

## Synergistic Effects of Copper and Butylic Ester of 2,4-Dichlorophenoxyacetic Acid (Esternon Ultra) on Amphibian Embryos

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**Abstract:** Cu<sup>2+</sup> and butylic ester of 2,4-Dichlorophenoxyacetic acid as Esternon Ultra (2,4-D) toxicity on *Bufo arenarum* embryos were evaluated by means of a short-term chronic toxicity test (AMPHITOX). The NOEC values for Cu and 2,4-D were 0.02 mg/L and 2 mg/L respectively. The toxicity profile curves for Cu and 2,4-D were reported. The interactions of the metal and the herbicide were evaluated by combined treatments with different concentrations of Cu and 2,4-D. Although in all cases, a synergistic effect between these chemicals was observed, the combination of concentrations exerting low level effects in isolated treatments resulted in more adverse embryonic survival. Considering that both products are extensively used in agroecosystems, this fact could be of concern for non target species like amphibians.

**Keywords:** 2,4-D, copper, amphibian embryos, synergistic effects.

### Introduction

Copper and butylic ester of 2,4-Dichlorophenoxyacetic acid as Esternon Ultra (2,4-D) are extensively employed as herbicides in the agroecosystems. Cu, an essential element, is involved both in the structure of several proteins and as enzymatic cofactor. At least 12 proteins require Cu in their structure [1]. However, in spite of its essential properties, concentrations of this heavy metal slightly higher than the homeostatic ones produce significant toxic effects mainly on reproductive processes, behaviour, skeleton and skin of different organisms. Concentrations as low as 1-2 µg Cu /L produce adverse effects on aquatic biota [2]. In this context, the Cu concentrations employed as herbicides could represent a risk for “no target” organisms [2, 3]. The main adverse effects of Cu at biochemical level occur on the structure and function of DNA and proteins, both in a direct way or mediated by means of oxidative stress mechanisms [4].

2,4-D is a phenoxyacetic herbicide that integrates one of the largest groups of herbicides sold in the world,

widely used in agriculture and forestry to destroy broad leaved weeds [5]. Although its higher toxicity is on autotrophic organisms there are numerous reports of adverse effects on a wide diversity of heterotrophic species [2]. Gorzinski et al showed a significant diminution in the gain weight and functional and structural alterations of kidneys of rats subchronically treated with 2,4-D (15-150 mg/kg/day for 13 days) [6], and it was also reported that different 2,4-D formulations produce neurotoxic effects such as ataxia and failures in neuromuscular coordination [7, 8] probably related to changes in various neurotransmitter systems, such as serotonin and dopamine [9]. Its oxidative stress effects were reported as the diminution of glutathion and thiol-proteins, lipoperoxidation [10], increases in superoxide dismutase activity, changes in catalase, glutathione peroxidase and Glutathione S-transferase activities [11]. It has also a desacoplant effect on the oxidative phosphorylation in mitochondria [12-13], and its genotoxicity was reported as chromatid and chromosome breaks, number of micronuclei and nuclear buds [5]. Most of these studies were conducted with 2,4-D in its technical grade form. In order to provide

more relevant information for environmental conditions, in this study we employed “Esternon Ultra” a commercial formulate of 2,4-D because it is well known that the formulated herbicide exerts higher toxicity than its corresponding technical grade chemical.

Since the toxicity of a substance or complex mixture depends on the concentration and exposure time, threshold values taking into account these parameters are reported as Toxicity Profile curves (TOP) providing, within a systemic toxicity approach, a more appropriate set of data for hazard and risk assessment purposes [14-3]. Taking into account that the two chemicals could be frequently present in the same agroecosystems, and both exert oxidative stress, it could be relevant to know the toxicity of these chemicals in simultaneous exposure conditions. In this study we report the toxicity of copper and 2,4-D as TOP curves and evaluate the interaction between these chemicals on *Bufo arenarum* embryonic survival by means of the short-term chronic AMPHITOX toxicity test [15-16]. The AMPHITOX test includes four different possibilities to evaluate adverse effects exerted by physico-chemical agents, including teratogenesis [15-16]. The short-term chronic AMPHITOX toxicity test is not appropriate for teratogenic studies because the embryonic stage employed is post early morphogenetic processes; however it is useful to evaluate lethality as well as synergistic or antagonistic effect because the susceptibility of the embryo to noxious agents remains almost constant during the whole test period.

