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Sensitivity of different stages of *Artemia franciscana* to potassium dichromate

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Abstract. Toxicity bioassays were carried out in order to determine the LC50 of potassium dichromate in fifteen stages of *Artemia franciscana*. Seven concentrations (250, 100, 50, 25, 20, 10 and 5 µg/mL) of potassium dichromate were used. The results show statistical differences between the LC50 for nauplius, metanauplius, juvenile and adults of artemia. The use of stages 2 and 3 is recommended because are faster to obtain and show an average sensitivity of the nauplius II and metanauplius .

Key words: toxicology, instar II, instar III, model organism, standardization

Resumen: Sensibilidad de diferentes estadios de *Artemia franciscana* al dicromato de potasio. Se realizaron bioensayos de toxicidad en quince estadios de *Artemia franciscana* con siete concentraciones (100, 50, 25, 20, 10 y 5 µg/mL) de dicromato de potasio. Los resultados muestran diferencias estadísticas las CL50 de los diferentes estadios que corresponden con los cambios morfológicos principales en el desarrollo de la Artemia nauplios, metanauplios, juveniles y adultos. Se recomienda el uso de los estadios 2 y 3 porque son más rápidos de obtener y presentan una sensibilidad promedio de los nauplios II y metanauplios.

Palabras clave: toxicología, instar II, instar III, organismo modelo, estandarización

Ecotoxicology is a scientific branch that analyzes the effects of a pollutant (physical or Chemical) on an organism or ecosystem. Studies of toxicity in the development of organisms are used to determine the effects of pollutants such as abnormalities in development or mortality. Through the analysis of the relationship between increasing concentrations and its effects, the median effect or lethal concentrations (LC50) are established (Serrano- Gallego 2003, García-González *et al*. 2006, Yu and Lu 2018). Several organisms are used as a model to perform toxicity tests, the use of mammals such as mice, rats, guinea pigs, rabbits (Hernández 2006) and pigs is common (Gullace and Caturini 2012). However, the reduction of their use is promoted by replacing them, among others, by fish embryos as *Danio rerio* (Muth-Köhne *et al*. 2013), invertebrates as *Daphnia* sp. (Díaz et al 2004) and artemias (Libralato *et al*. 2016, Yu and Lu 2018).

The use of the genus *Artemia* for acute and developmental toxicity tests usually includes the *Artemia franciscana* and *Artemia salina* species that are crustaceans belonging to the Branchiopoda Class, Anostraca Order, Artemiidae Family. *Artemia* have a brown color and soft body, their reproduction can be ovoviviparous, or oviparous (determined by salinity, pH and temperature) (Naceur *et al*., 2011). Oviparous reproduction occurs when adverse environmental conditions are present, embryonic development stop in gastrulation, and cysts are produced and released. Full development of embryo and hatching occurs only when conditions are optimal. Artemias grow up to 10 mm in length through 15 molts with four mainly distinguishable life stages nauplii, metanauplius, juvenile, and

adults; throughout their lives feed on protozoa, microalgae, and bacteria (Sorgeloos *et al*. 1986).

Artemia is so far the only organism with a cryptobiotic stage (cysts), which is widely distributed commercially with low cost. The cysts allow a constant availability of organisms because they hatch in a wide range of temperatures and salinities of artificial seawater (Vega-Villasante *et al*., 2013) or in NaCl solutions (Rahman *et al*., 2013). These characteristics allow the widespread use of Artemia in the toxicological evaluation of pesticide chemical compounds and natural medicines in short and long term, acute and chronic toxicities, respectively (Sorgeloos *et al*. 1978, Libralato *et al*. 2016, Yu & Lu 2018). The acute toxicity is determined with the median lethal concentration test (LC50) which is the concentration of the substance that kills 50% of the organisms at a given exposure time. With this test, the toxicity of diatoms (Caldwell *et al*. 2003), heavy metals (Jiménez *et al*. 2006), plant extracts (Fernández-Calienes *et al*. 2009), cyanobacteria (Hisem *et al*. 2011) and fungi were evaluated (Vega-Villasante *et al*. 2013), among others. Furthermore, the LC50 in *A. salina* of plants extracts correlates with the median lethal dose (LD50) in mice (Lagarto *et al*. 2001). Additionally, the use of this genus is according to the directives that point the use of invertebrates, for example, Council Directive 86/609/EEC of November 24th, 1986. All above make, the genus *Artemia* as a good replacement in toxicity tests.

