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Fungicide Exposure Induces Sensitivity Differences in Aquatic Life Stages of European Common Frogs (*Rana temporaria*)

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ABSTRACT.—**Pesticides have been identified as one of the major reasons for the worldwide decline of amphibian populations. In terms of aquatic amphibian exposure, most ecotoxicological studies concentrate on testing tadpoles in Gosner stage (GS) 25. To examine the representativeness of GS25, we exposed five aquatic stages of European common frogs (*Rana temporaria*) to the viticultural fungicide folpet (Folpan® 80 WDG) in a dose–response setup. The 96-h mortality data identified the hatchling stage GS20 as the most sensitive aquatic stage (LC_{50} of GS15: 2.68 mg/L, GS20: 1.01 mg/L, GS25: 1.22 mg/L, GS36: 2.11 mg/L, GS42: > 2.6 mg/L). Because these results suggest that the commonly used stage is not sufficient for a protective environmental risk assessment of pesticides, comprehensive investigations are needed to provide adequate regulatory guidance.**

Amphibian populations are declining worldwide at alarming rates; approximately one third of all amphibian species are considered threatened or in decline (Stuart et al., 2004). Among the large variety of contributing stressors, chemical pollutants like pesticides have been identified as a major factor for the global amphibian decline (Collins and Storfer, 2003). These pesticides can be present in breeding ponds such as rain retention ponds, drainage ditches, and other small temporary ponds in the agricultural landscape (Knutson et al., 2004) but also in natural habitats in remote areas (Davidson, 2004; Smalling et al., 2013). Therefore, aquatic amphibian life stages can be exposed to pesticides entering their breeding ponds via spray drift (Crossland et al., 1982), runoff (Edwards et al., 1980), drainages (Brown and van Beinum, 2009), or long-range transport (LeNoir et al., 1999).

Even though numerous studies have demonstrated lethal and sublethal effects of aquatic pesticide exposure (e.g., Relyea, 2005; Johansson et al., 2006; Relyea, 2009), amphibians remain underrepresented in ecotoxicology and environmental risk assessment (ERA) procedures (Sparling et al., 2010). To date, ecotoxicological data of surrogate species like fish and aquatic invertebrates are used in ERA to cover the sensitivity of aquatic stages of amphibians to pesticides, neglecting unique characteristics of amphibians like their special skin structure and the hormonal pathways during metamorphosis. Most studies that compare amphibian and fish sensitivities use free-swimming, feeding tadpoles with internal gills in Gosner stage (GS; Gosner, 1960) 25. However, several studies already identified sensitivity differences in aquatic developmental stages of amphibians when exposed to insecticides and herbicides (Greulich and Pflugmacher, 2003; Biga and Blaustein, 2013). This indicates that GS25 may not adequately represent sensitivity across all aquatic amphibian stages.

In contrast to the frequent use of herbicides and insecticides in ecotoxicological amphibian studies, the exposure to and effects of fungicides have received less attention, although they can be found throughout the entire growing season because of their constant prophylactic application (Zubrod et al., 2019). Several studies identified severe lethal and sublethal effects of fungicides on amphibians at field-relevant concentrations. One of the most investigated fungicide groups in aquatic ecotoxicology are strobilurins, which disrupt the metabolism of fungi by inhibiting electron transfer in mitochondria. The strobilurin pyraclostrobin induced 100% mortality at one-tenth of the label rate for corn (Belden et al., 2010) and effects on the development of *Bufo cognatus* tadpoles (Hartman et al., 2014). Li et al. (2016) examined four strobilurins in the frog embryo teratogenesis assay-*Xenopus* (FETAX) and identified lethal and teratogenic effects as well as malformations at actual exposure concentrations. Hence, strobilurins have been demonstrated as having high toxicity to the aquatic stages of amphibians. Several studies have been performed with the chloronitrile fungicide chlorothalonil and tropical amphibian species in the egg or larval stage. Chlorothalonil is a widely used nonsystemic broad-spectrum fungicide that prevents spore germination and zoospore motility. Méndez et al. (2016) and Yu et al. (2013) identified sublethal effects, that is, effects on growth, development, and biochemical biomarker after the exposure to environmentally relevant concentrations of chlorothalonil. In a study of Ghose et al. (2014) chlorothalonil was found to be very highly toxic at environmentally relevant concentrations resulting in a tadpole mortality of up to 100%. Based on toxicity assays and a meta-analysis of literature data, they found that fungicides are generally more toxic to amphibians than herbicides. This indicates the urgent need for more information about fungicide toxicity.

