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Roundup Biactive Modifies Cadmium Toxicity to *Daphnia carinata*

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In previous investigations (*Zalizniak PhD Thesis, submitted*), we found that technical grade glyphosate (Gly) in low concentrations (around 5-10% of its 48-h LC_{50}) improved population parameters including number of offspring per female and time to the first brood, of *Daphnia carinata* in sea salt medium (SSM from Barry 1999), but not in the balanced M4 medium specially designed for daphnids (Elendt and Bias 1990). It is known that Gly creates complexes with metals (Subramanian and Hoggard 1988, Wang et al. 2004) by binding them to one of its three chemical groups - amine, carboxylate and phosphonate (Pearson 1963). Artificial sea salt could lack some essential elements, for example, selenium, which was the most likely cause of daphnids' long-term poor reproduction performance in our earlier experiments in long-term culture. Because we did not observe an improved performance in the balanced M4 medium, we speculate that Gly in SSM facilitates the delivery of essential elements, resulting in better performance of the animals in low concentration Gly treatments compared with control. To test this hypothesis we investigated the effects of cadmium, which is highly toxic to daphnids, alone and with the addition of a low concentration (5 mg/L) of a Gly-based herbicide Roundup® Biactive (RB).

MATERIALS AND METHODS

A detailed description of *D. carinata* culture maintenance and feeding is provided in Zalizniak and Nugegoda (2006), and only briefly outlined here. Daphnids were maintained individually in M4 medium (Elendt and Bias, 1990), and fed with green alga *Pseudokirchneriella subcapitata* (formerly *Raphidocelis subcapitata* formerly *Selenastrum capricornutum*), which was cultured in Keating MS medium (Keating, 1985).

Roundup® Biactive with Gly concentration of 336 g/L was obtained from Monsanto (batch # 728408). All concentrations are nominal, expressed as concentrations of the active ingredient. Cadmium chloride $CdCl_2$ $2.5H_2O$ (Sigma) was used for Cd exposures. To ensure that Cd concentration was maintained at nominal level, stock solution concentration was occasionally measured with a Flame Atomic Absorption Spectrophotometer. It was found to be within 5% of

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the nominal level for the duration of experiment, therefore nominal concentrations were used throughout the study.

Initially 48-h LC_{50} for Cd was determined in acute toxicity tests (OECD, 1996) with four replicates of 5 animals (20 total) in each exposure concentration (volume per 5 animals was 25 mL). Concentrations of Cd used were 50, 100, 250, 500, 600, 700, 800, 900, 1000, 1100, 1200 and 1300 μg/L. The LC_{50} was determined to be 899 (95% CI 851-947) μg/L of Cd. Based on this result, five concentrations of Cd (in terms of proportion of the 48-h LC_{50}) were chosen for the long-term (21 days) toxicity tests using two successive generations of daphnids: '0.01 LC_{50}' (or 9 μg/L), '0.05 LC_{50}' (45 μg/L), '0.1 LC_{50}' (90 μg/L), '0.5 LC_{50}' (450 μg/L) and '1 LC_{50}' (900 μg/L). To test our hypothesis the concentration of RB where daphnids demonstrated an improved performance in our previous experiments – 5 mg/L (*Zalizniak and Nugegoda, submitted*) was added to another set of Cd exposures and also tested using two successive generations of *D. carinata*. Concentrations units were chosen and expressed as proportion of the LC_{50} of Cd to evaluate the relative toxicity and for ease of comparison of the different results (Zalizniak and Nugegoda, 2006).

Individual culture of *D. carinata* was chosen as an alternative to the OECD (1996) procedure (which requires testing of cohorts) for toxicity experiments (see Zalizniak and Nugegoda (2004) for details). 15 juvenile females per treatment/controls (age <24 hours) were placed individually in 25-ml McCartney bottles and exposed for 21 days. Mortality and reproduction parameters were recorded daily and daphnids transferred to new treatment media with algae (3.5×10^5 cells/cm^3), which were prepared daily, just before use. At the end of exposure, body length of surviving females (from the top of the crest to the base of a tail-spine) was also measured with an eyepiece micrometer under the microscope to the nearest 0.05 mm.

