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Co-formulants and adjuvants affect the acute aquatic and terrestrial toxicity of a cycloxydim herbicide formulation to European common frogs (*Rana temporaria*)

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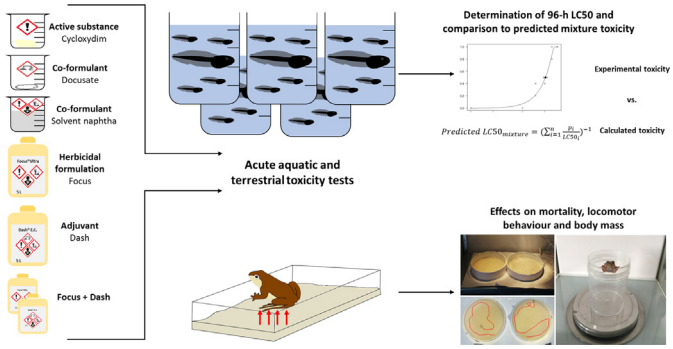
HIGHLIGHTS

- Formulation toxicity depends on co-formulants and the addition of adjuvants.
- The terrestrial exposure to these can lead to lethal and sublethal effects.
- Aquatic toxicity does not predict terrestrial toxicity to amphibians.
- Formulation toxicity needs special consideration in the amphibian risk assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

While pesticides are generally recognized as contributing to amphibian declines, there is a lack of knowledge about effects of co-formulants that are present in pesticide formulations and adjuvants which are mixed with these formulations. Since aquatic and terrestrial stages of amphibians can be exposed to these substances, adverse effects cannot be excluded. We investigated acute aquatic and terrestrial effects of the herbicide formulation Focus® Ultra, its active substance cycloxydim, its co-formulants solvent naphtha and docusate as well as the stabilizing adjuvant Dash® E.C. on larval and juvenile *Rana temporaria*. Aquatic toxicity was determined as 96-h LC50 values. Cycloxydim was the least toxic and solvent naphtha the most toxic substance of the formulation. The addition of Dash® E.C. increased the formulation toxicity substantially. Terrestrial toxicity was determined as lethal effects after a 48-h exposure to contaminated soil with 100% of the recommended field rate (FR) and as sublethal effects after the exposure to 10% of the recommended FR. The exposure to solvent naphtha and docusate at 100% FR led to mortalities of 42–100% probably due to their inhalation toxicity and dermal as well as eye irritation, respectively. Cycloxydim, Focus® Ultra and Dash® E.C. did not lead to any mortality. Sublethal effects on juvenile locomotor activity (i.e. moved distance) were observed for cycloxydim and the combined exposure of Focus® Ultra and Dash® E.C. Juvenile body masses declined significantly for all substances except for cycloxydim.

The present results show that aquatic sensitivity does not predict terrestrial sensitivity. It was shown that pesticide toxicity for amphibians can highly depend on the presence and amount of co-formulants and added adjuvants. Therefore, substances included in pesticide formulations which are known to be toxic by inhalation or harmful to eyes or skin should be specifically considered in the environmental risk assessment for amphibians.

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1. Introduction

Pesticide formulations are mixtures of one or more active substances and co-formulants (EU, 2009). While pesticides are generally recognized as contributing to amphibian declines (Stuart et al., 2004), there is a lack of knowledge of all chemicals present in pesticide formulations to which aquatic and terrestrial stages of amphibians are exposed. Following European Union terminology, co-formulants are substances or preparations which are used in pesticide formulations or adjuvants (EU, 2009). They are neither active substance nor synergists or safeners, which enhance the activity of the active substance or reduce phytotoxic effects of pesticides on certain plants, respectively. Co-formulants that have been proven to induce harmful effects on animal health or unacceptable effects on the environment shall not be accepted for inclusion in pesticide formulations (EU, 2009). Adjuvants are substances or preparations consisting of co-formulants and are placed on the market separately to be mixed with a pesticide formulation before application to enhance efficacy (EU, 2009). Thus, co-formulants and adjuvants are introduced in addition to active substances to the environment, potentially exerting adverse effects. However, the necessary investigations to disentangle toxicity of formulation components are complicated due to limited data access regarding proprietary information. Scientists of independent research institutions do not frequently have access to the composition of pesticide formulations and not all chemicals comprised in formulations are necessarily described in the safety data sheets.

Several studies indicate that co-formulants can be toxic themselves or enhance the toxicity of pesticide formulations to amphibians (e.g. Brühl et al., 2013; Hooser et al., 2012; Wagner et al., 2015). Amphibians might be highly sensitive to co-formulants and adjuvants because of their biphasic life-cycle and thus a combined aquatic and terrestrial exposure. Moreover, their permeable skin that enables water regulations (Wells, 2007) also facilitates the uptake of larger molecules such as pesticides through the dermal barrier (Kaufmann and Dohmen, 2016; Quaranta et al., 2009). Several studies observed increased dermal absorption of pesticide formulations and co-formulants in comparison to their active substances alone (Baynes and Riviere, 1998; Brand and Mueller, 2002; Reifenrath, 2007). Therefore, amphibians are especially threatened due to their high dermal uptake capacity.

