ACUTE AND CHRONIC EFFECTS OF A MIXTURE OF FORMULATED PESTICIDES AND ITS CORRESPONDING ACTIVE INGREDIENTS IN *Daphnia magna*

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ABSTRACT

Agricultural practices worldwide include the use of pesticides either as single-chemical or multiple-chemical applications aiming higher efficiency in controlling grass weeds and animal pests. Pesticides may easily reach and contaminate surface waterbodies mainly through drainage and spray drift, and hence are currently viewed as a major contamination issue. Following recent discussion on the ability of adjuvant chemicals added to marketed formulations to contribute for pesticide toxicity, this study focuses the acute and chronic toxicity of the insecticide Lannate® and the herbicide Stam Novel Flo®, as well as that of their active ingredients Methomyl and Propanil, to the freshwater cladoceran *Daphnia magna*. Furthermore, acute and chronic toxicity of mixtures is addressed, through the evaluation of *D. magna* responses to joint exposures comprising both active ingredients and both commercial formulations. Regarding acute exposures, the mixture comprising commercial solutions was shown to have a more than additive action, while the opposite (less than additive action) was found when testing the joint action of the active ingredients. Chronic exposures can provide additional information on chemicals` toxicity in reproductive endpoints, which is of particular relevance when considering that pesticides are often repeatedly applied within a single agricultural season. Life-history endpoints assessed under mixture exposures indeed indicated higher long-term than short-term toxicity, and showed a distinct pattern of joint action when compared to acute toxicity assessments. These results suggest that long-term exposures can provide relevant complementary information on mixtures' toxicity that should be taken into account within pesticide risk assessment procedures.

KEYWORDS: *Daphnia magna*; mixtures toxicity; Methomyl; Propanil; immobilisation; life-history.

INTRODUCTION

Agricultural practices often involve usage of combinations of several pesticides in the same crop, which contributes for generating pools of complex chemical mixtures that can reach nearby waterbodies, through several types of physical transport. Pesticide mixtures are likely to promote hazardous effects in non-target aquatic organisms. Studies have already shown that toxicity of chemical mixtures is generally higher than that of its individual components alone [1, 2]. Knowledge on the toxicity of chemical mixtures to aquatic organisms significantly increased recently [1, 3-10]. The analysis of joint effects of xenobiotics generally follows the assumption of simple additivity *i.e.* they are expected to act as dilutions of each other in their contribution to the overall toxicity. However, chemical combinations displaying synergistic and/or antagonistic behaviour have also been reported in literature [4, 5, 8, 11-13].

Due to their ecologically relevant role in the basis of the aquatic food web and its life-cycle characteristics, cladoceran species are promising model organisms for ecotoxicological assessments (e.g. [14]). The effects in *Daphnia magna* of binary mixtures comprising the insecticide Methomyl and the herbicide Propanil, as well as their commercial solutions Lannate® and Stam Novel Flo®, were evaluated. Propanil (3,4-dichloropropioanilide) is extensively used in the post-emergent treatment of rice [15, 16]. This acylanilide belongs to the phenylamide class and acts on target species as a photosynthetic inhibitor [17]. Methomyl [S-methyl-N-(methylcarbomoyloxy) thioacetimidate] is a broad-spectrum carbamate insecticide, hence acting as cholinesterase inhibitor [18]. Considering the distinct nature of their targets, Methomyl and Propanil can be applied jointly in crops to control simultaneously insect pests and grass weeds. With this study, we intended to address the toxic effects of binary mixtures comprising the referred commercial solutions or the respective active ingredients in *Daphnia magna*. Both short- and long-term binary exposures were carried out for this purpose.

MATERIALS AND METHODS

Toxicants

Technical-grade Methomyl (99.5% purity) was donated by Makhteshim Agan®, Portugal) while technical-grade Propanil (Propanil Pestanal®, 99.7% purity) was purchased from Sigma Aldrich®, Germany. The commercial solutions Lannate® (Sapec Agro®, Portugal), 200 g L^{-1} Methomyl concentrated, and Stam Novel Flo® (Dow®, Portugal), 480 g L^{-1} Propanil concentrated, were obtained from local suppliers; for text clarity convenience Stam Novel Flo 480® will hereinafter be referred as Stam. Main stock solutions were prepared on a weekly basis during testing procedures, by dilution or dissolution of commercial solutions or technical-grade chemicals, respectively, and stored in the dark at 4 ºC.