The same protocol was applied to experiments with the second generation of *D. carinata*. On the third or fourth day of reproduction the offspring from the first generation were taken for second-generation testing. Depending on the individual females' start of reproduction, the first-, second- and third-brood offspring were combined without distinguishing between broods. Offspring for the second-generation test were taken on the day when females produced enough young to start testing simultaneously in all treatments. Though it is a common practice to take the second or third brood only for experiments, Klein (2000) found no differences between the sensitivity of different broods to a reference toxicant potassium dichromate. Thus, in order to minimize the duration of the experiment, a mixture of several brood offspring of the age <24 h were used in our experiments. Offspring from daphnids exposed to 0.01 LC_{50} (Cd) in the first generation were exposed to 0.01 LC_{50} (Cd) in the second generation and so on for each treatment. The same end-points were observed as for the first generation.

Survival and fecundity values were calculated in all experiments and used in the computation of the intrinsic rate of natural increase r, which is determined from the formula (Lotka, 1913):

$$\Sigma\, l_x m_x e^{-rx} = 1,$$

where l_x is the proportion of individuals surviving to age x,
m_x is the age specific fecundity (number of females produced per surviving female at age x),
x is days.

The second-generation offspring (effectively – the third generation of daphnids) from all exposures were tested using the acute 48-h test protocol to determine if their sensitivity to Cd changed because of exposure of their parents and to compare the results from two sets – Cd exposure only and with addition of RB. Cd concentration range in this 48-h exposure was 500-1000 µg/L. This test was conducted according to OECD guideline for testing of chemicals (OECD, 1996). Volume of treatment solution was 25 ml for 5 animals. The LC_{50} values were determined separately for each pre-exposure concentration of Cd only and Cd+RB and plotted against these pre-exposure concentrations.

All tests were conducted at room temperature (21±1°C), photoperiod 16 hours day:8 hours night. Water quality parameters for M4 medium are: total hardness 2.5 mmol/L, alkalinity 0.9 mmol/L, conductivity 610 µS/cm, pH=8.2±0.1. The M4 medium is buffered, so the addition of acidic RB did not change the pH of exposure solutions.

Data were analysed using analysis of variance with the SPSS® 11.0 computer package. The pairwise comparisons of the values with and without RB in long-term experiments were performed using t-test assuming unequal variances (SPSS®). The LC_{50} values were determined using PROBIT analysis (SPSS®). The mean value of the intrinsic rate of natural increase and its standard error were determined using a jackknife approach as described by Taberner *et al.* (1993). Standard error was used throughout the results unless otherwise specified.

RESULTS AND DISCUSSION

In first generation '1 LC_{50}' exposures for both Cd and Cd+RB all daphnids died by day 6 before they started to reproduce. In '0.5 LC_{50}' Cd only exposure animals died by day 14 without reproducing, and in Cd+RB by day 16 after producing a few offspring. Since death limited the data for these exposure concentrations, the results for these are not presented. Combined results for the first generation of *D. carinata* are presented in Table 1. The control ('0' Cd treatment) animals performed better when a small amount of RB was added. Though the survival and time to the first brood were not significantly different, the animals were bigger and the number of offspring was greater in RB spiked control, resulting in a higher r-value (Table 1). The results for '0.01 LC_{50}' and '0.05 LC_{50}' did not show much difference in pairwise comparisons, except that in '0.05 LC_{50}' survival and size of animals were greater in RB spiked treatments compared to those in RB-

free. However, it did not affect their overall performance (the *r*-values are not statistically significantly different, Table 1). Surprisingly, the results for '0.1 LC_{50}' are the same as for '0' treatment with even greater differences between RB-spiked and RB-free exposures ($P<0.00001$).

