ACUTE AND CHRONIC EFFECTS OF COPPER, CHROMIUM AND INSECTICIDE-ENDOSULFAN ON LITTORAL CLADOCERA, *PSEUDOSIDA VARIABILIS*

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ABSTRACT

Recent works have emphasized on the serious problems caused by the toxicity of pesticides and heavy metals on aquatic ecosystems due to human activities. The aim of this work was to evaluate the toxicities of an insecticide with endosulfan as active element and the metals copper and chromium on the littoral cladocera ctenopoda, *Pseudosida variabilis*. The lethal and sublethal effects on eight biological endpoints were analyzed paying special attention to the intrinsic rate of increase (*r)*. The EC_{50} values to copper, chromium and endosulfan were: 29; 133.2 and 1.75 µl l^{-1} at 24 h and 12; 52.5 and 1.04 µg l^{-1} at 48 h respectively. A comparison with other freshwater cladocerans revealed that *P. variabilis* would be an appropriate species to be used as a test organism in ecotoxicological studies. A detailed analysis of each life history trait showed that copper, chromium and endosulfan had negative effects on several life history parameters. However, the *r* value was not the most appropriate endpoint of copper and chromium toxicity, when it was compared with other individual and population parameters. Survival, longevity, age of first reproduction and mean brood size were the most appropriate parameters for both metals. On the contrary, in case of endosulfan, this endpoint was severely affected, and the population consequences are discussed. The results suggest that multiple biological endpoints and an extended period of exposure are needed in order to achieve a better screening of metal and insecticide toxicity.

KEYWORDS: Pseudosida variabilis; metals; pesticide; life cycle; toxicity tests.

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1. INTRODUCTION

In South America, agricultural activities and industries are the major sources of aquatic pollution. In this line, several works have emphasized on the serious problems caused to the biota by the toxicity of many substances employed in that practices and released into the environment, such as pesticides and metal [1-5].

Among aquatic zooplankton microcrustaceans, the most commonly used species in ecotoxicology is the cladoceran *Daphnia magna* Straus [6; 7] and some species of the genus *Ceriodaphnia* [8; 9], both from the Suborder Anomopoda. However, *D. magna* does not belong to the regional fauna of South America, and its ecological relevance as a test specie can be criticized [10; 11]. Moreover, since *D. magna* and *Ceriodaphnia* are free living species in the limnetic areas, researches on vegetation-associated species are missing. Therefore, we suggest the littoral cladocera ctenopoda *Pseudosida variabilis* to be a suitable organism since the combination of several of its biological features, such as valid representative of Neotropical fauna [12], high fecundity and relative easiness to be cultured in laboratory, make it a promising test organism for littoral environments and shallow lakes.

The purpose of the present work was to assess the impact of a commercial insecticide, with endosulfan as active element (Zebra Ciagro™), and the metals copper and chromium on the mortality and life cycle of *P. variabilis.*

Endosulfan was selected because it is one of the most widely used insecticide in agricultural activities [2]. Endosulfan not only has been characterized as highly toxic and persistent in the environment by the USEPA, it has also been demonstrated that the mixture comprising commercial pesticides could be more toxicant that the single-active ingredient [13]. Both copper and chromium were chosen because of their persistence in waterbodies associated with industrialized areas, and the high toxicity they represent to different zooplankton organisms [5].

As a first step, the acute toxicity of the three chemicals was determined using *P. variabilis* s a bioassay species. Then, through a series of complete life cycle toxicity tests, the sublethal effects on several biological parameters of *P. variabilis* were analyzed.

2. MATERIALS AND METHODS

2.1. Stock culture and test organisms

P. variabilis used in the experiments was obtained from a population living in unpolluted waterbodies from the alluvial plain of the Paraná river (31º38'23.7''S; 60º40'53.3''W). Organisms were colected with a planktonic net (200 µm) and culture in a 1000 l tank located outside the Instituto Nacional de Limnología (CONICET-UNL).