Despite these investigations at environmentally relevant concentrations, the effects of fungicides on tadpoles have not been analyzed in a dose–response relationship with different stages. One of the most fungicide-intensive crops in central Europe is viticulture. For instance, up to 10 protective applications are performed per season to cope with fungal diseases in Germany (Roßberg, 2009). Because multiple fungicide applications coincide with multiple states of larval amphibian development in spring and summer, fungicides may cause adverse effects on aquatic amphibian life stages (Mann et al., 2009).

The objective of the present study was to investigate if GS25 is the most susceptible aquatic life stage to fungicide exposure. Because the European common frog *Rana temporaria* is one of the most widespread amphibian species and can be found in a wide range of habitats in central Europe (Gasc, 2004; Kuzmin et al., 2015), we used it as surrogate for European anuran species.

We performed the tests with a formulation of the most common German viticultural fungicide folpet (i.e., Folpan® 80

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TABLE 1. Nominal exposure concentrations (c_1–c_5) for respective Gosner stages (GS15–GS42) and initiation dates of the 96-h acute toxicity tests.

GS	Initiation date	c_1 (mg/L)	c_2 (mg/L)	c_3 (mg/L)	c_4 (mg/L)	c_5 (mg/L)	c_6 (mg/L)
15	22 Apr 2016	0	1.0	3.0	5.0	7.0	9.0
20	27 Apr 2016	0	0.7	0.9	1.1	1.3	1.5
25	30 Apr 2016	0	0.8	1.0	1.2	1.4	1.6
36	13 Jun 2016	0	1.6	1.8	2.0	2.2	2.4
42	26 Jun 2016	0	1.8	2.0	2.2	2.4	2.6

WDG). Folpet is an organochlorine phthalimide that is used as a protective, broad-spectrum fungicide to control fungal pathogens by inhibiting cell division of many microorganisms. We used the formulation instead of the technical grade active ingredient. In the European Union, the authorization of both active ingredients as well as formulations are required. The active ingredients need to be authorized by the European Food Safety Authority before the pesticide formulation can be authorized at national level. However, the use of the formulation instead of the active ingredient simulates a more realistic exposure scenario. Moreover, previous studies have demonstrated that additional ingredients (e.g., surfactants) in pesticide formulations may influence toxicity in amphibians (Puglis and Boone, 2011; Brühl et al., 2013).

MATERIALS AND METHODS

To analyze the sensitivity of different aquatic stages, we determined the 96-h median lethal concentrations (LC_{50}) of five stages (GS15, GS20, GS25, GS36, GS42) in a semistatic dose–response setup. We selected these stages because they represent different developmental levels with major morphological changes. GS15 is a late embryonic stage with a protective eggshell, which was left around the embryo during the tests. GS20 is the first hatchling stage with an external gill circulation, whereas the internal gill circulation starts in the tadpole stage GS25. In GS36 the tadpole hindlimbs are developed, and in the metamorph stage GS42, forelimbs emerge. Because of the external gills, the small size, metabolic and physiological differences, as well as the lack of a protective eggshell, we hypothesized that newly hatched tadpoles (GS20) are more sensitive than embryonic and later developmental stages.

In March 2016 we collected parts of three freshly laid (up to 24 h old) *R. temporaria* egg clutches from a temporary wetland in the Bienwald forest (49°01′19"N, 8°10′46"E, WGS 84) in the southeast of Germany. The sampled temporary wetland is located in a landscape conservation area in the Rhine river plain. This location was chosen to minimize the likelihood of previous pesticide exposure. We mixed the egg clutches and randomly assigned them to aerated 30-L aquaria (32 × 24 × 20 cm) filled with filtered tap water (0.2 µm Supor, Pall Corporation, Port Washington, New York, USA). We conducted housing and experiments in a laboratory with a 16/8-h light/dark cycle at 19 ± 1°C. Water renewal took place three times per week.

We assessed embryo and tadpole stages according to Gosner (Gosner, 1960) using a binocular (Leica KL300 LED, Wetzlar, Germany). As soon as the larvae reached the free-swimming GS25, we fed them ad libitum on a daily basis with commercially available rearing food (Sera® Micron, Sera GmbH, Heinsberg, Germany). No feeding took place during the experiments. Once the individuals reached the desired test stage (Table 1), we randomly caught the respective number of individuals with a dip net and used them for the experiments.