The second-generation results followed the same trend as the first one. The control animals performed better in RB-spiked exposure in terms of size, number of offspring per female and time to the first brood, resulting in a higher r-value (Table 2). Unlike the first generation, the second did not show any differences between RB-free and RB-spiked in '0.1 LC_{50}' exposures for observed endpoints, however, the resulting difference for r-value was significant in pairwise comparisons (Table 2). At concentration '0.05 LC_{50}' (Table 2) all endpoints were 'better' in RB-spiked exposure, indicating that animals of the second generation respond at a lower concentration of cadmium if the media is spiked with RB.

There were no differences between 48-h LC_{50} in RB-spiked and RB-free Cd exposures (Table 3). Though the values were higher for Cd only pre-exposed animals than for animals pre-exposed to RB-spiked treatments (except in '0.1 LC_{50}'), the differences were insignificant in all cases (their CI are overlapping).

Based on the results of chronic sublethal exposures of two generations of *D. carinata* to RB-spiked and RB-free Cd treatments we conclude that addition of a small amount of RB (5 mg/L) reduces the toxicity of Cd to *D. carinata* (Table 1) contrary to our original hypothesis. In first generation exposures both '0' Cd treatment animals and those in '0.1 LC_{50}' (and in some cases in '0.05 LC_{50}') showed improved performance in RB-spiked Cd treatments compared with RB-free. Surprisingly, the lowest Cd treatment of 0.01 LC_{50} did not show any improvement in RB-spiked exposures – values for all endpoints were practically identical in pairwise comparisons. The greatest differences were observed in '0.1 LC_{50}' for number of offspring per female ($P=0.000004$) resulting in a large difference in the intrinsic rate of natural increase, suggesting that the number of offspring was the main factor contributing to the difference in *r*-value.

Similar to the first generation, the second-generation animals also demonstrated hormesis in '0' Cd treatment (all endpoints). However, contrary to the first, the improved performance in RB-spiked treatments shifted towards a lower Cd exposure of '0.05 LC_{50}' (Table 2). All endpoints indicated presence of hormesis at '0.05 LC_{50}' Cd concentration in RB-spiked treatments compared with RB-free. It is worth noting that the differences were not as great as in the first generation. Our results suggest that with the increase of time (generation) of exposure, hormesis becomes less pronounced, however it still can be detected, though at a lower exposure concentration. Judging from this trend it is possible that with further exposure (for several more generations) hormesis could be eliminated altogether. This is perhaps reflected in the results of the third-generation toxicity testing. The RB-free and RB-spiked treatments did not show any differences at any given Cd concentration (Table 3), indicating that at least in terms of survival there was no hormesis present. This might indicate that whatever advantage was given to the

Table 1. Response of the first generation of *D. carinata* exposed for 21 days to different concentrations of Cd only and with addition of 5 mg/L of RB (Mean±SE, N=15).

Endpoint	Cd exposure concentration (proportion of LC_{50})							
	0	**0+RB**	**0.01**	**0.01+RB**	**0.05**	**0.05+RB**	**0.1**	**0.1+RB**
Time to the 1st brood (days)	8.3±0.2	8.4±0.2	8.3±0.2	8.3±0.2	8.3±0.2	8.1±0.1	8.5±0.2	8.3±0.2
Body length at day 21 (mm)	4.00^{a}±0.14	4.30^{b}±0.05	4.26±0.04	4.19±0.06	3.95^{a}±0.04	4.13^{b}±0.03	3.55^{a}±0.16	3.86^{b}±0.05
Number of offspring per female	78^{a}±5	104^{b}±7	75±8	76±7	63±5	76±5	31^{a}±4	65^{b}±4
Cumulative survival (%)	80	80	87	87	53	80	67	73
Intrinsic rate of natural increase, (day^{-1})	0.311^{a}±0.009	0.330^{b}±0.006	0.311±0.007	0.317±0.008	0.307±0.008	0.318±0.006	0.226^{a}±0.013	0.308^{b}±0.007

Different superscripts denote values that are significantly different from each other (P<0.05) in a pairwise comparison (without RB-with RB).

Table 2. Response of the second generation of *D. carinata* exposed for 21 days to different concentrations of Cd only and with addition of 5 mg/L of RB (Mean±SE, N=15).

