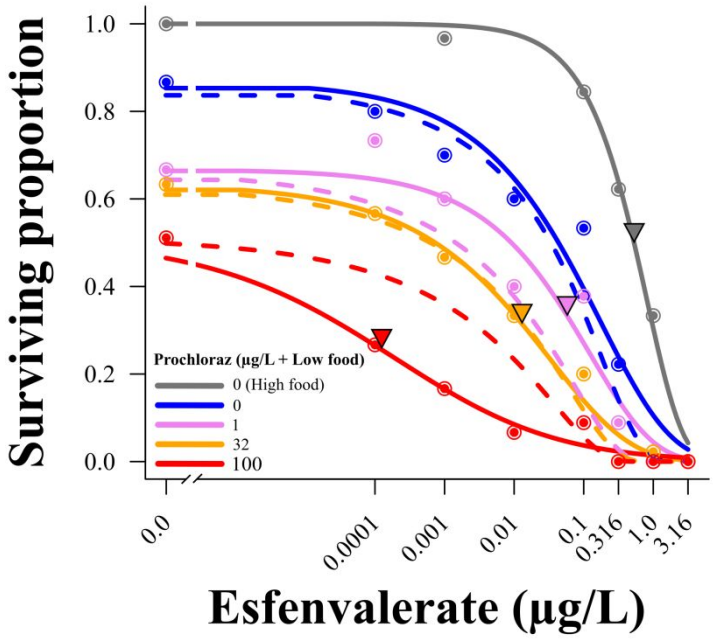

Figure 2. Survival and concentration-response curves of *Daphnia magna* exposed to a mixture of esfenvalerate and prochloraz and low food as an additional stress (interaction of three stressors). Data points represent an average survival based on three experimental repetitions that was calculated relative to the initial number of individuals. Organisms exposed to esfenvalerate alone under high food conditions were considered as control. The solid lines show the observed concentration-response relationships, whereas the dashed lines represent the modelled concentration-response relationships under the additional stress using the stress addition model (SAM). Triangles denote LC_{50} values for different concentration-response curves.

Table 1. Experimental observations and predictions of *Daphnia magna* exposed to esfenvalerate alone and in combination with prochloraz under high and low food conditions.

	Prochloraz (µg/L)	[1]Observed LC_{50} [2]95% CI	[3]Predicted LC_{50} 95% CI	Significance of synergism	MDR		
					CA	EA	SAM
High food	0 (high food)	0.529 (-0.023–1.367)	–	–	–	–	–
	1	0.647 (0.087–1.419)	0.529 (-0.023–1.367)	–	0.82	0.82	0.82
	32	0.272 (0.146–0.323)	0.556 (-0.065–1.363)	–	2.05	1.95	0.36
	100	0.247 (0.189–0.317)	0.54 (0.001–1.366)	–	2.18	2.14	0.71
Low food	0 (low food)	0.0746 (0.036–0.245)	–	–	–	–	–
	1	0.0576 (0.035–0.165)	0.15 (0.076–0.299)	–	2.6	1.3	0.0042
	32	0.0127 (0.003–0.04)	0.167 (0.084–0.292)	*	13.2	5.9	0.0123
	100	0.000125 (-0.03–0.093)	0.241 (0.11–0.32)	*	1925	597	0.2742
[4]Combination of three stressors†	0 (high food)	0.529 (-0.023–1.367)	–	–	–	–	–
	0 (low food)	0.0746 (0.036–0.243)	0.577 (0.244–0.584)	*	7.7	7.1	0.743
	1	0.0576 (0.035–0.165)	0.628 (0.182–0.899)	*	10.9	9.2	0.287
	32	0.0127 (0.003–0.04)	0.636 (0.176–0.802)	*	50.2	41.8	1.093
	100	0.000125 (-0.03–0.093)	0.664 (0.207–0.749)	*	5312	4229	58

Values are based on the data from day seven after pesticide exposure for 24 h.

[1]The observed LC_{50} was calculated using the mean survival of the three experimental repetitions.

[2]The 95% CI is based on three LC_{50} values calculated for separate rounds.

[3]The predicted LC_{50} was calculated using CA model and 95% CI is based on three values calculated for separate repetitions.

[4]Organisms exposed to esfenvalerate alone under high food conditions were considered as overall control (optimal laboratory condition). Synergism was considered significant if the 95% confidence intervals of observed and predicted LC_{50} did not overlap.

In addition, the increase in toxicant sensitivity due to the combined effect of the three stressors compared to the survival under exposure to esfenvalerate alone, was quantified as the shift in LC_{50} (LC_{50}/LC_{50}*). The LC_x shifts modelled by the SAM and observed in different experiments

were significantly correlated (LC_{50}: adjusted $R^2 = 0.83$, p-value = 0.006, $n = 6$; LC_{10}: adjusted $R^2 = 0.64$, p-value = 0.01, $n = 7$; Figure S4).