We prepared all test solutions and control media using filtered tap water. Because we did not know the toxicity of the test substance towards aquatic amphibian stages, range-finding tests were performed for each stage to provide guidance on the concentrations to be tested in the final tests. For these 48-h static range-finding tests, we tested three concentrations of the formulation (Appendix Table A1) with three replicates of one individual. Thereby, we modified the concentrations across Gosner stages based on the endpoint concentrations for fish and daphnia (0.22 mg/L and 0.68 mg/L, respectively; Adama, 2016) as well as the mortality results of the preceding test stage and our expectations of sensitivity differences to define the subsequent test concentrations. In addition to the test solutions, a control was set up for each pretest. Mortality was recorded after every 24 hours and the final concentrations were concluded.

For the final tests, 10 replicates of one individual of GS15, 20, 25, 36, and 42 were exposed to Folpan® 80 WDG (Table 1). We conducted the tests of GS15, 20, 25, and 36 in 250-mL glass beakers and GS42 individuals in plastic aquaria (7 × 16 × 22 cm, 3 L, BraPlast, Bergheim, Germany). We arranged the aquaria in a sloped position to allow for dryland refuge preventing drowning of metamorphing tadpoles. Because folpet does not heavily adsorb to plastic ($K_{OC} = 304$; Adama, 2016) it can be assumed that the aquatic exposure is similar in both test systems. All test systems were filled with 200 mL of the respective treatment solutions. Because of the fast degradability of folpet in water ($t_{1/2}$: 2.6 h–2 d, EFSA, 2009), we renewed the treatment solutions completely after 48 h to keep concentrations as stable as possible. After 96 h, we terminated the experiments and euthanized all surviving larvae with a 0.1% buffered MS-222 solution.

For statistical analyses and the generation of figures we used the software R for Windows (R Development Core Team, 2008, Version 3.3.2) and the extension package drc (Ritz et al., 2015). Based on the obtained binomial mortality data, we determined 96-h LC_{50} values by fitting dose–response models for each of the investigated Gosner stages. We selected the best-fitting models based on Akaike's information criterion and visual judgment (Appendix Fig. A1 and Tables A2–A3). Moreover, we assessed the LC_{50} values for statistically significant differences among Gosner stages via LC_{50} ratio test after Bonferroni correction (Wheeler et al., 2006, Ritz et al., 2015). Therefore, the 95% confidence intervals were calculated with the drc package in R. They were calculated as asymptotic-based confidence intervals using the method "delta" as interval settings. If 95% lower and upper confidence intervals of the difference between two LC_{50} values did not include zero in the ratio test, the difference was judged statistically significant (Appendix Table A4).

RESULTS

Even though we observed mortality over 96 h, highest mortality rates occurred within 24 h after test initiation; that is, on average 84% of the final mortality took place within this period (Appendix Tables A5–A9). The determined LC_{50} values ranged from 1.01 mg/L up to 2.68 mg/L and were significantly different from each other after executing a confidence interval ratio test (Fig. 1). GS20 was the most sensitive life stage, with a 17.2% lower LC_{50} than the commonly studied GS25 (1.01 mg/L