In addition to enhanced absorption, increased toxicity of formulations or toxicity of co-formulants themselves were observed in several studies on lethal and sublethal level of amphibians. Increased mortality of aquatic stages after exposure to formulations of the insecticide permethrin (Boone, 2008) and the herbicide glyphosate (Howe et al., 2004) has been observed in comparison to the active substances alone. Lethal formulation effects of the fungicide pyraclostrobin and glyphosate have been observed for early terrestrial amphibian stages (Brühl et al., 2013; Relyea, 2005). Effects of formulations were also observed on a sublethal level such as effects on the aquatic development (Howe et al., 2004) and in vitro neurotoxic effects (Swann et al., 1996). These studies show that knowledge about the toxicity of active substances does not per se allow a prediction about the effect of pesticide formulations.

Another example for formulation effects was investigated by Wagner et al. (2015) who determined a significantly higher mortality and malformation rate of embryos and early-stage larvae of the African clawed frog (*Xenopus laevis*) after exposure to the herbicide formulation Focus® Ultra in comparison to exposure to the active substance cycloxydim alone. However, effects of this formulation, its active substance and its co-formulants on aquatic and terrestrial stages of European amphibian species remain unstudied. One of the co-formulants, solvent naphtha, has already been shown to potentially increase the toxicity of pyraclostrobin fungicide formulations on juvenile *Rana temporaria* at environmentally relevant concentrations (Brühl et al., 2013), thus suggesting the possibility for similar increased toxicity of Focus® Ultra compared to the active substance.

Currently, the risk assessment of pesticide effects on aquatic amphibian life stages is assumed to be covered by the use of data of surrogate species (Weltje et al., 2013) such as the standard temperate fish species rainbow trout (*Oncorhynchus mykiss*). Although dermal pesticide exposure of postmetamorphic juveniles and adults is highly likely (Lenhardt et al., 2015), this pathway is not yet considered in the environmental risk assessment of pesticides and ecotoxicological studies investigating the sensitivity of terrestrial amphibian stages to pesticides are considerably rare (e.g., Adams et al., 2021a; Brühl et al., 2013; Leeb et al., 2020; Relyea, 2005). This scarcity might be due to ethical restrictions regarding animal testing and the unavailability of standardized test guidelines. Moreover, it is not known whether the effects of active substances, co-formulants, adjuvants and pesticide formulations are comparable between aquatic and terrestrial amphibian stages.

Based on these uncertainties, the aims of the present study were (i) to investigate the aquatic toxicity differences between the herbicide formulation Focus® Ultra, its active substance cycloxydim, its two co-formulants solvent naphtha and docusate as well as the adjuvant Dash® E.C., that is part of the combination package Focus® Aktiv-Pack, (ii) to compare the experimentally determined formulation and package toxicity to predicted toxicity values based on a concentration addition model, and (iii) to determine lethal and sublethal effects of environmentally relevant concentrations of each substance to terrestrial juvenile amphibians. As the European common frog (*Rana temporaria*) is one of the most widespread amphibian species (Sillero et al., 2014) and it has been investigated in previous aquatic and terrestrial amphibian toxicity tests (e.g., Adams et al., 2021a; Adams and Brühl, 2020; Brühl et al., 2013) we used it as surrogate for European anuran species.

2. Material and methods

2.1. Test substances

The combination package Focus® Aktiv-Pack including the herbicidal pesticide formulation Focus® Ultra (hereafter Focus) and the adjuvant Dash® E.C. (hereafter Dash; both manufactured by BASF SE, Ludwigshafen, Germany) was purchased from a local distributor. Ingredients of Focus according to the safety data sheet are the active substance cycloxydim (10.8%) and the co-formulants docusate (dioctyl sodium sulfosuccinate, <5% according to BASF, 2018b, 2.4% w/w according to Wagner et al., 2017), and solvent naphtha (< 60% according to BASF, 2018b, 50% w/w according to Wagner et al., 2017, 47.2% w/w according to BVL, 2015). The formulation is applied once per season with a maximum field application rate of 5.0 L/ha in 150–300 L water/ha (BASF, 2018a; BVL, 2015). Fish LC50 values for the formulation, its co-formulants and adjuvant are given in Table 2.

The active substance cycloxydim was purchased as technical grade standard (<100% purity, Merck KGaA, Darmstadt, Germany). Cycloxydim is a cyclohexenone belonging to the HRAC-group A (Herbicide Resistance Action Committee, www.hracglobal.com), which inhibits acetyl-CoA carboxylases in sensitive plants leading to a decreased fatty acid synthesis in plastids and membrane formation. The co-formulant docusate was purchased by Merck KGaA as sodium salt (<100% purity, Darmstadt, Germany). Docusate is used in medicine as laxative and stool softening agent due to its characteristic as surfactant, allowing water to pass intestine membranes by decreasing their surface tension (Brunton et al., 2018). Solvent naphtha, also known as Solvesso, is a fraction of aromatic hydrocarbons that is generated during the distillation of high temperature coal tar or petroleum. It was purchased by DHC Solvent Chemie GmbH (Mülheim a. d. Ruhr, Germany) as Hydrosol A200 ND that consists of C10-aromatic hydrocarbons with a low content of naphthalene (<1%). The adjuvant Dash is used as an additive to stabilize the efficacy of herbicides and fungicides. For this, the adsorption and wetting behaviour on plant surfaces is optimized by decreasing the pH and surface tension of the spray solution. It is applied with a maximum application volume of 1.0 L/ha (BASF, 2020).