## Material and Methods

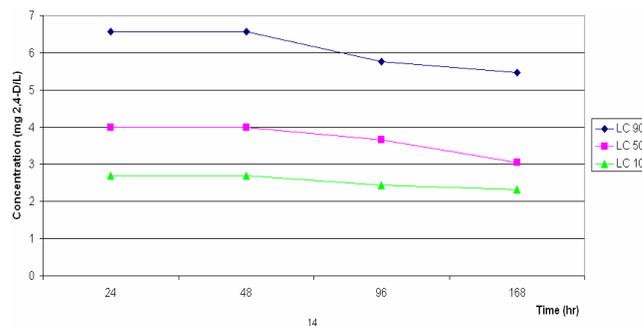
Ovulation of *Bufo arenarum* females was induced by an i.p. injection of homologous hypophysis preserved according Pisanó [17]. Oocytes were fertilized “in vitro” with a sperm suspension in AMPHITOX Solution (AS); composition (in mg/L): NaCl: 36; KCl: 0.5; CaCl<sub>2</sub>: 1 and NaHCO<sub>3</sub>: 2. Embryos were maintained in AS at 20±1°C until the complete operculum stage (S.25), [18]. At this developmental stage embryos were selected for the toxicity study conducting a 7 day-exposure test (short-term toxicity of AMPHITOX) [16]. Duplicate batches of 10 embryos were placed in covered 10cm glass Petri dishes containing 40 mL of AS with copper, butylic ester of 2,4-Dichlorophenoxyacetic acid as Esternon Ultra (2,4-D) and copper plus 2,4-D in different concentrations at 20±1°C. Chemical concentrations were selected according to results obtained in preliminary studies (NOEC values for 2,4-D and Cu<sup>2+</sup> for 7 days of exposure were 2 mg/L and 0.02 mg/L respectively). To evaluate Cu and 2,4-D toxicity, *Bufo arenarum* embryos were treated with Cu<sup>2+</sup> (in mg/L): 0.03; 0.04; 0.06; 0.075 and with 2,4-D (in mg/L): 2.5; 3.5; 4.5. The toxicity of Cu and 2,4-D was reported as TOP curves from 24 and up to 168 hr. To evaluate the combined effects of the two chemicals, embryos were exposed to solutions containing Cu and 2,4-D in all the possible combinations of the concentrations stated above. Controls were duplicate groups of 10 embryos maintained in AS without

additions. Survival was evaluated hour by hour during the first 12 hr of treatment and then each 24 hr up to 168 hr (7 days). Experimental solutions were prepared in AS from stock solutions of Cl<sub>2</sub>Cu (385 mg Cu<sup>2+</sup>/L), measured by means of atomic absorption spectrophotometry with flame and the commercial formulate herbicide “Esternon Ultra” (butylic ester of 2,4-dichlorophenoxyacetic acid: 797 g Eq. ac/L). Lethal concentrations were obtained by means of PROBIT analysis [19].

## Results and Discussion

AMPHITOX is a standardized test employing amphibian embryos that can be used to evaluate toxicity for acute, short-term chronic, chronic, and early life stage exposure to hazardous substances and samples [15]. By means of AMPHITOX the toxicity of different substances and environmental samples such as surface, groundwater, soils, leaches and industrial effluents can be evaluated by adjusting the exposure period to the toxicity of the sample [16-20].