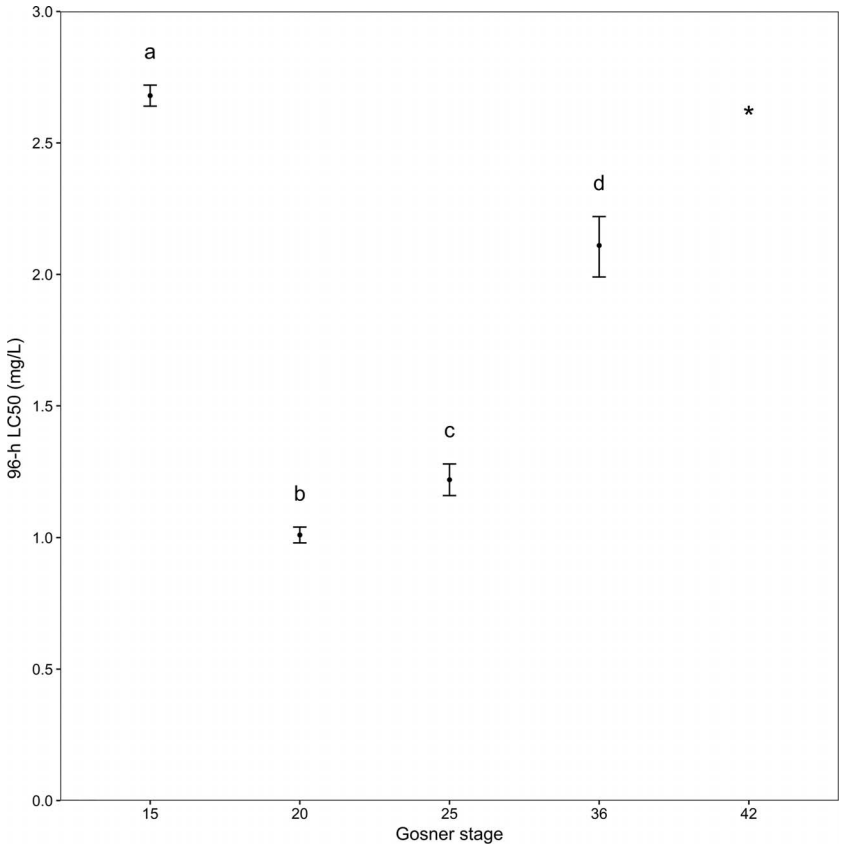

FIG. 1. Acute sensitivity of aquatic Gosner stages of *Rana temporaria*. 96-h LC_{50} values ± 95% confidence interval (CI) are represented. Significant differences among LC_{50} values are indicated by different letters. The asterisk indicates the highest tested concentration of GS42 because no mortality of 50% was achieved, indicating a LC_{50} higher than this value.

and 1.22 mg/L, respectively). Moreover, the GS20 LC_{50} value was 62% lower than the least sensitive embryonic GS15 (2.68 mg/L), the tadpole stage GS36 (2.11 mg/L), and the metamorph stage GS42. For the metamorph stage GS42 no mortality of 50% was achieved, but only 30% for the highest exposure concentration, and therefore no dose–response model could be fitted to the data. Consequently, we defined the LC_{50} as > 2.6 mg/L, which represents the highest tested concentration for GS42.

DISCUSSION

The significant sensitivity differences between the tested developmental stages detected in this study highlight the crucial role of the developmental stage when testing pesticide sensitivity of amphibians. In contrast to other aquatic organisms like fish, limited data on the metabolism of pesticides are available for amphibians. Pesticides can be taken up by aquatic amphibian stages via passive diffusion through gills, skin, or via the dietary route, i.e., the gastrointestinal tract. Because no feeding took place during the experiment, the uptake via the gastrointestinal tract can be excluded. After uptake via the gills or the skin, pesticides are either adsorbed to a lipid phase or metabolized by enzymes and finally excreted as molecules or metabolites (Katagi and Ose, 2014). Because of the external gill circulation of GS20, a higher fungicide amount may be internalized than via internal gills, which are present in GS25. Moreover, tadpoles in GS25, 36, and 42 exhibit an enhanced metabolism compared to GS20, which goes along with a faster detoxification of xenobiotics, for example, via the glutathione-S-transferase pathway (Katagi and Ose, 2014). Furthermore, sensitivity differences between the larval stages can be explained by the increasing body size, which can be negatively correlated with pesticide sensitivity (Hall and Swineford, 1980; Howe et al., 1998) and the change in skin structure during the larval and premetamorphic development (Yoshizato, 1990).

The finding of the late embryonic GS15 reacting least sensitive is in line with our assumptions that the eggshell left around the embryos during the test could act as a protective barrier (Muñoz et al., 2014; Mesléard et al., 2016). However, other studies have shown that eggs were harmed significantly by the exposure of, for example, pyrethroid insecticides, indicating insufficient protection by the jelly coat surrounding the embryo (Berrill et al., 1993; Greulich and Pflugmacher, 2003). Baier et al. (2016) found similar results for the sensitivity of *Bufo bufo* to the herbicide glyphosate, because embryos showed a higher sensitivity than tadpoles. Thus, the protectivity may depend on pesticide class and test species, and it cannot be concluded in general that jelly coat–exhibiting egg stages are less sensitive than larval stages. Because ecotoxicological studies comparing sensitivity differences of developmental stages are too few, and many other factors like pesticide class and test species (Bridges and Semlitsch, 2000) play a crucial role for the toxicity of pesticides, no general conclusion can be drawn about stage-specific sensitivities. However, our results underline the need for more data on stage-specific sensitivity.