2.2. Animal collection and husbandry

In March 2019, we collected parts of egg clutches of *R. temporaria* from a forest pond in the Palatinate forest in Southwest Germany (49.25475 N, 7.96182 E, WSG84). The pond was expected to be uncontaminated because of its distance to any agricultural area. Water samples analysed in the course of another study (Adams et al., 2021b) revealed no pesticide residues. The eggs were kept in aerated aquaria (32 × 24 × 20 cm) filled with filtered tap water (0.2 μm Supor, Pall Corporation, Port Washington, USA) in a laboratory with a 16:8-h light:dark cycle at 21 ± 1 °C. Water renewal took place every other day. As soon as the tadpoles reached the free-swimming and feeding Gosner stage (GS) 25 (Gosner, 1960), they were fed ad libitum on a daily basis with commercially available rearing food (Sera Micron, Sera GmbH, Heinsberg, Germany) until metamorphosis. Juveniles were kept in terraria (32 × 24 × 20 cm) filled with moisturized forest soil, moss, leaves and a water supply. Every other day, juveniles were fed ad libitum with *Drosophila melanogaster* and *D. hydei* obtained from an in-house culture.

2.3. Acute aquatic toxicity tests

Aquatic acute toxicity tests were performed in a climate chamber (WK 19´/+15-35, Weiss Technik GmbH, Reiskirchen, Germany) with a 16:8-h light:dark cycle at 21 ± 1 °C. Tests were performed with the non-feeding hatchling stage GS20 as this tadpole stage was shown to be most sensitive (Adams and Brühl, 2020). For concentration range finding, 48-h tests with three treatment concentrations of each chemical and a control group with three replicates of one individual per species were performed to provide guidance on the final test concentrations. Final tests were performed as 96-h tests to allow comparison to the 96-h LC50 values for fish. For each chemical (cycloxydim, docusate, solvent naphtha, Focus, Dash, Focus + Dash), five treatment groups and one control group with five replicates of five individuals (150 randomly selected tadpoles per test) were examined in 1.7 L glass jars filled with 1 L test solution prepared with FETAX medium (Table 1). Next to a control group with FETAX medium, an additional ethanol control group was added because cycloxydim had to be pre-dissolved in 0.001% (*v*/v) ethanol. Solvent naphtha was tested as a water accommodated fraction, for which the test solutions were stirred for 24 h with a magnetic stirrer to break up the oil into small droplets that mix more easily with the FETAX medium before introducing the test individuals. A slow stirring was continued until test termination to ensure the presence of solvent naphtha in the water phase. pH-values of all test concentrations were measured using a WTW multiparameter MultiLine Multi 340i and a WTW SenTix pH-electrode (WTW, Weilheim, Germany). No feeding took place during the exposure period and dead tadpoles were removed every 24 h. To determine 96-h median lethal concentrations (LC50), mortalities were assessed after 96 h. After test termination, tadpoles were euthanized using a 0.1% buffered MS-222 solution.

2.4. Dermal soil exposure tests

Terrestrial soil exposure tests were performed in a laboratory at 21 ± 1 °C with a 16:8-h light:dark cycle. A study length of 48 h was chosen to prevent juveniles from dehydration. Freshly metamorphosed juveniles (seven to ten days old) were kept randomly and individually in clear, lockable plastic terrariums (22.5 × 16.5 × 7 cm, Braplast, Bergheim, Germany) filled with 250 g artificial soil that consisted of 70% industrial sand (particle diameter: 50–200 μm; Euroquartz, Dorsten, Deutschland), 20% kaolin clay (Carl Roth, Karlsruhe, Germany), and 10% sphagnum peat (sieved through 2 mm mesh; Florafort, Floragard, Oldenburg, Germany).

Lenhardt et al. (2015) determined that up to 17% of a reproducing population of the German amphibian species *Bombina bombina* can encounter a herbicide application during bare soil stage in maize. At the leaf development stage with 25% plant interception, about 12% of the *B. bombina* population coincided with herbicides on maize fields. Therefore, a worst-case scenario with 100% of the maximum recommended field rate (FR) was tested. To increase the environmental relevance and to consider pesticide exposure mitigation by interception of crops, a second test using 10% of the maximum recommended FR was performed. For logistical reasons, 10% FR and 100% FR treatments were not tested in parallel, but within the same week. 100% FR were applied with an application volume of 5.0 L Focus/ha and 1.0 L Dash/ha in 200 L water/ha. Since no exact amounts of solvent naphtha and docusate are provided in the safety data sheet of Focus, and due to the varying information according to Wagner et al. (2017) and BVL (2015), 60% and 50% of solvent naphtha (600 mg/L and 500 mg/L) as well as 5% and 2% of docusate (50 mg/L and 20 mg/L) were applied. Cycloxydim was only tested for the 10% FR tests because it was not soluble within the specified limit of solvent use for toxicity testing (0.01%, OECD, 2019) in the 100% FR tests. In the 10% FR tests, 0.5 L Focus/ha and 0.1 L Dash/ha were applied. To reduce vertebrate testing, only the highest concentration for solvent naphtha (6%, 60 mg/L) and docusate (0.5%, 5 mg/L) were tested in the 10% FR tests. Solvent naphtha spray solutions were mixed for 6 h with a magnetic stirrer before use. For each treatment group (control, cycloxydim, docusate, solvent naphtha, Focus, Dash, Focus + Dash) twelve individual replicates were tested. To keep vertebrate testing at a minimum, six control and six solvent controls (0.01% ethanol) were used in the 10% FR tests. Consequently, 84 and 72 individuals were used for the 100% FR and 10% FR tests, respectively.