Organisms and testing

Monoclonal bulk cultures of *Daphnia magna* (clone A *sensu* [19]) have been continuously reared in our lab under a 16 h^L: 8 h^D photoperiod, at 20 ± 2 ºC. The organisms were cultured in the synthetic medium ASTM hard water [20], supplied with an organic additive [19]. The renewal of the cultures occurred every other day, as well as the food supply ($3.0x10^5$ cells ml^{-1} of the green algae *Pseudokirchneriella subcapitata*). All the organisms used in tests were newborns ageing less than 24 h, and yielded by cultured females within 3^{rd}-5^{th} brood.

The acute assays were conducted in general accordance with the related protocol by OECD [21]. As there is recommended, each treatment comprised four replicates with five individuals each, held in glass vessels containing a final test volume of 100 ml. The test solutions were not renewed and the organisms were not fed during the tests; dissolved oxygen and pH were monitored at the beginning and end of the test for validation purposes. The incubation conditions were kept as described for culturing procedures. Immobilized daphnids were counted after 48-h exposure. Nominal concentrations used in single-toxicant acute exposures were as follows: (i) 18.6, 24.2, 31.4, 40.9, 53.1 and 69.1 μg L^{-1} for technical-grade Methomyl; (ii) 750, 1125, 1688, 2531, 3797 and 5675 μg L^{-1} for technical-grade Propanil; (iii) 16.9, 22.0, 28.6, 37.1, 48.3 and 62.7 μg L^{-1} of Methomyl in Lannate®; (iv) 1250, 1875, 2813, 4219, 6328 and 9492 μg L^{-1} of Propanil in Stam. Following the approaches of Bailey et al. [22] and Banks et al. [5], mixture concentrations (binary mixtures of Methomyl and Propanil; Lannate® and Stam) were prepared using the same concentrations as those in their respective concurrent single-exposure tests. In preliminary range-finding tests, we found, however, that this dose design was not suitable: high mortality rates were recorded in the lower-dose treatments and, hence, mixture-acute toxicity data could not be feasibly fitted to any concentration-response function for EC calculations. Therefore, we lowered proportionally the treatment ranges by combining half of the concentrations used in the concurrent single-exposure tests.

The chronic assays were conducted according to the related OECD guideline [23]. Daphnids were individually exposed to the chemicals in 50-ml glass vessels and ten replicates were used per treatment. Tests were carried out along the standardized 21 days under the incubation conditions as described for the culture rearing. The synthetic medium was renewed every other day, and the organisms were fed daily with *P. subcapitata* at the concentration used for culturing. Tests were screened daily and, when present, neonates were counted for fecundity-related calculations and immediately discarded. The daily growth rate, g (day^{-1}), of tested females was calculated using the following equation:

$$g = ln\mathrm{BLf} - ln\mathrm{BLi}/\Delta t$$

where BL_i and BL_f stands for the body length of the females (mm) at the beginning and at the end of the test, respectively, and Δt for the time range (days). Measurements were taken under a stereoscope in accordance to Pereira et al. [24]. The population growth (r, day^{-1}) was iterated from the Euler-Lotka equation:

$$1 = \sum_{i=1}^{n} e^{-rx} l_x m_x$$

where x stands for age class (day), l_x is the probability of surviving to age x, and m_x represents age-specific fecundity. Uncertainties were calculated according to the Jackknife technique [25].