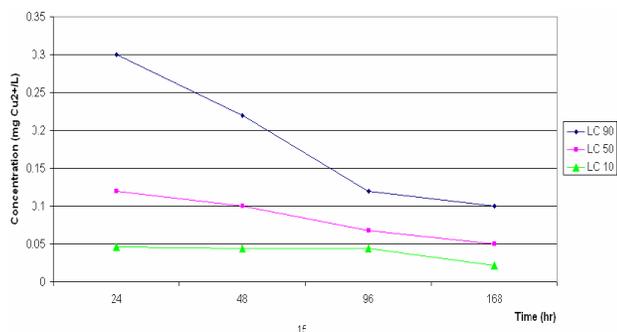
Figure 1 shows the TOPs curves of Cu from 24 to 168 hr for *Bufo arenarum* embryos. For copper, the LC<sub>50</sub> 24 and 168 hr were 0.12 and 0.05 mg Cu<sup>2+</sup>/L respectively. The LCs are within the range obtained in previous studies [3-21]. The LC<sub>10</sub> for 168 hr resulted in 0.022 mg/L. For freshwater invertebrates, 48-h L(E)C<sub>50</sub> range from 5 µg Cu/L for a daphnid species to 5300 µg Cu/L for an ostracod [2]. For freshwater fish 96-h LC<sub>50</sub>s range from 3 µg Cu/L (artic grayling) to 7340 µg Cu/L (bluegill), [2]. Although the concentrations exerting adverse effects on *Bufo arenarum* embryos are within the range reported as maximal value found in unpolluted aquatic ecosystems, that is from 1 to 20 µg Cu/L [22], the bioavailability of copper as well as antagonistic effect of other compounds like Zn [3] could explain the presumably no adverse effect on amphibians in those pristine environments due to copper toxicity.



**Figure 1:** Toxicity profile (TOP) curves of copper for *Bufo arenarum* embryos at complete operculum stage (S.25).

Figure 2 shows the TOPs curves of 2,4-D from 24 to 168 hr for *Bufo arenarum* embryos. The LC<sub>50</sub> 24 and 168 hr resulted in 4 and 3 mg 2,4-D/L respectively. Morgan et al., reported for another amphibian, *Xenopus laevis*, a LC<sub>50</sub> of 254 mg/L 2,4-D [23]. This value, almost 70 times higher than the obtained for *Bufo arenarum* embryos in this study, could be related to different susceptibility inherent to each

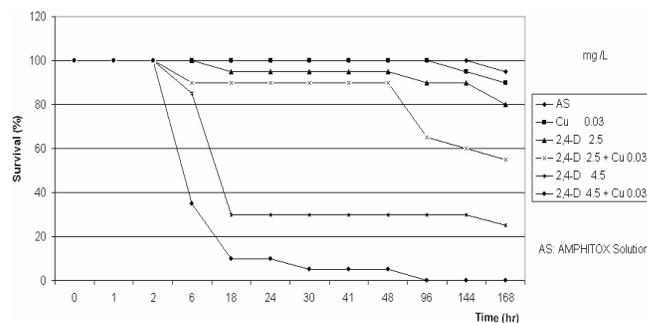
species. In a recent contribution, the difference in the susceptibility to physico-chemical stress was partially related to the record in living organism of evolutionary events during their phylogenetic process [24]. In addition, the fact that the *Xenopus laevis* study was conducted with FETAX [25], a different toxicity test, could also explain at least partially this difference by taking into account: i) the high salinity of the solution employed in FETAX test which usually reduce toxic effects [16]; ii) FETAX is a 96 hr test conducted at  $24\pm 2^{\circ}\text{C}$  while the AMPHITOX test employed is for 168 hr at  $20\pm 1^{\circ}\text{C}$ . In mammals, different formulations of 2,4-D administered in unique oral doses, result in a  $\text{LD}_{50}$  of 553-1090 mg/kg depending on its chemical form, while the NOEL value was 15 mg/kg/day [6]. As a whole, for *Bufo arenarum*, the results point out that copper is very significantly more toxic than 2,4-D (for example, 60 times at 168 hr of exposure).