Treatment and control solutions were prepared in filtered tap water. pH-values of all spray solutions were measured using a WTW multiparameter MultiLine Multi 340i and a WTW SenTix pH-electrode (WTW, Weilheim, Germany). Before application of treatment and control solutions, the soil of each terrarium was pre-wetted with tap water (40 mL/box) to prevent dehydration of juveniles during the test period. The solutions were applied by using a laboratory spray application system (Try Spray Cabinet, Schachtner Gerätetechnik, Ludwigsburg, Germany) with singular nozzles (TeeJet TP80). Juveniles were placed in the terraria 2 h after application for 48 h and were not fed during the exposure period. Mortality of juveniles was assessed after 48 h. To investigate sublethal effects in the 10% FR tests, juveniles were weighed prior to test initiation and after the exposure period to calculate the relative body mass decline. Moreover, their locomotor behaviour after 48 h was assessed. Juveniles were filmed individually for 10 min after an acclimatization period of 3 min in a circular arena (glass dish with a diameter of 20 cm and height of 5 cm) using eight camera modules (SC15–1, Kuman Ltd., Shenzhen, China) connected to single-board computers (Raspberry Pi 3 Model B, Raspberry Pi Foundation, Cambridge, United Kingdom). The video tracking software EthoVision XT (Noldus Information Technology, 2017) was used to analyse the total distance each juvenile moved. After test termination, juveniles were euthanized using a 0.1% buffered MS-222 solution.

Table 1
Nominal concentrations of test substances used in aquatic 96-h acute toxicity tests.

Substance	Test concentration [mg/L]					
	1	2	3	4	5	6
Cycloxydim	0	20.0	40.0	60.0	80.0	100
Docusate	0	60.0	62.0	64.0	66.0	68.0
Solvent naphtha	0	5.0	7.5	10.0	12.5	15.0
Focus	0	26.0	27.0	28.0	29.0	30.0
Dash	0	4.0	4.2	4.4	4.6	4.8
Focus + Dash	0	2.2	2.4	2.6	2.8	3.0

2.5. Statistical analyses

For statistical analyses the software R for Windows (R Core Team, 2020,Version 4.0.2) was used. For all statistical tests, the criterion for significance was set to $\alpha = 0.05$. The extension package "drc"

(Ritz and Streibig, 2005) was used to fit a dose-response model for each tested substance (SI Table A1). Candidate models were log-normal functions (LN.2, LN.3, LN.4), log-logistic functions (LL.2, LL.3u, LL.4, LL.5), and Weibull-functions (W1.2, W1.3, W1.4, W2.2, W2.3, W2.4). Models were selected based on Akaike information criterion (AIC). After the calculation of 96-h LC50 values for each component, they were compared via LC50 ratio test after Bonferroni correction as described by Wheeler et al. (2006). For this, asymptotic-based 95% confidence intervals were calculated using the method "delta" as interval settings. If 95% lower and upper confidence intervals of the calculated differences did not include zero, the differences were judged statistically significant (SI Table A2).

In a review of the European Commission (Kortenkamp et al., 2009), the use of a concentration addition (CA) model was proposed as most relevant concept of mixture toxicity. Therefore, the predicted aquatic mixture toxicities for the combination of cycloxydim, solvent naphtha and docusate as well as for the combination of Focus and Dash were calculated according to Eq. (1) and compared to the measured LC50 values of Focus and the combination of Focus and Dash, respectively.

$$Predicted\ LC50_{mixture} = \left(\sum_{i=1}^{n} \frac{p_i}{LC50_i}\right)^{-1} \quad (1)$$

where:

n = number of mixture components
i = index from 1 to n mixture components
p_i = the i^{th} component as a relative fraction of the mixture composition
$LC50_i$ = LC50 of component i

Afterwards, Eq. (2) was used to calculate the model deviation ratio (MDR) according to EFSA (2013). The MDR can be used to countercheck the calculated and measured mixture toxicity of the formulation as well as the combination of the formulation and the adjuvant and to determine if the components act more (i.e. synergistically) or less (i.e. antagonistically) than expected by the CA. The observed and calculated mixture toxicities are considered in agreement if the MDR is between 0.2 and 5. If the MDR is higher than 5, a synergistic mixture toxicity is indicated. An MDR below 0.2 indicates an antagonistic mixture toxicity.

$$MDR = \frac{LC50_{mixture,calculated}}{LC50_{mixture,measured}} \quad (2)$$

Body mass decline and distance moved data of the terrestrial juveniles were checked for normality and homogeneity of variances. Tukey's method was used to identify and remove outliers ranging above and below the 1.5 × IQR (Kannan Senthamarai et al., 2015). Differences in moved distance between treatment groups were compared using analysis of variance (ANOVA) with consecutive post-hoc Tukey's test. Non-parametric Kruskal-Wallis test with consecutive Dunn's test was applied for the body mass decline data. *p*-values were adjusted using the Benjamini-Hochberg method.