A parallel stock culture was maintained for several months under laboratory conditions (photoperiod: 16 h light-8 h darkness; light intensity: 2200 (± 244) Lux; temperature: 21 ±2 ºC). The organisms were placed in glass containers with twice filtered (50 µm), aerated (24 h) and autoclaved pond water, in a density of 50 ind.l^{-1}. This water was also used for acute and life cycle experiments, since it represents the real medium where the organisms live. Physico-chemical characteristics were measured at the beginning and at the end of each experiments, according to the Standard Methods for the Examination of Water and Wastewater [14].

The organisms were daily fed *ad libitum* with a *Chlorella* sp. concentrate (algal density: 3.7 x 10^5 cel.ml^{-1}). The algae were cultured following Borowitzka [15] considerations and concentrated by centrifugation at 500 rpm for 7 min. The resuspension and homogenization was conducted in the same medium used for the cladocerans, with gentle shaking. The algal quantifications (ind. ml^{-1}) were determined using a invert microscope Wild at 400X following the Utermöhl [16] method. Both acute and chronic bioassays were begun with newborn (<24 h) neonates obtained from the third brood progeny of *P. variabilis* from the laboratory stock culture.

2.2. Chemicals

The metallic salts used in this study were copper sulphate ($CuSO_4.5H_2O$) and potassium dichromate ($K_2Cr_2O_7$), Ciccarelli® (Santa Fe, Argentina). A stock solution of both metals were prepared by dissolving the salts in distilled water respectively. The insecticide employed was Zebra Ciagro™ (Ciagro, S.A. Buenos Aires, Argentina), containing 35% of organochlorine endosulfan as active ingredient (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a ,6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3, benzodioxanthiepine-3-oxide). As the USEPA (1980) specify, commercial products with endosulfan contain a mixture of isomers α-endosulfan and β-endosulfan in a ratio of 2:1–7:3. In order to prepare a stock solution, the mentioned product which was in liquid form, was diluted with distilled water taking into account the concentration of the active ingredient in the formulation.

Toxicant concentrations in water were quantified at the beginning of each (acute and chronic) experiment, within 72 h after the exposition. Endosulfan concentration was measured by GC-ECD, according to EPA [17]; and metals concentrations was quantify according to Martin et al. in EPA [18].

2.3. Acute toxicity assays

Static bioassays were conducted in order to determine the 24 and 48 hours EC_{50} values and its 95% confident limits for the three toxicants. Prior to acute assay, the ovigerous females were isolated in 20 ml glass containers with the control medium. After egg hatching, females were removed and 20 neonates were randomly isolated in groups of five (4 replicates) with 12 ml of each test concentration or the blank control (a total of 320 specimens were used for the determination of 24 and 48 h EC_{50}). The experiments were conducted under the same temperature as described for the stock culture conditions (21 ±2 ºC), but in absence of light in order to avoid photodegradation of the chemicals. No food was added during the assays. The Effective Concentration (EC) was considered when an animal ceased to move and it no longer responded to mechanical stimulation.

The concentrations tested were prepared by dissolving the stock solutions in the control water immediately before to each experiment. The range of each chemical was established considering values of other zooplankton cladocerans and carrying out numerous preliminary tests. The final tested concentrations were for copper: 3, 6, 12.5, 25 and 50 µgl^{-1}; for chromium: 9.5, 19.5, 39, 78, 156 and 312 µgl^{-1}; and for endosulfan: 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 µg l^{-1}.

2.4. Chronic toxicity assay

The EC_{50} values were used to determine the final concentrations to life cycle experiments. The effects on survival, life span and reproduction were monitored throughout all *Pseudosida* life cycle (29-62 days, depending on the toxicity). The concentrations tested for each chemical were: 2.5; 5; 10; 20 µg l^{-1} for copper; 3.3; 6.5; 13; 26 µg l^{-1} for chromium and 0.018; 0.037; 0.075; 0.15 µg l^{-1} for endosulfan, all plus the blank control (0 µl l^{-1}). The test solutions were partially (50 %) renewed daily. Experiments were conducted under the same temperature and photoperiod regimes as described for the stock culture conditions.