For ERA purposes the use of GS25 sensitivity data for the comparison to fish data would not have been appropriate for the presented study because GS20 was significantly more

sensitive than GS25. Thus, the risk for amphibians would have been underestimated, which confirms the necessity to find conformity in the stage to be tested in ecotoxicological studies. Although the 96-h LC_{50} value for fish (0.22 mg/L; Adama, 2016) would cover all stages tested in this study, it cannot be generally stated that fish data can be used to cover amphibian sensitivity because of their unique permeable membrane, their dual aquatic–terrestrial life cycle, and the special hormonal pathways during metamorphosis. Therefore, further information as well as a specification of the appropriate test species and stage is needed to establish protective uncertainty factors, which can be used in ERA. Beyond that, an appropriate ERA needs to cover amphibian specific sublethal pesticide effects under chronic, low-concentration exposure, especially on metamorphosis, which cannot be surrogated by other test organisms. Because no general conclusion for all pesticide classes can be drawn from the present study, we highly recommend performing more aquatic amphibian tests with several developmental stages. This research would provide valuable data for a better risk assessment of effects related to pesticide classes and test species.

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APPENDIX

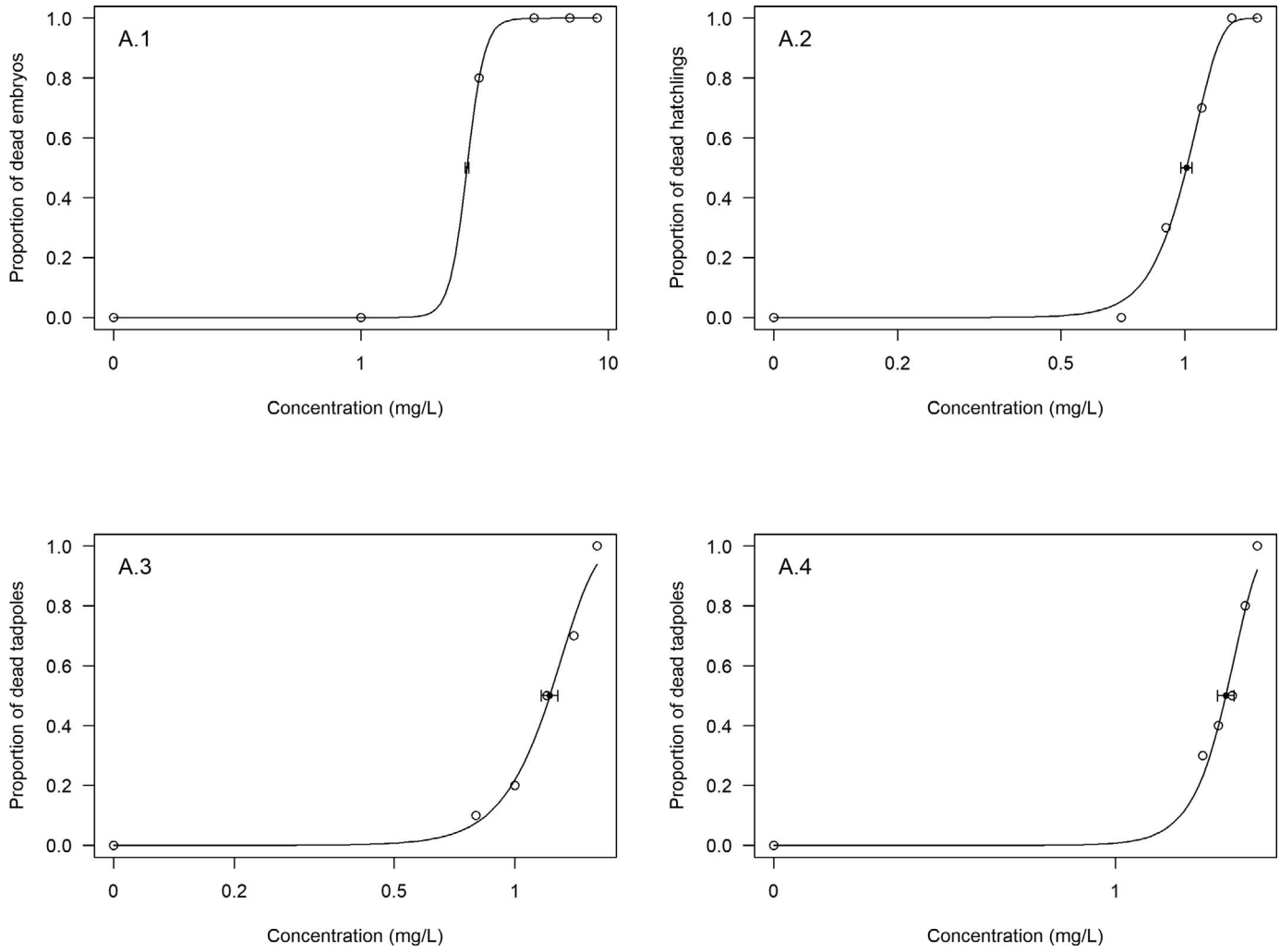

FIG. A1. Dose–response curves underlying the 96-h LC_{50} calculations for different developmental stages. Filled circles = mean mortality with 95% confidence interval. (A.1) GS15; (A.2.) GS20; (A.3.) GS25; (A.4.) GS36.