2.6. Animal welfare

The experiments were approved by the Federal Investigation Office of Rhineland-Palatinate (Landesuntersuchungsamt, Koblenz, Germany) to § 8a of the German law for animal welfare with the approval number 23 177-07/G18-20-009, and the Struktur- und Genehmigungsdirektion Süd (Neustadt an der Weinstraße, Germany, license number 42/553-254/455-18).

3. Results

3.1. Aquatic toxicity

Since no mortality was observed in either the control or ethanol groups, the results of the water control and solvent control were combined as recommended by Green and Wheeler (2013). Because the limit of solubility was reached for cycloxydim and no mortality was observed at the highest tested concentration of 100 mg/L, no dose-response model could be fitted to the data and no LC50 was obtained. The determined LC50 values of the other substances ranged from 2.44–62.4 mg/L (Table 2) and were significantly different from each other (SI Table A2): The active substance cycloxydim was least toxic (LC50 > 100 mg cycloxydim/L). The two co-formulants led to LC50 values of 62.4 mg docusate/L and 10.2 mg solvent naphtha/L. The exposure to the formulated product Focus resulted in a LC50 of 29.4 mg Focus/L which is six times higher than the LC50 of the adjuvant Dash (4.56 mg Dash/L) and twelve times higher than the LC50 of the combined exposure to Focus and Dash as an equitoxic mixture (2.44 mg mixture/L). Measured pH-values of the test solutions were 7.6 (control), 7.5 (cycloxydim), 7.5 (docusate), 7.4 (solvent naphtha), 7.5 (Focus), 7.2 (Dash), and 7.3 (Focus + Dash).

To allow predictive mixture toxicity calculations, the LC50 of cycloxydim was set to the highest tested concentration of 100 mg/L with a ratio of 10.8% of the formulations. Since the safety data sheet of Focus only provides imprecise content ratios, the calculations were conducted using ratios of 50% and 60% for solvent naphtha as well as 2% and 5% for docusate. Therefore, two difference predicted LC50 values were obtained for each co-formulant (Table 3). The different ratios of docusate did not influence the outcome, but solvent naphtha ratio changes did. The predicted LC50 values ranged from 16.5 (60% solvent naphtha, 2% and 5% docusate) to 19.9 mg/L (50% solvent naphtha, 2% and 5% docusate) which is 32–44% lower than the measured LC50 of Focus. The predicted mixture toxicity of Focus and Dash in an equitoxic mixture led to a predicted LC50 of 7.90 mg/L, which is three times higher than the measured LC50 value for the combined exposure of Focus and Dash. The calculated MDR values (Table 3) ranged from

Table 2
Measured aquatic 96-h LC50 values of investigated with 95% confidence intervals and standard error for *Rana temporaria* and literature 96-h LC50 values for fish. Because no mortality of 50% for cycloxydim was achieved, no dose-response model could be fitted to the respective data. The determined LC50 values were significantly different from each other.

Substance	LC50 [mg/L]	Lower 95% CI [mg/L]	Upper 95% CI [mg/L]	Standard error [mg/L]	Fish LC50 [mg/L]
Cycloxydim	>100	NA	NA	NA	>220[a]
Docusate	62.37	61.99	62.75	0.19	49[b]
Solvent naphtha	10.22	9.56	10.88	0.32	2.0[c]
Focus	29.40	27.58	31.22	0.89	20.4[d]
Dash	4.56	4.30	4.83	0.13	22[e]
Focus + Dash	2.44	2.43	2.45	0.01	NA

[a] Determined for *Oncorhynchus mykiss* (Agriculture and Environment Research Unit of the University of Hertfordshire, 2013).
[b] Determined for *Danio rerio* (Sigma-Aldrich, 2018).
[c] Determined as LL50 (lethal loading rate of water accommodated fractions; water accommodated fractions are media prepared via low energy mixing of a poorly soluble test material such as oil; Aurand and Coelho, 2005) for *O. mykiss* (DHC, 2018).
[d] Determined for *O. mykiss* (BASF, 2018a).
[e] Determined for *O. mykiss* (BASF, 2016).

Table 3
Predicted aquatic mixture LC50 values and calculated model deviation ratio (MDR) for the combination of the active substance cycloxydim and the co-formulants solvent naphtha and docusate with different content ratios as well as for the combination of Focus and Dash.

	SN:D ratio 60:5 and 60:2	SN:D ratio 50:5 and 50:2	Focus:Dash ratio 50:50
Predicted mixture LC50 [mg/L]	16.5	19.9	7.90
Measured mixture LC50 [mg/L]	29.4	29.4	2.44
MDR	0.56	0.68	3.24

SN = solvent naphtha, D = docusate.

0.56 to 3.24, thus indicating neither a synergistic nor an antagonistic effect.

3.2. Terrestrial mortality

After the exposure to 100% FR of the substances, no mortality was observed for the control, Focus, Dash and both in combination. The dermal exposure to 5% and 2% of docusate lead to 67% and 42% mortality. Solvent naphtha led to 100% mortality after 60% and 40% exposure. After the exposure to soil contaminated with 10% of the recommended FR, no juveniles died. pH-values of all spray solutions ranged from 7.1 (10% FR Dash) to 7.6 (control) except for 100% FR solvent naphtha (4.3), 100% FR Focus (6.2), 100% FR Dash (2.1), and 100% FR Focus and Dash (2.3).