In all cases 25 replicates for control and concentrations -of copper, chromium or endosulfan- was used. Each one consisted on a neonate (<24 h) which was individually allocated to glass containers with 20 ml of the test solution. During the experiment, the organisms were daily fed with a *Chlorella* sp. concentrate (3.7 x 10^5 cell ml^{-1}). The algae was cultured following the same methodology described in the stock culture section [15].

Survival and longevity was daily monitored and the endpoints used to determine the effect of copper, chromium and endosulfan on reproduction were: total eggs per female, mean brood size, mean age to first reproduction (AFR), the net reproductive rate (*R*o), generational time (T) and the intrinsic rate of increase (*r*). Ro was calculated according to Lotka [19] and T and *r* were calculated according to Laughlin [20].

2.5. Statistical Analysis

The EC_{50} values and their 95% confidence limits for 24 and 48 h were estimated with the Standard method of Probit Analysis as described by Finney [21].

The Kruskal-Wallis test was used to evaluate the effect of the chemicals on *P. variabilis* survival. A posteriori Dunn' test was done to determine significant differences between two treatments, differences were considered significant at $p<0.05$.

To test the significance of each concentration of both metals and the insecticide on *P. variabilis* life history traits (development time, growth, first reproduction time and fecundity), a one-way ANOVA was employed followed by a post hoc Dunnett's test. Differences were considered significant at $p<0.05$. Prior to ANOVA, data were tested for normality and homogeneity of variances using the Kolmogorov-Smirnov and Levene test respectively.

The LOEC (lowest concentration that produced a significant toxic response) and the NOEC (highest concentration that did not produce a significant toxic response) were determined using as endpoints the longevity, age of first reproduction (AFR), total eggs/female and mean brood size. LOEC and NOEC was calculated by testing the responses in each concentration group and comparing responses with those of the control group (Dunnet test). The subchronic value (SChV) was computed as the geometric mean of the highest NOEC and the lowest LOEC values. The SChV is an estimate of the chronic value and represents the hypothetical toxic threshold between the NOEC and LOEC for a given endpoint [22].

3. RESULTS AND DISCUSSION

3.1. Water quality parameters

The mean values (and ± SD) registered to water quality parameters throughout the toxicity tests were: dissolved oxygen: 6.4 (±0.8) mg l^{-1}; pH: 8.39 (±0.24); conductivity: 245.33 (±28.18) µS/cm; nitrates: <0.1 (±1.2) mg l^{-1}; nitrites: 0.01 (±0.01) mg l^{-1}; ammonium: 0.29 (±0.13) mg l^{-1}; chlorides: 3.5 (±4.3) mg l^{-1}; sulphates: 8.3 (±0.75) mg l^{-1}; total alkalinity: 77 (±8) mg l^{-1} $CaCO_3$; bicarbonates: 94 (±3) mg l^{-1}; sodium: 7.7 (±1.85) mg l^{-1}; magnesium: 6.8 (±2.5) mg l^{-1}; calcium: 12.9 (±3.45) mg l^{-1}; potassium: 1.8 (±0.2) mg l^{-1}; COD: 10 (±1.48) mg l^{-1}; BOD: 0.08 (±0.02) mg l^{-1}. Measured toxicants concentrations for the experimental test conditions were 75.69 (±27), 95.6 (±54) and 80 (±20) % of nominal concentrations for copper, chromium and endosulfan, respectively.

3.2. Acute toxicity tests

No mortality was observed in controls at the end of the 48 hours assays, but both metals, copper and chromium, and the pesticide had a direct toxic effect on the survival of *P. variabilis* at tested concentrations. The EC_{50s} and their 95% confidence limits at 24 and 48 h are shown in Table 1.

TABLE 1 - 24 and 48 h EC_{50} values for copper, chromium and the insecticide-endosulfan. The values are based on the inmobility and/or absence of responses to mechanical stimulation, n= 20 for each treatment. The 95% confidence limits are given in parentheses.