Nominal concentrations used in single-toxicant chronic exposures were adjusted relatively to concentration ranges used in acute exposures in order to ensure the detection of statistically significant sub-lethal reproductive effects relatively to controls, and prevent for egg mortality in Propanil exposures (see Pereira et al. [26] for details on this issue). Thus, severe reductions in Propanil concentration ranges were done and slight reductions were operated for establishing Methomyl related ranges: (i) 6.9, 10.4, 15.6, 23.3 and 35 μg L^{-1} of technical-grade Methomyl; (i) 19, 29, 43, 65 and 98 μg L^{-1} of Propanil in Stam Novel Flo®; (ii) 4.2, 6.3, 9.5, 14.2 and 21.3 μg L^{-1} of Methomyl in Lannate®; (iii) 24, 36, 55, 82 and 123 μg L^{-1} of technical-grade Propanil. As to the mixture chronic exposures, we experimentally found adequate touse of the same approach as in the acute assays, by combining half of the concentrations used in the concurrent single-toxicant tests (see above).

Data analysis

All the 48-h EC_{50} values and respective 95% confidence limits were determined using Probit analysis [27]. As to chronic toxicity data, one-way analysis of variance followed by the Dunnett *post-hoc* test, when applicable, was used to detect and assign significant differences between control and treatments, for each life-history endpoint. A significance level (α) of 0.05 was always used.

Mixture-acute effects were addressed following e.g. Woods et al. [4]. Interactions between the pesticides pre-

vailing in the acute exposures were hence analyzed using a toxic units (TU) approach, which is based on the comparison between EC_{50} values obtained for chemicals under single and joint exposures. The TU for a binary mixture (TU_{mix}) is given by the following equation:

$$TU_{mix}=EC_{50}A(mix)/EC_{50}A(single)+EC_{50}B(mix)/EC_{50}B(single)$$

where A and B stand for the chemicals involved, and EC_{50}(mix) and EC_{50}(single) represent the toxicity of each chemical within mixture or single exposures, respectively. If the model outcome equals 1 ($TU_{mix} = 1$), the toxicity of the mixture would be additive, while values below or above 1 denote a more or less than additive behaviour of the mixture, respectively. Since concentration-response curves yielded from chronic assays could not be feasibly fitted to any model for EC-related calculations, we could not use a TU approach to address chemical interactions within tested mixtures.

RESULTS AND DISCUSSION

Either Methomyl or Propanil are fairly water-soluble and are not likely to adsorb strongly to soil particles (low Koc) [28]. There is, thus, an enhanced theoretical possibility for the pesticides to reach and contaminate water bodies standing nearby the local of application. Moreover, joint application of Propanil- and Methomyl-based pesticides in crops to control animal pests and grass weeds is very likely, which increases the relevance of testing effects of their mixture in aquatic non-target organisms such as *Daphnia*. In fact, under natural conditions aquatic organisms are typically not exposed to single substances but rather to multiple mixtures of xenobiotics [3]; these chemicals can act singularly but often interact with each other [5], which may affect their overall toxicity to non-target aquatic organisms. Furthermore, pesticide effects on aquatic organisms are often investigated in lab using single toxicants and studies focusing interactions between toxicants still scarce. In this study, responses obtained after single (see additionally [29]) and mixture exposures were distinct; both the mixture of commercial solutions (Stam plus Lannate®) and the mixture of its active ingredients (Propanil plus Methomyl) impaired the assessed acute and chronic endpoints at low toxicant concentrations i.e. mixtures comprising these two pesticides seem highly toxic to non-target *D. magna*.

Table 1 summarizes the immobilisation EC_{50} values obtained after a 48-h acute exposure to the single toxicants and to the binary mixtures of the commercial solutions and the active ingredients. Lannate® and Methomyl exhibited very high and comparable acute toxicity to *D. magna* (EC_{50} values of 31 and 24 μg L^{-1}, respectively). These toxicity records were slightly more conservative than those reported previously in literature [11, 30]. Propanil and Stam were fairly less acutely toxic than Methomyl and Lannate®, and their EC_{50}s were also less comparable: Propanil was more toxic than Stam to *D. magna* (EC_{50}s of 2109 and 3554 μg L^{-1}, respectively). Regardless the solution purity, Propanil acute toxicity was consistent to the range expected from literature [31, 32]; the difference noticed between toxicity values yielded from exposures to the active ingredient and commercial solution should be due to the adjuvant chemicals that add the pesticide in the commercial solution. Marketed pesticides are composed of the active ingredient and a number of other chemicals (generally called inert ingredients) that support its mixing, dilution, application, and stability [33], and can contribute for the overall toxicity profile of the pesticide [e.g. 31].