3.3. Locomotor behaviour

Because the control and solvent control data did not differ significantly, the results were combined as recommended by Green and Wheeler (2013). The distance moved by juveniles in 10 min after the 48-h exposure to soil contaminated with 10% FR of the investigated components ranged from 88.9 cm (cycloxydim) to 167.8 cm (Focus, SI Table A3). The exposure induced statistically significant differences to the control ($df = 6$, $F = 5.99$, $p < 0.001$). Significant declines were observed for the active substance cycloxydim (67% reduction, $p < 0.01$) and the combined exposure of Focus and Dash (63% reduction, $p < 0.01$) in comparison to the control (Fig. 1, SI Table A4). Moreover, animals in the cycloxydim treatment group and the combined Focus and Dash treatment group moved significantly less than the individuals of the Focus treatment group (89% and 84% reduction, respectively, $p < 0.001$).

3.4. Body mass decline

The 48-h dermal exposure to soil contaminated with 10% of the FR of the tested substances led to mean mass declines ranging from 8.8% (control) to 15.1% (Dash, SI Table A5). The Kruskal-Wallis test revealed statistically significant body mass declines ($X^2 = 32.74$, $df = 6$, $p < 0.001$). Exposure to the different substances induced significantly increased body mass declines in juveniles for docusate ($p < 0.01$), solvent naphtha ($p < 0.05$), Focus ($p < 0.001$), Dash ($p < 0.001$) and the combination of Focus and Dash ($p < 0.05$) when compared to the control group (Fig. 2, SI Table A6).

4. Discussion

4.1. Acute aquatic toxicity

Due to the low acute fish toxicity (Table 2), the active substance was expected to have a low toxicity to *R. temporaria* tadpoles which was confirmed by a LC50 > 100 mg/L. As indicated by the fish LC50, docusate did not lead to high aquatic toxicity in tadpoles. Solvent naphtha was expected to show high toxicity due to the low fish LC50 and its GHS (Globally Harmonized System of Classification and Labelling of Chemicals) classification as harmful for aquatic organisms, which was confirmed by the lowest determined LC50 value of the formulation components. Due to the higher fish LC50 values of Focus and Dash, a moderate toxicity was expected for both substances. This assumption was confirmed for Focus as the exposure of tadpoles to the formulation led to a 1.4-times higher LC50 than for fish. However, the exposure to Dash led to a LC50 of 4.56 mg/L, which is five times lower than the fish LC50. Because no LC50 value of the combination of Focus and Dash is given in the safety data sheet, a comparison to amphibian sensitivity is not possible.

Wagner et al. (2015) determined a four-times lower toxicity of cycloxydim (96-h LC50 of 4.0 mg/L) than of Focus (96-h LC50 of 0.9 mg/L) to early larval stages of *X. laevis*. These findings indicate that *X. laevis* tadpoles were 33-times and 25-times more sensitive than *R. temporaria* tadpoles towards the formulation and the active substance, respectively. Thus, the results confirm the lower toxicity of the active substance in comparison to the formulation but also emphasize

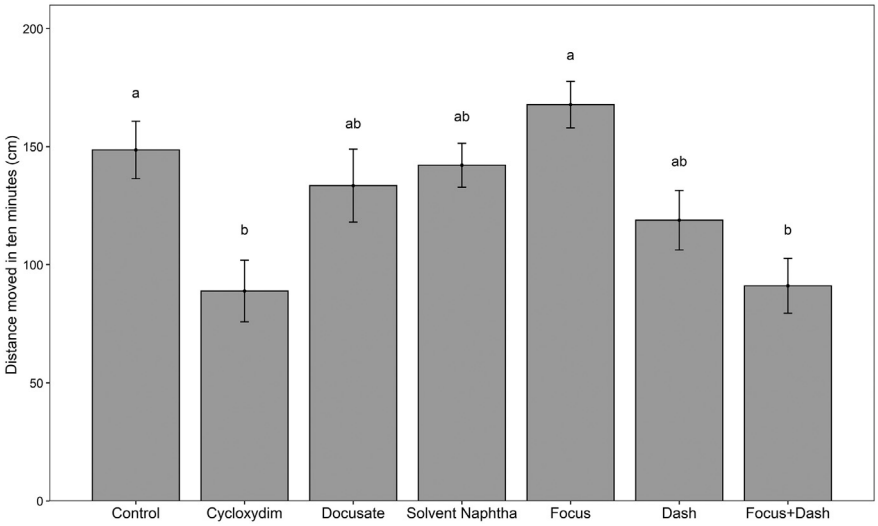

Fig. 1. Total mean moved distance ± standard error of juvenile *Rana temporaria* after 48-h exposure to 10% of the field rate of the investigated substances. Letters represent statistically significant differences ($p < 0.05$).