	EC_{50} (µg l^{-1})	
	24 h	**48 h**
Copper	29 (17.7-29.8)	12 (9.4-15.4)
Chromium	133.21 (99.55-181.59)	52.47 (18.66-141.4)
Insecticide (endosulfan)	1.754 (0.67-7.86)	1.041 (0.59-1.76)

Although no available information exist about the acute toxicity of copper, chromium or the pesticide Zebra Ciagro® to other cladocerans from the Suborder Ctenopoda (Family Sididae), values obtained in this assays can be compared with information on different organisms of the Suborder Anomopoda (Family Daphniidae). The cladocera *D. magna* registered acute values from 60 to 210 µgl^{-1} for chromium and from 1.4 to 18.5 µgl^{-1} for copper [23-28]. In case of endosulfan, the 48 h LC_{50} was 62 µgl^{-1} [29].

The 48 h LC_{50} to *Ceriodaphnia dubia* for chromium was 144 µgl^{-1} [30] and for copper, from 35 to 79 µgl^{-1} [31]. However, Gagneten and Vila [8] found lower acute values for copper, ranging from 5 to 20 µgl^{-1} at different levels of pH. The acute toxicity of endosulfan in the same specie varies from 53.3 to 490 µgl^{-1} [32; 33].

Information regarding the toxicity to freshwater copepods is scarce, but Wong and Pak [34] found 48 h LC_{50} to the nauplii *Mesocyclops pehpeiensis* of 75 µg copper l^{-1} and 510 µg chromium l^{-1}. Adults of *Cyclops abyssorum* registered 48 h LC_{50} to chromium and copper of 10000 and 2500 µgl^{-1}, respectively and *Eudiaptomus padanus* registered 48 h LC_{50} values to Cr and Cu of 10100 and 500 µgl^{-1}, respectively [35]. A representative calanoid copepod from the Neotropical region, *Notodiaptomus conifer,* registered 48 h LC_{50} values ranging from 42 (to nauplii) to 62 µgl^{-1} (to adults) for copper and 170 (to nauplii) and 230 µgl^{-1} (to adults) for chromium [36].

Among higher crustaceans, *Gammarus pseudolimnaeus* registered values between 94.1 and 67.1 µg chromim l^{-1} [37]. Regarding the effects on decapods, in case of copper *Macrobrachium rosenbergii*, Li et al. [38] registered values between 530 and 450 µgl^{-1}. To endosulfan, *Palaemonetes argentinus* registerd 48 h LC_{50} between 14.1 and 6.28 µgl^{-1} [39], *Trichodactylus borellianus* between 1827.66 and 1984.26 µgl^{-1} [40] and the prawn *Macrobrachium malcolmsonii* from 0.16 to 0.19 µgl^{-1} [41; 42].

The above comparisons indicated that lethal toxicity to *P. variabilis* for both metals and the insecticide with endosulfan occurred in general at lower concentrations than those reported to different freshwater crustaceans. Although further information is needed about the responses of other freshwater organisms to copper, chromium and endosulfan, the high sensitivity of *P. variabilis* make it an appropriate species to be used as a test organism in ecotoxicological studies. Moreover, considering the particular complexity and heterogeneity of littoral aquatic environments [43], the use of *P. variabilis* in ecotoxicological tests, as representative of such areas, will allows to obtain more suitable information about the real ecological damage of pollutants in that regions.

3.3. Chronic toxicity tests

Figure 1 shows the survival curves of the organisms from the control group and each treatment with copper, chromium and endosulfan, during the first 35 days. *P. variabilis* exposed to copper registered a high significant mortality (from 16 to 60 %) in the three higher concentrations (KW=17.23, p=0.001), however, the groups exposed to chromium and endosulfan registered a significant mortality only in the higher concentrations (55% and 32 % respectively) (chromium KW=61.39, p< 0.001; endosulfan KW=33.97, p<0.001).