In the mixture combining the commercial solutions, EC_{50}s were broadly half of their concurrent in the single-toxicant exposures (Table 1); *e.g.* 15μg L^{-1} of Methomyl in Lannate® and 1495μg L^{-1} of Propanil in Stam were needed to immobilize 50% of the tested individuals. This seems to suggest an additive behaviour of the chemicals to produce the overall effect in the organism. In fact, a TU_{mix} of 0.905 was found for this mixture, which would mean a more than additive behaviour of the chemicals according to the TU model [4]. This conclusion should, however, be held carefully given that the difference between the TU_{mix} found and 1 (additivity) is actually very little. Conversely, the combination of the technical-grade pesticides provided a TU_{mix} of 1.568, which denotes a less than additive interaction of the mixed chemicals. The herbicide Propanil and the insecticide Methomyl belong to different chemical classes, and exert their toxic effects through distinct mechanisms since the former is a photosynthetic inhibitor with no known neurotoxic activity [28], while the

TABLE 1 - EC_{50} values (μg L^{-1}) found for single and combined acute exposures of *Daphnia magna* to Methomyl and Propanil (technical-grade ingredients) and within the commercial formulations (Lannate® and Stam), with the respective 95% confidence limits ($CL_{95\%}$). Data showed for commercial solutions (Stam and Lannate®) refer always to concentrations of the active ingredient in test solutions.

		EC50	Lower $CL_{95\%}$	Upper $CL_{95\%}$
Single exposures	Methomyl	24	21	27
	Propanil	2109	1876	2404
	Lannate®	31	28	32
	Stam	3554	3165	4061
Joint exposures	Propanil	1375	1302	1458
	+ Methomyl	22	21	24
	Stam	1495	1137	1979
	+ Lannate®	15	13	16

latter is a potent anticholinesterase carbamate insecticide [17, 18]. It is unlikely thought that their joint effect in *Daphnia* denotes more than additive or synergistic interactions [34]. In this way, the slight and unexpected more than additive effects found in our joint acute exposures to commercial compounds are likely to be due to the action of the (unknown) adjuvant complex. As long as the complete list of the adjuvant chemicals or the proportions within this list in commercial pesticide solutions remains unknown, further conclusions on this issue would be fairly inconsistent.

Considering that long-term effects of pesticide mixtures have been rarely if ever evaluated, this study may be a relevant contribution for ecotoxicological databases regarding pesticides. In general, the long-term exposures did not impair survivorship of *D. magna* (as expected given the reproductive scope of the tests) and eventual mortality was considered as an integrated record for calculation of population growth rates. Methomyl was able to significantly impair *D. magna* fecundity and growth rates at very low concentrations both in exposures concerning the active ingredient and the commercial formulation (Fig. 1; Table 2). These three endpoints were also significantly affected in the exposure to Stam; Propanil (active ingredient) promoted significant reduction in *Daphnia* fecundity and somatic growth rate, and a slight decrease in the corresponding population growth rate (Fig. 1; Table 2). The noticed reduction in offspring production as pesticide concentrations increased may actually be explained by an impairment of the females growth in juvenile stages, which is consistent with the decrease of somatic growth rates observed in all exposures. Hanazato [35] concluded that toxic chemicals such as pesticides often reduce the growth rate of neonates, and cause them to mature at a smaller size and, subsequently, with fewer eggs. Hence, the number of offspring per clutch should not be directly affected by the toxicant chemical but rather, it is a function of the maternal body size, which, in turn, is governed by the growth rate during the juvenile stage [34, 35]. On the other hand, under adverse conditions, such as chemical stress, and once reproduction is onset, daphnids generally allocate energy at higher rates to the progeny production instead of investing in somatic growth [37, 38], which may contribute for additional reduction of growth rates.