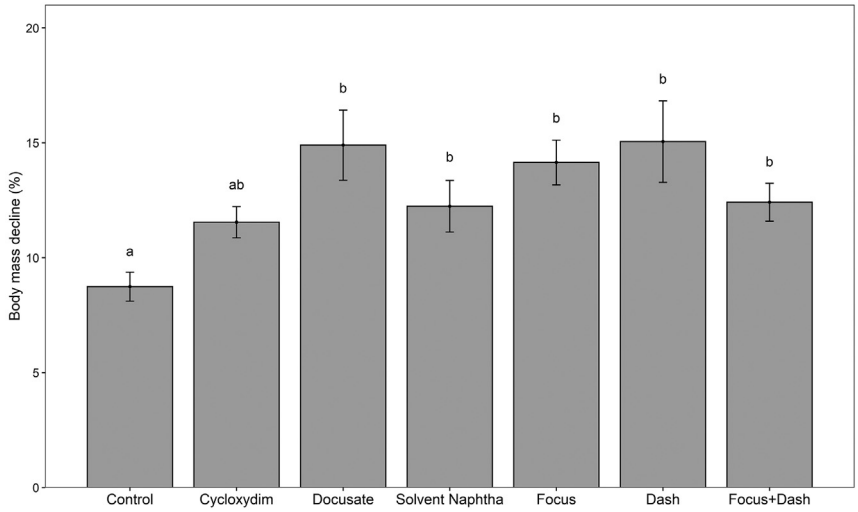

Fig. 2. Relative mean body mass decline ± standard error of juvenile *Rana temporaria* after 48-h exposure to 10% of the field rate of the investigated substances. Letters represent statistically significant differences ($p < 0.05$).

the consideration of species sensitivity differences. As the pH-values of the aquatic test solutions were all in the range of 7.2–7.6, they are not the reason for different observed toxicities, but rather the systemic toxicity of the tested compounds.

4.2. Acute aquatic environmental risk assessment considerations

Except for Dash, all determined amphibian LC50 values were lower than the fish LC50 values. Despite the five times lower LC50 for Dash, for acute environmental risk assessment purposes, the fish LC50 values would cover the determined amphibian sensitivity after the application of the recommended uncertainty factor of 100 for acute aquatic toxicity (EFSA, 2013).

Depending on the co-formulant contents, the predicted aquatic mixture toxicity of the formulation components was 1.8-times and 1.5-times lower than the measured LC50 for Focus. Thus, the predicted Focus LC50 value would cover the aquatic amphibian sensitivity. However, it needs to be considered that the content of solvent naphtha clearly affects the toxicity of the formulation because of its low measured LC50 but also because of its decreasing effect on the predicted, calculated LC50. Therefore, co-formulants exhibiting high toxicities themselves should be considered with a priority in the risk assessment of pesticide formulations. These results show that the calculation of the predicted mixture toxicity might be a good tool to assess formulation toxicity to aquatic stages of amphibians. However, the predicted aquatic mixture LC50 of the combination of Focus and Dash was three times higher than the measured LC50, thus underestimating the toxicity of combined exposure of the formulation and the adjuvant. This underestimation might be due to the additional toxicity of Dash that consists of a non-ionic surfactant (ethoxylated alcohol), nonfatty acid methyl ester, and oleic acid (BASF, 2016). Lewis (1992) investigated toxicity trends for mixtures containing surfactants and pesticides. They found that it is difficult to generalize or predict synergistic, antagonistic or additive toxicities of these mixtures. The interaction of surfactants and other chemicals has been found to affect different functions and multiple cellular response targets, which generate a complex cascade of events in organisms that cannot be easily summarized (Wei et al., 2009). Therefore, an uncertainty factor should be applied in the environmental risk assessment of formulations and adjuvants to ensure no unacceptable risk to amphibians.

In the European risk assessment a predicted environmental concentration (PEC) in surface water of 4.6 mg Focus/L was determined for spray drift (BVL, 2015). Moreover, the PEC considered for entry via runoff is 0.22 μg cycloxydim/L, and for drainage 0.51 μg cycloxydim/L or 0.17 μg cycloxydim/L in autumn/winter or spring/summer, respectively. As these environmentally relevant concentrations are considerably lower than the determined LC50 values, no acute amphibian toxicity is expected for the active substance and the formulation in the environment.

4.3. Terrestrial toxicity

The mortality results after exposure to soil contaminated with 100% FR show that the sole exposure to the co-formulants docusate and solvent naphtha is most toxic as it led to 42–100% mortality whereas no mortality was observed in other treatment groups. Because the juvenile frogs were not directly oversprayed and test solutions were likely buffered by the artificial soil, the different pH values of the test solutions probably did not have an acute effect on amphibian survival. A more probable reason for lethality might be an effect on the juveniles' skin. As docusate acts as a surfactant, it decreases the surface tension and thus the barrier of membranes, allowing penetration of water and therefore potentially also of the test solution into the body (Brunton et al., 2018). This membrane modification might have led to docusates GHS classification as “causes serious eye damage” and “causes skin irritation”. The high acute toxicity of solvent naphtha might be caused by its GHS classification as “may be fatal if swallowed and enters airways”. On the one hand, inhalation toxicity is especially relevant for amphibians because of their high respiration rate based on their metabolic rate which is increased due to their poikilothermy (Halsey and White, 2010). On the other hand, the entire skin of terrestrial amphibian stages is a respiratory organ and for small individuals with a high surface-to-volume ratio, skin breathing covers an essential part of respiration (up to 30% of O_2 uptake and 70% CO_2 elimination; Burggren and Moallf, 1984). Thus, adverse effects on lung as well as dermal respiration might be the reason for the high toxicity of solvent naphtha. Interestingly, no mortality was observed after exposure to the formulation including docusate and solvent naphtha. This might either indicate an interaction of compounds in the formulation or altered for example volatility or surfactant properties of the co-formulants in the formulation. Future research should verify concentrations of the co-formulants in soil to allow further interpretation of this finding.