Mean longevity in the control group was 32.5 days. This endpoint was negatively affected in organisms exposed to all copper concentrations, reducing it between 20 and 24 days (ANOVA, F=122, p<0.01). In case of chromium, longevity was significantly reduced to 20 days in the highest concentration (Dunnet=2.821, P<0.05). The life span of the organisms exposed to endosulfan was reduced to 25.9 and 25.3 days in the two highest concentrations, however no statistical significant differences were found in relation to controls (ANOVA, F=7.37, p>0.05).

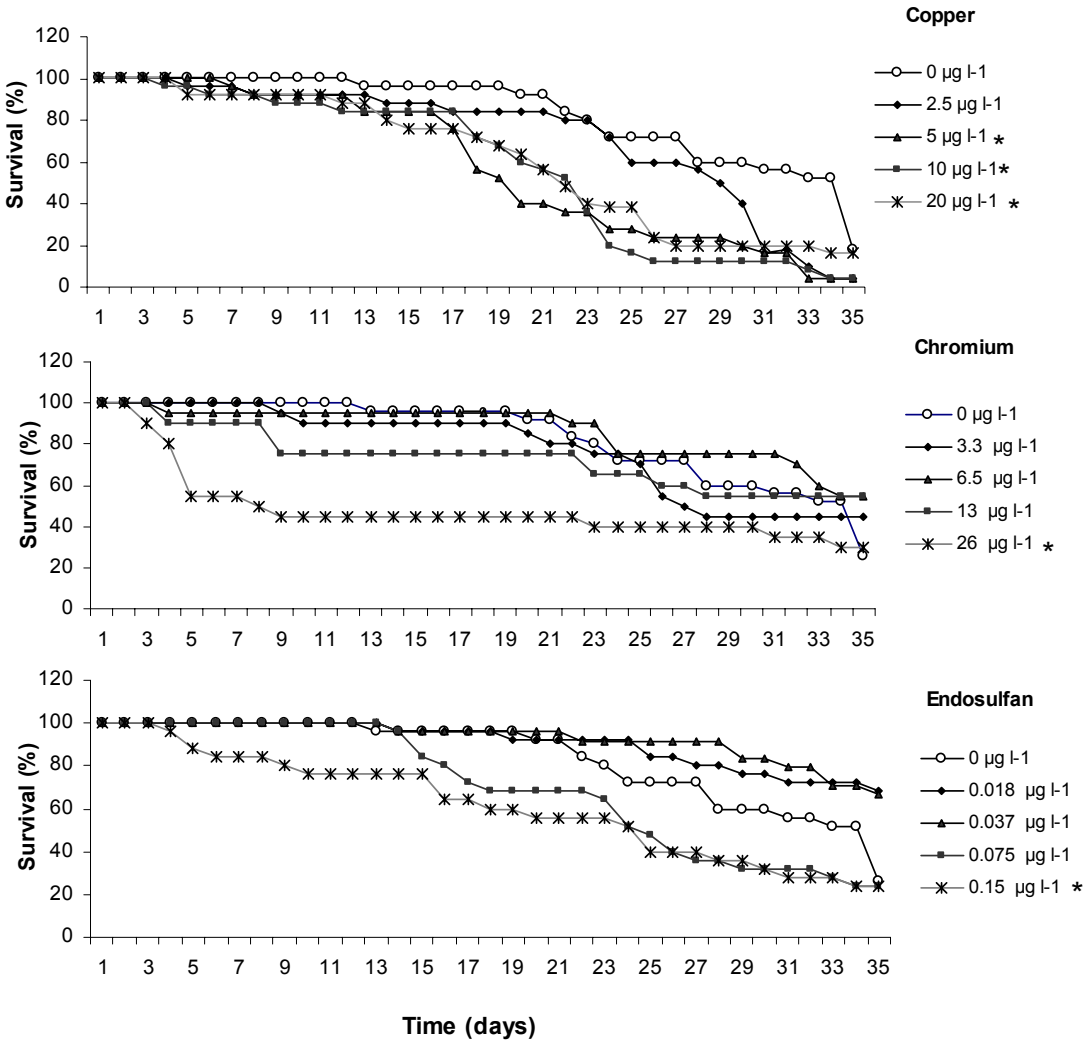