TABLE A1. Nominal exposure concentrations (c_1–c_3) for respective Gosner stages (GS15–GS42) of the 48-h range-finding tests.

GS	c_1 [mg/L]	c_2 [mg/L]	c_3 [mg/L]	c_4 [mg/L]
15	0	0.1	1.0	10
20	0	0.1	1.0	2.0
25	0	0.1	1.0	2.0
36	0	1.0	2.0	2.5
42	0	1.5	2.0	2.5

TABLE A2. LC_{50} values with 95% confidence intervals of 96-h acute toxicity tests of *Rana temporaria* at five different Gosner stages. Because no mortality of 50% for GS42 was achieved, no dose–response model could be fitted to the respective data.

GS	LC_{50} [mg/L]	Lower 95% CI [mg/L]	Upper 95% CI [mg/L]	Standard error [mg/L]
15	2.68	2.64	2.72	0.02
20	1.01	0.98	1.04	0.01
25	1.22	1.16	1.28	0.02
36	2.11	1.99	2.22	0.04
42	>2.6	N/A	N/A	N/A

N/A = Calculations not possible because no mortality of 50% was achieved

TABLE A3. Model specification on which each 96-h LC_{50} value is based (Ritz et al., 2015). 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). Because no mortality of 50% for GS42 was achieved, no dose–response model could be fitted to the respective data.

GS	Function	R function
15	Two parameter log-logistic	LL.2
20	Two-parameter Weibull	W2.2
25	Two-parameter Weibull	W2.2
36	Two-parameter Weibull	W2.2

TABLE A4. Contingency table of LC_{50} comparisons via CI ratio testing (Ritz et al., 2015). Because no mortality of 50% for GS42 was achieved, the comparison via confidence interval ratio testing was not possible for this stage. However, it can be assumed that the LC_{50} of GS42 would significantly differ from GS20 because it is higher than GS36.

Comparison	Estimate	Standard error	Lower	Upper
GS15–GS25	2.20	0.04	2.10	2.30
GS20–GS25	0.83	0.02	0.78	0.87
GS36–GS25	1.72	0.05	1.61	1.84

TABLE A5. Mortality results of 96-h acute toxicity test of Gosner stage 15.

Concentration [mg/L]	Percentage mortality			
	24 h	48 h	72 h	96 h
0	0	0	0	0
1	0	0	0	0
3	80	80	80	80
5	80	80	100	100
7	100	100	100	100
9	100	100	100	100

TABLE A6. Mortality results of 96-h acute toxicity test of Gosner stage 20.

Concentration [mg/L]	Percentage mortality			
	24 h	48 h	72 h	96 h
0	0	0	0	0
0.7	0	0	0	0
0.9	10	30	30	30
1.1	50	60	70	70
1.3	60	70	100	100
1.5	100	100	100	100

TABLE A7. Mortality results of 96-h acute toxicity test of Gosner stage 25.

Concentration [mg/L]	Percentage mortality			
	24 h	48 h	72 h	96 h
0	0	0	0	0
0.8	10	10	10	10
1.0	20	20	20	20
1.2	50	50	50	50
1.4	60	60	70	70
1.6	100	100	100	100

TABLE A8. Mortality results of 96-h acute toxicity test of Gosner stage 36.

Concentration [mg/L]	Percentage mortality			
	24 h	48 h	72 h	96 h
0	0	0	0	0
1.6	20	20	30	30
1.8	30	30	40	40
2.0	30	40	50	50
2.2	70	70	70	80
2.4	100	100	100	100

TABLE A9. Mortality results of 96-h acute toxicity test of Gosner stage 42.

Concentration [mg/L]	Percentage mortality			
	24 h	48 h	72 h	96 h
0	0	0	0	0
1.8	0	0	0	0
2.0	10	10	10	10
2.2	10	10	10	10
2.4	20	20	30	30
2.6	20	20	20	30