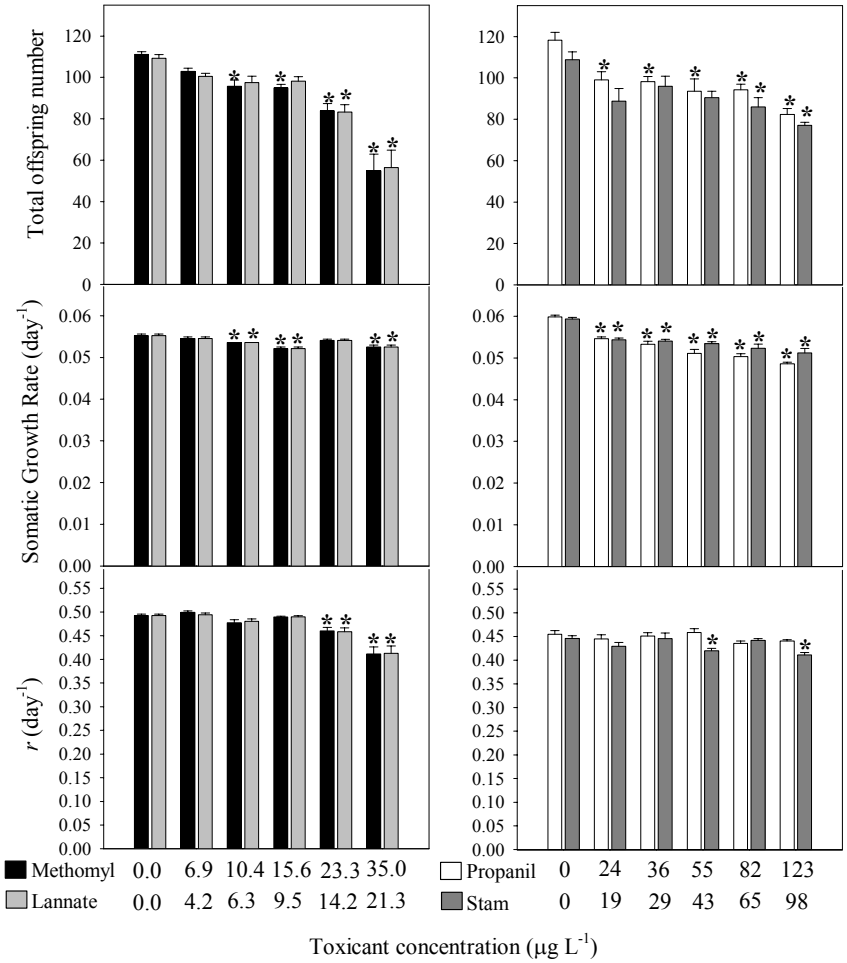

FIGURE 1 - Fecundity (total offspring number) and growth rates (somatic and population growth rate, *r*) of *Daphnia magna* after long-term single exposures to Methomyl and Lannate® (left pane plots), Propanil and Stam Novel Flo 480® (right pane plots). Error bars represent standard error, and "*" indicates statistically significant differences of the toxicant treatments relatively to control ($P < 0.05$).

TABLE 2 - One-way ANOVA summary regarding the life-history responses of *Daphnia magna* to single and combined exposures of the active ingredients (Methomyl and Propanil) and commercial formulations (Lannate® and Stam) (df – degrees of freedom). LOECs were added to the ANOVA summary (μg L^{-1}); as to endpoints assessed after exposure to pesticide mixtures, concentrations within the treatment which was statistically depicted as LOEC are clarified (Methomyl concentration + Propanil concentration within mixture treatment; μg L^{-1}). Data on commercial formulations refer always to concentrations of active ingredient in test solutions.