FIGURE 1 - Survival curves of *P. variabilis* from the control group and each treatments with copper, chromium and endosulfan, during the first 35 days. The panels shows mean values (n=25). The asterisks indicate a significant difference between control and treatment (p<0.05; Dunn's multiple comparisons test).

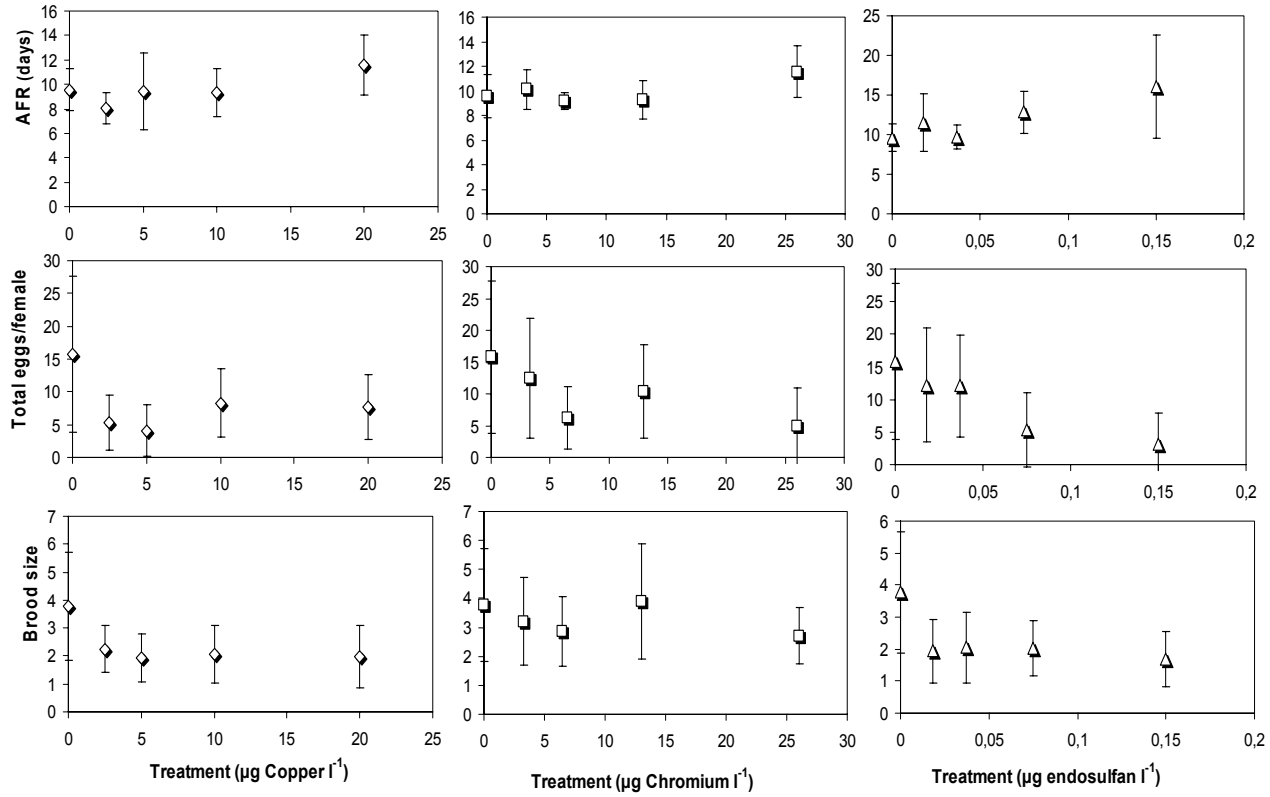

FIGURE 2 - Reproductive endpoints of *P. variabilis* exposed to four concentrations of copper (left panels), chromium (central panels) and the pesticide with endosulfan (right panels). Error bars represent ± SD (n=25).