Increased toxicity with increasing solvent naphtha content in pesticide formulations was also indicated by Brühl et al. (2013). They determined 100% mortality of juvenile *R. temporaria* after direct overspray with a fungicide formulation containing the active substance

pyraclostrobin and 67% solvent naphtha. On the contrary, only 20% of the juveniles died after overspray with a pyraclostrobin formulation containing <25% solvent naphtha. These results confirm our findings that solvent naphtha is highly toxic for terrestrial stages of amphibians.

Sublethal effects of formulation co-formulants and adjuvants after the exposure to 10% FR were different than indicated by the determined lethal effects after 100% FR exposure. Moved distance was affected by the exposure to cycloxydim and the combination of Focus and Dash. In this context, the non-significant gradual decrease in moved distance by juveniles exposed to Focus, Dash and the combination of Focus and Dash became apparent, indicating an enhanced sublethal toxicity by the addition of the adjuvant Dash. Lower activity might play an important role due to the juveniles´ key role in the dispersal of amphibians (Cushman, 2006). Thus, the reduced activity might further contribute to local amphibian declines. Moreover, it could lead to an impaired predation behaviour as it was observed by Adams et al. (2021a) for juvenile *R. temporaria* after exposure to the fungicide folpet. Such an effect might lead to a decreased survival that further impairs overall population survival chances. In general, most studies investigating amphibian behavioural responses to pesticides focus on larval amphibian stages (Sievers et al., 2019) emphasizing the underrepresentation of terrestrial amphibian stages in ecotoxicological studies (Brühl et al., 2011). Most studies investigating pesticide effects on terrestrial stages did not find behavioural alterations (see review of Brühl et al., 2011) that might be due to a lack of standardized methods and endpoints to analyse such responses (Leeb et al., 2020).

Statistically significant body mass declines were observed after the exposure of every test solution except for cycloxydim. A smaller body mass might represent an increased risk of predation and low survivorship at maturity (Berven and Gill, 1983; Smith, 1987). Adams et al. (2021a) determined a non-significant overall body mass decline of *R. temporaria* juveniles after 48-h exposure to the fungicide folpet. In contrast, Webber et al. (2010) did not find an effect on the growth of juvenile Great Plains toads (*Anaxyrus cognatus*) after exposure to the insecticide carbaryl. The herbicide atrazine was shown to affect the body mass of Gray tree frogs (*Hyla versicolor*) at metamorphosis (Diana et al., 2000). These results show that effects on body mass also depend on the pesticide that amphibians are exposed to.

The observed declines might have been developed as hydration loss due to irritated or damaged skin caused by the co-formulants. With respect to the great importance of water for amphibians, this hydration loss might indicate stress regarding the osmoregulation and thus affecting vital functions of the juveniles (Shoemaker and Nagy, 1977). Cusaac et al. (2017) did not find an adverse effect of dehydration on the mortality of terrestrial stages of two North American toad species after exposure to a pyraclostrobin fungicide formulation. In contrast, they determined a reduced mortality in comparison to hydrated toads. This difference might have been attributed to behavioural and physiological adaptations to dehydration in toads (Cusaac et al., 2017). They observed that juvenile toads kept their ventral seat patch elevated to avoid water loss. Furthermore, they observed reduced activity of the dehydrated juveniles, who frequently aggregated in the corners of the aquaria, a behaviour that is consistent with conserving water. A hydrated stratum corneum in the membrane of the investigated juveniles may have been substantially more permeable than dehydrated skin (Trommer and Neubert, 2006). This might be an explanation for the toxicity differences between the test compounds. Moreover, as solvent naphtha and docusate directly affect the amphibian membrane also in the pesticide formulation, cycloxydim alone might have been excluded from any body burden in the single compound exposure.

5. Conclusions

The present study showed that pesticide toxicity in amphibians can highly depend on the presence and amount of co-formulants in formulations and added adjuvants. Because detailed information on formulation composition is difficult to obtain, more information on the types of co-formulants and adjuvants included in or added to pesticide formulations and their fate in the environment is required. The knowledge about the presence of co-formulants and adjuvants would greatly aid in assessing the exposure and potential toxicity for amphibians and other non-target organisms. As adverse effects on the skin of terrestrial amphibian stages might not be identified in aquatic tests, we recommend including the toxicity of co-formulants and adjuvants which are known to be harmful to eye or skin or toxic by inhalation in the toxicity evaluation of pesticides for terrestrial amphibian toxicity. Ultimately, the use of substances such as solvent naphtha in pesticide formulations should be avoided to reduce adverse effects on amphibian populations.

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Data availability statement

Data are available by contacting E. Adams (adams@uni-landau.de).

CRediT authorship contribution statement

Elena Adams: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Verena Gerstle:** Investigation, Writing – review & editing. **Tobias Schmitt:** Investigation, Writing – review & editing. **Carsten A. Brühl:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147865.

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