Potassium dichromate (PD) is a reddish-orange, water-soluble crystalline solid, which often used as a reference toxic substance. González-Pérez & Aportela-Gilling (2001) evaluated the ecotoxicological impact that PD has on the environment and determined acute toxicity in *A. salina* nauplii. With PD as reference Hondal *et al*. (2003), run toxicity tests in *A. franciscana*, of surface sediment extracts from nine sites of the Cauto hydrological basin in the Gulf of Guacanayabo, Cuba. In the same way, Moshafi *et al*. (2010) carried out bioassays on the cytotoxicity of essential oils and several extracts of the plant *Heracleum persicum* on *A. salina* and used PD as a positive control. However, studies using *Artemia* as a test organism and PD as a positive control, do not always mention the specific life stage of this crustacean in the bioassays, even though they are distinguishable with an available, usual and simple methodology. There are different values reported of LC50 of the PD in *A. franciscana*, probably by the sensitivity of each developmental stage of this organism. The objective of this work is to determine the LC50 of the DP in the fifteen life stages of *A. franciscana* to establish the sensitivity of each of them, and to determine if there is a significant difference of the LC50 between the stages evaluated. In order to standardize toxicity tests in relation to the life stage of Artemia.

The toxicity bioassays were carried out in the Laboratorio de Calidad de Agua y Acuicultura Experimental (LACUIC) located at the Centro Universitario de la Costa, of the Universidad de Guadalajara campus in Puerto Vallarta. Artificial seawater was made with commercial salts (Red Sea®) at 40 psu (practical salinity units), which is suitable for the *A. franciscana* (34-55 psu) (Soundarapandian & Saravanakumar 2009). To obtain organisms, cysts of the trademark Biogrow were used with an average hatching range of 225,000 nauplii / g.

Artemia franciscana cysts (0.1g) were hydrated in 5 mL of artificial seawater for 5 minutes. Then were transferred to a vessel with a volume of 1 L of seawater with constant illumination (fluorescent light 60 watts), aeration with an air pump (Elite ®), and with heater with thermostat (Elite Radiant ®), which kept the water temperature at 28.0 ± 1.0 ° C for 24 hours. Then, the nauplii were collected and put in a plastic container with 20 L of artificial seawater, at the same previous conditions. After 24 h of hatching, the organisms were fed *Spirulina maxima* dehydrated at 10 mg / L. The organisms were collected of this container and used for the bioassays of LC50. The organisms taken between 0 and 24 hours after hatching were defined as stage 1, between 24 and 48 hours as stage 2, and so on. A theoretical stage was established every 24 hours, until reaching the 15 mentioned stages.

Potassium dichromate solutions were prepared at 250, 100, 50, 25, 20, 10 and 5 µg/mL in artificial seawater at 40 psu in 10 ml in test tubes per sextuplicate (Vega-Villasante *et al*., 2013). As a negative control, artificial seawater at 40 psu was used. Ten *A. franciscana* organisms of each development stages were introduced per test tube. At the end of 24 h of exposure, dead organisms were recorded (no movement of appendages for 10 seconds). No food was added during the exposure to avoid interactions between the PD and the spirulina.

LC50 was calculated with of probit regression using IBM® SSPS® Statics software, as well as the standard error of the LC50 for the stages following formula indicated by Randhawa (2009). In order to

evaluate the differences between the LC50 of the different stages, the confidence interval at 95 % of estimated LC50 with the standard error was used.

Table I shows the PD LC50 in the stages of development of *A. franciscana*. The LC50 is variable along the different stages of its development. At the first 24 hours after hatching, the LC50 is one of the highest found in this study (21 µg/mL). Afterward decreases to 15 µg/mL at the second stage and remains unchanged until the fifth stage, with no significant differences. In the sixth and seventh stages, an LC50 increase to 21 µg/mL with a further increase to 25 µg/mL at the eighth stage, which represents the peak value of the LC50 in this study. A decreasing trend in LC50 in the 10th and 11st stages with values of 21 and 16 µg/mL, respectively, was observed. From the 12nd to the 15th stage, the LC50 values were not significantly different between them.