Among reproductive parameters, age of first reproduction (AFR) was the least affected. Control organisms reproduced for the first time, in average, at the 9.6 day. The presence of copper and chromium delayed significantly this trait only in the highest concentration (copper: Dunnet= 3.089, $p<0.01$; chromium: Dunnet=3.88, $P<0.01$), but the insecticide delayed it in the two highest concentrations (Dunnet= 3 and 5.7 respectively; $p<0.05$) (Figure 2).

The total number of eggs per female was significantly reduced in all concentrations of copper (Dunnet=5.4, 6.1, 3.9, 4.2, $p<0.01$ in all cases), the concentrations 6.5 and 26 μgl^{-1} of chromium (Dunnet= 3.7 and 4.2, respectively; $p<0.05$ in both cases) and the two highest concentrations of endosulfan (Dunnet=4.5 and 5.4 respectively; $p<0.01$) (Figure 2).

The mean brood size was significantly lowered in all copper and endosulfan concentrations (Dunnet $p<0.01$, in all cases). In case of chromium, only 6.5 and 26 μgl^{-1} were significantly different from control (Dunnet=3.02 and 3.46 respectively; $p<0.05$ in both cases) (Figure 2).

Table 2 shows Ro, T and *r* values. The net reproductive rate (Ro) decreased in the presence of the three toxics, but the most important reduction was clearly observed in the organisms exposed to all copper concentrations and in the higher concentrations of chromium and endosulfan.

Generational time (T) did not show important variations in the organisms exposed to both metals. However, a delay between 7 to 11 days was observed when organisms were exposed to all endosulfan concentrations. The intrisic rate of natural increase (*r*) was positive in all cases ($r>0$), indicating that in spite of the toxicity, the population exposed did not suffer a decrease during the experimental period. Nevertheless, it could be tested that the highest concentration of endosulfan caused an important decrease in *r*.

At present, many ecotoxicological studies, based on several life history traits of the species, frequently conclude that the estimate of intrinsic rate of natural increase (*r)* is the ultimate parameter of interest as integrates all components of the life history. Additionally, it also has been widely used for many authors as a measure of fitness since Birch [44]. However, the results obtained in this work allow to recognize that although most life cycle parameters of *P. variabilis* were affected, the single statistician *r* was not altered by copper and chromium.

According to the ecophysiological theories [45], it has been reported that under stress situations, the organisms could establish *trade offs* between conflictive energetic demands in order to maintain their fitness [46]. In this sense, it is possible to hypothesize that the maintenance of *r* would be the result of multiple energetic compensations between survival, longevity and reproduction.

TABLE 2 - Net reproductive rate (Ro), generational time (T) and the intrisic rate of natural increase (*r*) values of *P. variabilis* exposed to four concentrations of copper, chromium and the insecticide-endosulfan.

	Ro	T	*r*
Copper (µg l^{-1})			
0	15.57	15.49	0.18
2.5	5.28	10.53	0.16
5	4.05	14.70	0.09
10	8.24	14.07	0.15
20	6.95	16.25	0.12
Chromium (µg l^{-1})			
0	15.57	15.49	0.18
3.3	12.08	17.96	0.14
6.5	6.25	11.91	0.15
13	10.40	11.75	0.19
26	4.89	13.06	0.12
Endosulfan (µg l^{-1})			
0	15.56	15.49	0.18
0.018	12.08	26.67	0.09
0.037	12.58	22.79	0.11
0.075	5.32	22.51	0.07
0.15	3.12	26.20	0.04

TABLE 3 - LOEC, NOEC and SChV values (in µg l^{-1}) calculated to four life cycle endpoints of *P. variabilis*: longevity, age of first reproduction (AFR), total eggs per female (eggs/female) and mean brood size.