**Figure 2:** Toxicity profile (TOP) curves of 2,4-D for *Bufo arenarum* embryos at complete operculum stage (S.25).

Figure 3 shows examples of the interactions evaluated for both chemicals (2,4-D: 2.5 and 4.5 mg/L with Cu: 0.03 mg/l) on the survival of *Bufo arenarum* embryos allowing to easily appreciate the pattern of Cu and 2,4-D synergistic effect. For example, for the more lower concentrations of both chemicals, at 168 hr of exposure, the toxicity increased approximately twice respect to the corresponding additive effect while for the highest concentrations evaluated, an earlier toxic effect was registered with an average increment of 20% of lethality from 18hs of exposure onwards. The synergistic effect of  $\text{CuCl}_2$  and a substance with 2,4-D (the Dimethylammonium 2,4-dichlorophenoxyacetate (2,4-D.DMA), usually knows as U46 D Fluid was also demonstrated *in vitro* employing human fibroblasts. In that study, the pretreatment with copper in subtoxic (or very slight toxic) concentrations did not affect cellular survival and the capability to generate colonies. However the treatment with both chemicals exerted cellular growth inhibition, diminution in the DNA synthesis and failure in the DNA reparation process, alterations that indicate a synergistic effect [26]. In a subsequent study the same formulation showed a synergistic effect with copper on DNA structure. Whereas 2,4-D.DMA alone or

$\text{CuCl}_2$  alone did not show any or only a negligible effect, 2,4-D.DMA plus  $\text{CuCl}_2$  induced strand breaks in PM2, probably by means of redox reaction of Cu (II) and 2,4-D [27]. Ferri et al showed in pups exposed to 2,4-D through dam's milk, that copper was the most altered ion (among others as Fe and Zn), increasing its level in serum, liver and some brain areas and decreasing in whole brain. A general decrease in the dam's body brain and liver weight was also reported [28]. They also observed that undernourished pups were more vulnerable to the 2,4-D effects. The interaction of Cu and 2,4-D at enzyme level was reported in the mechanisms involved in the detoxification of the herbicide by a TfdA, a non-heme iron enzyme which catalyzes the first step in the oxidative degradation of 2,4-D [29].



**Figure 3:** Survival of *Bufo arenarum* embryos treated with 2,4-D and copper evaluated by means of the short-term toxicity of AMPHITOX test.

The main purpose of this study was to report lethality and the eventual interactions of Cu and a formulate of 2,4-D in a native amphibian specie from South America. Thus, the AMPHITOX test selected (short-term chronic), although appropriate for that purpose is not indicated for teratological studies. It is noteworthy that both chemicals exert teratogenic effects in different species including mammals and amphibians. For *Xenopus laevis* the  $\text{EC}_{50}$  was 0.16 mg  $\text{Cu}^{2+}/\text{L}$  with eye, gut, facial, notochord, and cardiac anomalies [30], while in mammals (e.g., mice), there are studies reporting adverse effects at developmental stages such as reduced body weight, hydrocephaly, encephalocoels, abnormalities of the ribs and vertebrae, from 1.3 up to 159 mg  $\text{Cu}/\text{kg}$  body weight per day [31, 32].

There is an increasing concern due to the decline of amphibian populations and the large number of malformations found in many geographic regions, a fact very probably related to the multiple developmental toxicants due to anthropogenic activities that decrease water quality [33-36]. For instance, by means of early life stage toxicity test of copper to endangered and surrogate species, a safety factor of 0.5 was recommended to apply to the current chronic water quality criterion (WQC) values in order to protect the most sensitive fishes [37]. A similar criterion could be of high value for amphibian species. With respect to 2,4-D, although there is very limited information on its levels in surface waters in South America, in the case of the Traiguén river basin in Southern Chile in October,

2003 it was registered 2.9 µg/L of this herbicide which is three orders of magnitude below the lethal concentrations for *Bufo arenarum* embryos as obtained in this study [5].

Amphibian embryos can be more susceptible to chemical stress than fishes [38] and moreover some studies point out that even water quality features in unpolluted places could be also related to adverse effects on amphibian embryos [39-40]. Taking into account that 2,4-D and copper could be employed in the same environmental scenarios, the information on their synergistic effect on no target species as in this case *Bufo arenarum* embryos, could be valuable for the protection of endangered species and for more customized environmental risk assessment.

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