DISCUSSION

In the present study, we revealed synergistic effects of the pesticide mixture of esfenvalerate and prochloraz under different food conditions. The results of our study show that synergistic effects between prochloraz and esfenvalerate were dramatically increasing under low food conditions. Based on CA, the threshold for synergy (MDR > 2) for both pesticides decreased from 32 µg/L prochloraz under high food conditions to 1 µg/L prochloraz under low food. This threshold concentration of 1 µg/L can be realistically expected in surface waters[53, 63, 64] and is lower than that reported in previous studies without additional stress. For example, Nørgaard and Cedergreen[20] identified synergistic effects of alpha-cypermethrin and prochloraz on *D. magna* at higher concentrations of prochloraz (≥ 99±8 µg/L). Bjergager and co-authors[19] exposed *Daphnia magna* to different combinations of esfenvalerate with 90 µg/L prochloraz in microcosms and observed up to a 14 fold increase in mortality compared to the mortality in the CA predictions. In comparison, Bjergager et al.[32] observed synergy of prochloraz and alpha-cypermethrin at 9.794 ± 4.897 µg/L prochloraz towards the immobilisation of *D. magna* under laboratory conditions. The authors also observed that the threshold of synergistic effects decreased to 5.651 ± 1.507 µg/L from 48 h to 14 days after contamination. This threshold concentration is still higher than that in our experiment, where we detected a synergistic effect at 1 µg/L prochloraz under low food conditions. In addition, Bjergager et al.[32] exposed daphnids to fungicides during the whole experiment, while we applied a simultaneous peak exposure to both pesticides for only 24 h. The short exposure in our study might have led to a higher detected threshold concentration of synergistic effects than those in studies with longer or continuous exposure.[65] Hence, this is

345 the first study to reveal strong synergistic effects of pesticide mixtures at environmentally
346 realistic concentrations under low food conditions.

347 In terms of the pyrethroid esfenvalerate, we recorded strong effects on the survival of *D. magna.*
348 The LC_{50} of esfenvalerate at low food conditions decreased with increasing concentrations of
349 prochloraz. At the nominal concentration of prochloraz (≥1 µg/L), the LC_{50} of esfenvalerate was
350 0.058, which is more than one order of magnitude lower than the concentrations frequently
351 detected in field.[51] Further, at higher concentrations of prochloraz (100 µg/L), the LC_{50} of
352 esfenvalerate decreased up to 0.000125 µg/L that is two orders of magnitude lower than the LC_{50}
353 (0.012 µg/L) reported by Bjergager et al.[19] for *D. magna* exposed to esfenvalerate and
354 prochloraz. In the present study, this lower LC_{50} could be due to the additional environmental
355 stress of low food.

356 MDR for the CA reference model underestimated the LC_{50} of esfenvalerate up to 5312 fold at
357 100 µg/L prochloraz and low food conditions compared to that of the control conditions without
358 prochloraz and food stress (high food control). The identified MDRs were also much stronger
359 than those detected for comparable concentrations of prochloraz.[20, 32] Until now, the highest
360 synergism between two pesticides has been reported for *Ceriodaphnia dubia* exposed to
361 cypermethrin in the presence of piperonyl butoxide with a 137 fold increase in toxicity by
362 Wheelock et al.[66] The high level of synergism of the pesticide mixture in the present study was
363 due to the additional impact of food stress. The presence of food stress alone without prochloraz
364 already increased the toxicity of esfenvalerate by a factor of seven. Starving organisms may have
365 low energy reserves for physiological defence against stress and therefore show more sensitivity
366 to contaminants.[67] As a possible consequence, some studies previously reported that the toxicity
367 of metals and pesticides on invertebrates increased due to food limitation.[34, 35, 68-70]

In the present study, we found that CA and EA generally underestimated the combined effects of the pesticide mixture under low food conditions as well as the interaction of all three stressors (Table 1, Figure S3). These results are not surprising for synergistic mixtures, because CA and EA assume additive effects. In contrast, the SAM, which is designed to predict synergism between toxicants and environmental stress, predicted the combined effects of both pesticides and food stress better than EA and CA (Figure 2, S4; Table 1). In general, SAM is able to predict a certain range of synergism with the most robust predictions for strong synergistic effects. However, even the SAM underestimated the combined effect of the pesticides and food stress at the highest concentration of prochloraz (100 µg/L). The underlying mechanisms for this high synergism should be the subject of future investigations.

The interactions of biotic- and abiotic stress factors are much more complex under field conditions, modifying the sensitivity of communities and populations to contaminants.[71-73] Recently, Delnat et al.[38] investigated the effect of a common environmental stressor – daily temperature variation – on the combined toxicity of an organophosphate chlorpyrifos and a biopesticide *Bacillus thuringiensis* var towards vector mosquito *Culex pipiens*. A high variation in daily temperature changed the combined effect of both pesticides from additive to synergistic. Similarly, Gandar et al.[74] reported higher toxic effect of a pesticide mixture towards molecular response of a goldfish (*Carassius auratus*) at 32 °C as compared to 22 °C. Other investigations also have reported synergistic interactions among various environmental and toxicants (Holmstrup, et al.[33] and calculated by Liess et al.[12]), however, only single toxicant exposure was considered.

As a conclusion, mixtures of pesticides and environmental stressors may act in a strong synergistic manner on non-target organisms. Environmental risk assessments should consider

391 these combined effects in order to be protective for the environment. Additionally, approaches
392 such as the SAM can improve the prediction of the combined effects of synergistic toxicant
393 mixtures and environmental stress.

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400 **AUTHOR CONTRIBUTIONS**

401 Study design: NS, SK; conducting experiments: NS; data analysis and interpretation of results:
402 all; drafting of the manuscript: NS; revising manuscript: all.

403 **SUPPORTING INFORMATION**

404 Tables showing description of experimental setup, and concentrations of pesticides analysed
405 during different experimental rounds. Figures showing the survival of *Daphnia magna* exposed
406 to a common mixture of esfenvalerate and prochloraz under high and low food conditions,
407 interaction of multiple stress (esfenvalerate, prochloraz and food limitation), and relationship
408 between LC_x—shifts modeled by SAM and observed in different experiments (PDF).

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