Endpoint	Chemical	NOEC	LOEC	SCh V
longevity	Copper	<2.5	2.5	<2.5
	Chromium	13	26	18.4
	Endosulfan	0.15	>0.15	<0.15
AFR	Copper	10	20	14
	Chromium	13	26	18.3
	Endosulfan	0.037	0.075	0.053
Eggs/female	Copper	<2.5	2.5	<2.5
	Chromium	3.3	6.5	4.6
	Endosulfan	0.037	0.075	0.053
Mean brood size	Copper	<2.5	2.5	<2.5
	Chromium	3.3	6.5	4.6
	Endosulfan	<0.018	0.018	<0.018

From the obtained results and taking into account this consideration, we can conclude that the parameter *r* is not always the most appropriate endpoint of toxicity, and more information about individual and populational impacts should be considered in order to achieve better conclusions. For example, in this work, a detailed analysis of each life history trait of *Pseudosida* showed that both metals had negative effects on important life history parameters which could, in a long term, generate negative population consequences in nature [47].

The aforementioned assumption is in accordance with many authors, which suggest that *r* is not always the most sensitive parameter in toxicity tests [48; 49]. Moreover, other authors suggests that *r* is not as sensitive as growth or reproduction [50-52], and they discuss the needed to obtain a better screening of pollutants toxicity by means of using multiple biological endpoints and an extended exposure time.

When the life cycle of *P variabilis* exposed to endosulfan was analyzed, a different pattern was observed. Mean brood size and *r* was severely affected, however survival, longevity and the other reproductive parameters were moderately altered. This may indicate that accumulation patterns and toxicity mechanisms would be different from metals.

In relation to the parameter *r*, many authors have emphasized on the ecological relevance of a reducing it near-zero by chronic stress, and it is suggested that the main consequence resides in the high possibility that population would disappear in a middle or long term [47].

The mean generation time was another parameter of *P. variabilis* highly affected when exposed to the pesticide-endosulfan. Despite the fact that concentrations were lower than lethal doses, this product would probably be affecting some physiological molt processes [53]. The consequences have been studied by Allan and Daniels [54],

who have demonstrated through mathematical models that the effect of the increase in generational time is an important reduction in the size of populations exposed to contaminants.

Despite T and *r* are key controlling traits of the species life cycles, it is no possible to make predictions about the influence of their changes on the community level [8]. Complex relations exist between species composition and the functional attributes of communities, especially if the littoral ones are considered [43]. Nevertheless, more information about the responses of other associated organisms to direct and indirect effects of pollutants may allow to have more real interpretations of the impact of pollutants on the community.

Table 3 shows the LOEC, NOEC and SChV values to each endpoints of *P. variabilis* life cyle. Field records of total Cu and Cr concentrations in natural environments of South America, show values of 11.3 μg l^{-1} and 8.3 μg l^{-1} respectively [4]. On the other hand, endosulfan concentrations in water bodies, near rice fields from the same region, ranged from 0.2 to 13.5 μg L−1 [55]. A comparison between these field data and the SChV of *P. variabilis* reveals that their populations could be greatly affected, especially considering that the longevity and reproductive parameters are main sensitive endpoints.

4. CONCLUSIONS

Data obtained from this study show that both metals (copper and chromium) and the insecticide-endosulfan are very toxic to several parameters of *P. variabilis* life history. It is probable that the latter mentioned substance has a different pattern of action with important energetic costs that can cause severe decline in *r*.

The calculated LOEC, NOEC and SChV indicate that *r*, survival, longevity, age of first reproduction and mean brood size are suitable endpoints to be analyzed. A comparison with field data indicates that *P. variabilis* populations could be seriously affected.

The highly sensitivity of this cladocera make it not only an appropriate species to be used as a test organism, but also a species of ecological relevance to littoral regions. However, this evaluation should be continued with multigenerational studies in order to avoid underestimate the effect of the chemicals [56] and validated with macroscale and multispecific assays to reach representative conclusions [57].

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