Endpoint		df	$MS_{residual}$	*F*	*P*value	LOEC
Total offspring number	Methomyl	5, 51	136.8	26.35	0.000	10.4
	Propanil	5, 53	142.2	9.08	0.000	≤ 24
	Methomyl+Propanil	5, 54	66.8	51.77	0.000	17.5 + 61.5
	Lannate®	5, 51	149.3	22.03	0.000	14.2
	Stam	5, 52	163.9	6.86	0.000	43
	Lannate® + Stam	5, 52	242.0	2.15	0.074	10.7 + 49
Growth rate	Methomyl	5, 51	$1.2x10^{-6}$	11.59	0.000	10.4
	Propanil	5, 53	$4.1x10^{-6}$	36.31	0.000	≤ 24
	Methomyl+Propanil	5, 54	$5.2x10^{-6}$	5.92	0.000	≤ 3.45 + ≤ 12
	Lannate®	5, 51	$1.2x10^{-6}$	11.59	0.000	6.3
	Stam	5, 52	$4.8x10^{-6}$	16.31	0.000	≤ 19
	Lannate® + Stam	5, 51	$5.0x10^{-6}$	23.17	0.000	≤ 2.1 + ≤ 9.5
r	Methomyl	5, 54	$5.84x10^{-4}$	18.15	0.000	23.3
	Propanil	5, 54	$4.87x10^{-4}$	1.58	0.182	> 123
	Methomyl+Propanil	5, 54	$2.55x10^{-4}$	41.98	0.000	11.7 + 41
	Lannate®	5, 54	$5.85x10^{-4}$	17.10	0.000	14.2
	Stam	5, 54	$5.02x10^{-4}$	4.28	0.002	43
	Lannate® + Stam	5, 54	$6.07x10^{-4}$	0.68	0.643	> 10.7 + > 49

The population growth rate integrates records concerning individual-level traits, namely survival, reproductive output and time-delays between successive offspring clutches. It generally predicts the population changes induced by given stimuli based on the individual life-history responses. Therefore, *r* is recognized as an important ecotoxicological endpoint, since it provides a more ecologically relevant measure of the toxicants' impact in natural populations [39]. Despite less expressively, *r* followed the trend depicted by the remaining life-history parameters in all single-toxicant exposures, except for Propanil, that had no statistically significant influence in population growth. Mortality and delay in brood releases were rarely noticed (data not shown) and these endpoints are of great influence when estimating population growth [40, 41]. Thus, changes in *r* should be mainly due to differences in reproductive output caused by the toxicants action.

Both binary mixtures (active ingredients and commercial solutions) have impaired significantly fecundity and somatic growth, and the mixture comprising Methomyl and Propanil has indeed promoted a significant decrease in population growth (Fig. 2; Table 2). When comparing effects on fecundity of single and joint exposures, the lower LOECs found for the latter exposure scenario indicate that single compounds induce more noxious effects on *Daphnia* reproduction (Table 2). The opposite trend was observed for somatic growth rates i.e. LOEC values show that daphnids growth in joint exposures was significantly reduced at lower concentrations (LOEC values of ≤ 3.45 μg L^{-1} Methomyl and ≤ 12 μg L^{-1} Propanil; and of ≤ 2.1 μg L^{-1} Lannate® and ≤ 9.5 μg L^{-1} Stam) than in single-chemical exposures (LOEC values of 10.4 μg L^{-1} Methomyl and ≤ 24 μg L^{-1} Propanil.; and of 6.3 μg L^{-1} Lannate® and ≤ 19 μg L^{-1} Stam). Furthermore, the somatic growth

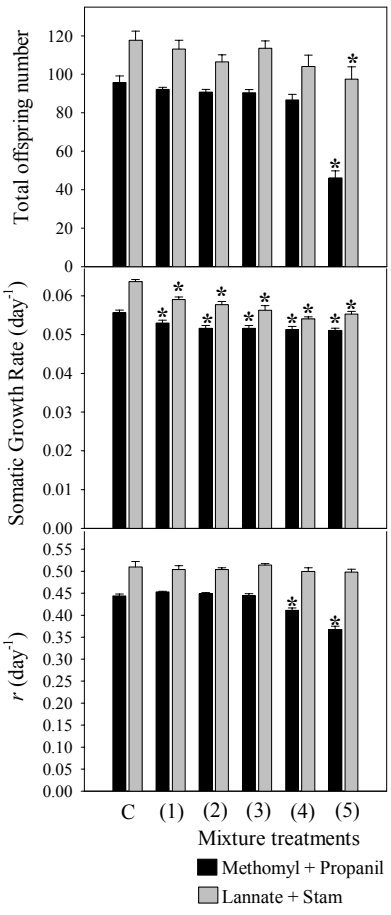

FIGURE 2 - Fecundity (total offspring number) and growth rates (somatic and population growth rate, *r*) of *Daphnia magna* chronically exposed to Stam Novel Flo 480® plus Lannate® and Propanil plus Methomyl. Error bars represent standard error, and "*" indicates statistically significant differences of treatments, (1)-(5), relatively to control, (C), ($P < 0.05$).