Table I. Median lethal concentration 50 LC50 of potassium dichromate on the stages of *Artemia franciscana.* The stages enclosed in each square have LC50 no statistically different between them.

The variations in LC50 on the studied stages (a theoretical stage every 24 hours) show a correspondence with the major morphology stages described for Artemia development. Nauplius I, which does not have the mouth and neither anus, showed one of the lowest sensibility to PD. Nauplius II and metanauplius showed and the same sensibility to PD, probably by the same metabolic state, and is the highest sensibility observed in this study. The increase in the sensitivity of the brine shrimp nauplii of 48 and 72 hours in relation to the newly hatched ones was observed with dichlorvos, guthion, coumaphos, dieldrin and p, p'-DDT (Sánchez-Fortún *et al.*, 1995).

Two of the three stages corresponding to juvenile have the same decreased sensibility to DP. Sexual development could be involved in lowering the sensibility, in fact, stage 8 corresponding to the last stage in juvenile showed the lowest sensibility to DP. The sensibility to PD in the 10Th stage is one of the lowest with a decrease in 11st stage after that The sensibility to PD in the adults that correspond to the stage 12 to 15 is the same.

The fact of variations in sensibility is related to development stage show that the effect is related to the metabolic state of Artemia. González-Pérez and Aportela-Gilling (2001) reported a PD LC50 of 12.5 µg/mL for *A. salina* but without mentioning the stage used; however, LC50 was not found in any of the stages used in this study, although it is close to

Table I. Median lethal concentration 50 (LC50) of potassium dichromate on the stages of *Artemia franciscana.* The stages enclosed in each square have LC50 no statistically different between them. LC50 = median concentration; SE = standard error. ND = No data

Days	LC50 µg mL^{-1}	SE	Morphology
1	21	0.4	Nauplius I
2	9,12	0.2	Nauplius II
3	15	0.3	Metanauplius
4	14	0.2	Metanauplius
5	15	0.2	Metanauplius
6	21	0.4	Juvenile
7	21	0.6	Juvenile
8	25	0.4	Juvenile
9	ND*	ND	Adult
10	21	0.3	Adult
11	16	0.4	Adult
12	18	0.4	Adult
13	17	0.4	Adult
14	20	0.4	Adult
15	20	0.6	Adult

the most sensitive (stage 4) with 15 µg/mL. Hondal *et al.* (2003), report an LC50 of 8.41 µg/mL for A. franciscana, lower than the lowest LC50 obtained in the present study. Moshafi *et al.* (2010) obtained an LC50 with PD of 27.75µg/mL, in nauplii of 48 h of life (stage 2). Its result with PD is similar to stage 1 with an LC50 of 27.6 µg/mL, obtained in our research.

Sorgeloos *et al*. (1978) mentioned that the sensitivity of artemia to different chemical products could have different variations depending on the geographical region and the strain of study. Therefore, it is suggested that when carrying out a toxicity study, the area where the cysts come from shall be mentioned. These authors evaluated the sensitivity of artemia in the first three stages against chromic acid, where they found that stage 1 is more resistant than stages two and three. These results are similar to the ones obtained in this study where stage 1 showed lower sensitivity and therefore greater resistance to the PD toxic effect. However, at this stage, the animal has its ventral face covered with a sclerite (hardened cuticle plate) and does not feed since its buccal and anus are not functional until stage 2 (Sorgeloos *et al*., 1978; Sorgeloos, 1986). Therefore, the possible toxicity of PD or other compounds to be evaluated would be at integumentary level only, and could not be incorporated through the digestive tract, which causes a bias in toxicity determination.

In order to assess the concentrations of LC_{50} of the different toxins or substances to be evaluated, it is recommended to use stages 2 and 3, since these stages show an average sensitivity (**table 1**), they are easy to obtain and identify.

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