Endpoint	Cd exposure concentration (proportion of LC_{50})							
	0	**0+RB**	**0.01**	**0.01+RB**	**0.05**	**0.05+RB**	**0.1**	**0.1+RB**
Time to the 1st brood (days)	9.3^{a}±0.3	8.3^{b}±0.2	8.6±0.2	8.7±0.3	9.2^{a}±0.3	8.3^{b}±0.2	9.0±0.3	9.6±0.6
Body length at day 21 (mm)	4.03^{a}±0.03	4.13^{b}±0.03	4.01±0.04	4.08±0.05	3.93^{a}±0.03	4.02^{b}±0.04	3.73±0.03	3.64±0.13
Number of offspring per female	80^{a}±4	98^{b}±4	68±6	76±6	63^{a}±5	78^{b}±4	33±4	41±6
Cumulative survival (%)	87	93	87	93	80	100	93	80
Intrinsic rate of natural increase, (day^{-1})	0.293^{a}±0.010	0.317^{b}±0.008	0.289±0.009	0.304±0.007	0.270^{a}±0.011	0.312^{b}±0.006	0.217^{a}±0.011	0.271^{b}±0.009

Different superscripts denote values that are significantly different from each other (P<0.05) in a pairwise comparison (without RB-with RB).

first generation by Gly addition did not last till the third generation. However this hypothesis requires further testing.

Tsui et al. (2005) studied the change in acute (48-h) toxicity of several heavy metals to *Ceriodaphnia dubia* in the presence of Gly. They found that the toxicity of Cd (as well as 6 other metals) was reduced in the presence of 2.88 mg/L of Gly (for Cd the reduction was 48% of the initial Cd toxicity). Gly clearly lowered the availability of Cd to daphnia. The authors explained this as due to the formation of insoluble metal complexes of Gly in hard waters and thus their reduced bioavailability. This was also confirmed in our long-term study, when the toxicity of Cd (provided in solution as Cd^{2+}) was lower in the presence of Gly. However it did not explain the enhanced performance of daphnids when no Cd was added but Gly was (control). Tsui et al. (2005) also reported that Se toxicity did not change with Gly addition, and mortality was 100% in both exposures with or without Gly present). Because they used relatively high concentrations of Se to cause 100% mortality, it is not known if the result was the same at low concentrations of Se. At low concentration of Se exposure it might be that with Gly present, the toxicity of Se could be altered. Alternately if Gly enhances the uptake of some essential elements when they are low/deficient in the solution, Gly may be beneficial to daphnids. This could be one possible explanation for the enhanced performance of daphnids in the presence of Gly without Cd in our experiments. Tsui et al. (2005) also measured Ag and Hg accumulation in the animals after 4-h exposure to metals with and without the addition of Gly. They found that with 100nM of Gly present there was a decrease in both metals in the solution, however uptake by the animals was only detected in Hg exposure, but not in Ag. Our results on reduction in Cd toxicity in the presence of Gly indicate that Cd uptake was possibly reduced in the presence of Gly similar to Ag. This suggests that different metals react differently with Gly, and need to be studied individually for changes in their toxicity in the presence of Gly.

The effect of Gly can have environmental implications when metal pollution is also an issue. Some metals (such as Cd) can have their availability reduced, but uptake of some (Hg) increases. Mercury uptake into aquatic organisms (fish in particular) is already at undesirably high levels, and this in turn can affect their consumers' health, including humans. Gly can also bind to the substrate, and its accumulation with later release can potentially increase Gly load in the environment. Though Gly is a herbicide, it is nevertheless toxic to some aquatic animals at environmentally realistic concentrations (Relyea 2005, Relyea *et al.* 2005).

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Table 3. Third-generation 48-h LC_{50} (μg/L) (Mean with 95% CI in brackets, N=4) for animals, whose parents and grandparents were exposed to different concentrations of Cd and Cd+RB.

Pre-exposure concentration (proportion of the initial LC_{50})	Cd only	Cd+RB
0	595 (537-639)	577 (512-621)
0.01	552 (481-598)	546 (483-587)
0.05	593 (376-753)	542 (396-638)
0.1	401 (256-499)	408 (221-525)

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