pattern depicted in mixture exposures was consistent with that found for single-toxicant exposures in the sense that once again growth rates tended to be affected by lower toxicant concentrations than was fecundity; this reinforces the evidence that fecundity and somatic growth rates in daphnids are strongly dependent on each other, and illustrates how pesticides can unbalance this relationship (see above the discussion on single-toxicant exposures). The mixtures tended to exert higher toxicity than single-exposures also over population growth (lower LOECs; see Table 2). Population growth calculations rely on the integration of fecundity-related endpoints and mortality [39]; delays on first reproduction have high weight on the *r* calculation algorithm [25], which is in line with the relevance of a first reproduction event to ensure survivorship of natural populations [41]. There is a critical size that females must reach to onset reproductive investment [32], and this is the point where somatic growth rates can indirectly influence *r*: if somatic growth is depressed by a given stressor, then more time will be needed to reach the critical size needed to yield the first brood. This may actually explain the consistent pattern found in mixture exposures between impairment of somatic growth rates and population growth rates.

Further inferences on the mechanisms behind the interaction between Methomyl and Propanil and related toxicity effects in *D. magna* would be fairly inconsistent. In fact, these pesticides are specifically designed to damage a given target species and few information exists regarding their toxicity mechanisms in non-target species such as *Daphnia* (particularly for Propanil). Apparently, no coincidence exists between molecular targets of Methomyl and Propanil in *Daphnia*. While carbamates such as Methomyl are neurotoxic by inhibiting the activity of acetylcholinesterase and other esterase neurotransmitters [18, 30, 43], amide herbicides such as Propanil act as photosynthetic inhibitors by interfering with the electron transport chains within the light reaction of photosynthesis [17]. If the toxicity mechanism of the former in *Daphnia* is quite straightforward, information is still needed to infer about it regarding Propanil. One may speculate that pathways involving electron transfer reactions in non-target species may be impaired by this chemical, and interactions with Methomyl neurotoxic pathways may occur, but no experimental evidences exist to support this hypothesis.

Either in single or in joint chronic exposures, the commercial pesticide formulations tended to promote higher impairment to *Daphnia magna* life-history than active ingredients i.e. lower LOEC values were generally found after exposures to commercial solutions relatively to those found in tests considering active ingredients (Table 2). These experimental evidences are consistent with considerations made by other authors in literature that have expressed concerns on the potential of adjuvant chemicals to contribute for pesticides toxicity in non-target organisms [e.g. 31]. In addition, our results reinforce the relevance of addressing commercial formulations rather than only active ingredients in estimations of pesticide environmental risks.

CONCLUSIONS

In general, results showed that the short-term and the life-history responses of *D. magna* in exposures to the mixtures of commercial solutions and active ingredients were distinct from responses obtained in single-toxicant exposures. Despite the pesticides have no common toxicological targets, experimental evidences suggest that some interaction exists between the pesticides either in acute or chronic joint exposures. The lack of consistent information on the toxicity mechanisms of Methomyl and Propanil in non-target organisms constrains more accurate analyses on these interactions. Even though, the relevance of addressing mixtures effects and their complex interactions is still not compromised: when more than additive effects are recorded, such as happened in this study, one should recognize that toxicity references based on single-chemical effects are not protective or environmental realistic. Moreover, our results in short-term mixture exposures were distinct from those yielded after chronic exposures. This suggests that assessments considering mixtures toxicity on the basis of acute exposures may underestimate the potential of pesticides to affect the aquatic ecosystem – in addition, if one considers that pesticides are repeatedly applied in crops and aquatic organisms are likely to be continuously exposed to these toxicants, the evaluation of effects of short exposure to highly concentrated pesticide solutions aiming environmental risks estimation seems senseless.

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