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In vitro Toxicity of Cadmium on the Development of Parthenogenetic Eggs of a Freshwater Cladoceran: *Daphnia magna*

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Abstract

This work aims to assess the effects of cadmium on a bioindicator of pollution, *Daphnia magna*, a freshwater cladoceran microcrustacea. In fact, the chronic toxicity test duration was 21 days for Daphnis, so it's a long and costy test. However, the development stages of *Daphnia magna's* eggs could be used as potential tests for chronic toxicity. Indeed, microscopic examination of eggs for 72 hours could be an alternative to chronic toxicity test using 24 hours neonate. The parthenogenetic eggs of *Daphnia magna* were e exposed *in vitro* to cadmium (60, 80 and 100 µg l^{-1}) revealed an embryotoxicity during the different stages of development, with appearance of abnormal neonates of various shapes and sizes, with few developed carapace and the disappearance of antennas. On the basis of these data, we suggested that cadmium had a negative environmental impact on aquatic invertebrates. Eggs proved to be a suitable alternative model for ecotoxicological studies and the assessment of water quality.

Keywords: *Daphnia magna*, heavy metals, acute toxicity, chronic toxicity, embryotoxicity.

Introduction

The freshwater cladoceran, *Daphnia magna*, have a recent interest as a model organism for bioassays, because of the need to develop alternative tests for the assessment of the ecotoxicological risk of dangerous pollutants improving the water quality criteria for drinking purposes and human health [1]. Water fleas (*Daphnia sp.*) have a relatively short life cycle, require little space, are easily adaptable to laboratory conditions and are sensitive to a wide range of aquatic contaminants [1-5]. Moreover, they reproduce by parthenogenesis of a single individual in laboratory conditions and therefore they are genetically identical [3, 6]. In acute and chronic toxicity tests using daphnia, neonates aged less than 24 hours are generally used. Although it is possible to obtain information on toxic lethal and sublethal effects, but the design of these trials can't provide information about the effects on embryo survival and morphological abnormalities during development [7]. However, embryonic abnormalities at later stages (stages 5 and 6) of development were not observed with ethylenethiourea [6], aniline derivates [8, 9] with egg bioassay tests of *D. magna*. Thus, these compounds may not have a specific toxic mode of action like ETU towards embryonic developmental processes. Till now, only a small number of investigations have been undertaken to determine the environmental pollutants effects on development stages of Daphnis. Researchers have defined various stages of abnormal embryonic development based largely on development of the daphnid's eye, carapace, and secondary antennae [10, 11].
Freshwater is the receptacle of most toxic substances including heavy metals, used in agriculture and rejected by the industry. Although aquatic ecosystems are equipped with a variety of physico-chemical parameters and biological mechanisms to eliminate or reduce adverse effects of toxic substances, toxicants may induce changes in development, growth, reproduction and behavior. They can even cause the death of freshwater organisms [12, 13].
Organic pollutants and heavy metals are considered to be a serious environmental problem for human health. The contamination of soils and aquatic systems by toxic metals and organic pollutants has recently increased

due to anthropogenic activity. [14, 15]. Among these metals, cadmium (Cd) has received considerable attention in recent years because its concentration in water body has been markedly increased by human activities such as sewage treatment, production of pulp and paper, and processing of metals [16-18]. Cd has toxic effects on the kidney, liver, blood, nervous system and reproductive tissues in vertebrates [19-22]. Cd is also carcinogenic and a known teratogen, which accumulates in vertebrates over a period of years [23]. As an endocrine disruptor, Cd has been reported to cause acute reproductive effects in mammals including testicular necrosis, ovarian hemorrhaging and delayed embryo implantation [24].
As a nonessential element, Cd may endanger the growth and development of aquatic life; benthic organisms are often victims of Cd pollution [25]. Cd has been shown to negatively impact growth and cellular energy allocation in Daphnia and recent work suggests that Cd may Affect molecular pathways, including oxidative response [26, 27].
Acute and chronic toxicity of Cd to cladocerans with particular reference to *D. magna* is well documented [12, 29, 30]. However, information of Cd on cladocerans embryonic developmental stages is not clearly reported so far. Because of the paucity of information on this subject, the present study was undertaken to determine the toxic effects of Cd on a freshwater cladoceran *Daphnia magna* developmental stages survival, morphological abnormalities, developmental arrests, and to establish easy to identify egg stages that could be potential endpoints for embryo-larval toxicity tests. The water flea *D. magna* was chosen for ecological reasons, as it is aubiquitous species found in Algerian lakes, ponds, and rivers and also a significant member of aquatic food chain(s). In the acute and chronic toxicity tests with heavy metals, cladoceran *D. magna* has been previously used [11, 29-31].

2. Materials and methods

The water flea *D. magna* were obtained from a culture in the Laboratory of Biology at 08 may 1945 University (Guelma) Northeast Algeria. They were kept in water at a temperature of 20 ± 2 °C under a light / dark cycle of 16/8h. Daphnia were fed two or three times a week with the yeast: *Saccharomyces cerevisiae* [31]. Just after the release of the third brood, the females were isolated and observed until the appearance of new eggs in the incubation pouch. This event was considered as time zero of egg development. Eight hours after time zero, the females were placed under microscope for the dissection. Dark brown eggs with round shape were withdrawn by puncture of the brood pouch Water flea (*D. magna*) embryos are capable of normal development outside of the brood pouch and are therefore suitable for *ex vivo* studies. Medium Elendt M4 was used for culture of isolated parthenogenetic eggs. Glass beakers of 2 l were used as the culture vessels including 1.5 l medium and 50–70 adult *Daphnia*. Gravid females were selected from the cultures and examined microscopically for the level of development (i.e., stages 1–2, as described below) were removed by applying gentle pressure to the posterior region of the brood chamber with a dissecting needle. Extruded embryos were collected and pooled. Eggs were washed several times, successfully adding and removing medium with a fine glass dropper. At the start of Cd exposure, 2–6 h old eggs (between stages 1 and 2) were used. Eggs were dark brown and round in shape (diameter approximately 400μm). Tests were performed in 24-well tissue culture plates and eggs were exposed individually in 2 ml test solution for each Cd concentration. Embryos were incubated at 20±1 ◦C and were examined microscopically every 24 h during the test period. We scored embryos for stage of development and recorded mortality and any abnormalities in development after exposure to a series of Cd concentrations [33].
Extruded, the embryos were collected and put together after being washed several times. Eggs witch the age is ranging between the first and the second step were incubated in Petri dishes with different concentrations of Cd (60, 80 and 100 μg l^{-1}).
Each egg was exposed to 2 ml of the test solution for each concentration of Cd. Embryos were incubated at 20 ± 2° C and were examined microscopically every 24 h during the experiment. The dissolved oxygen, hardness, and pH of the test solutions during the trial period were 5.8-6.5 mgl^{-1}, 230-245 mgl^{-1} of $CaCO_3$, and 7.3 to 7.6 respectively. The test water was not renewed during the trial period.
Embryo development was observed at 24, 48 and 96 h to detect any embryos toxicity that can result in malformations. The eight stages of embryonic development described by Le Blanc et al., and by Khangarot and Das [1, 34] were monitored and controlled under microscope. The various discernible stages of the development were recorded by an Olympus digital camera placed on the microscope.

3. Results and discussion

3.1 Controls:

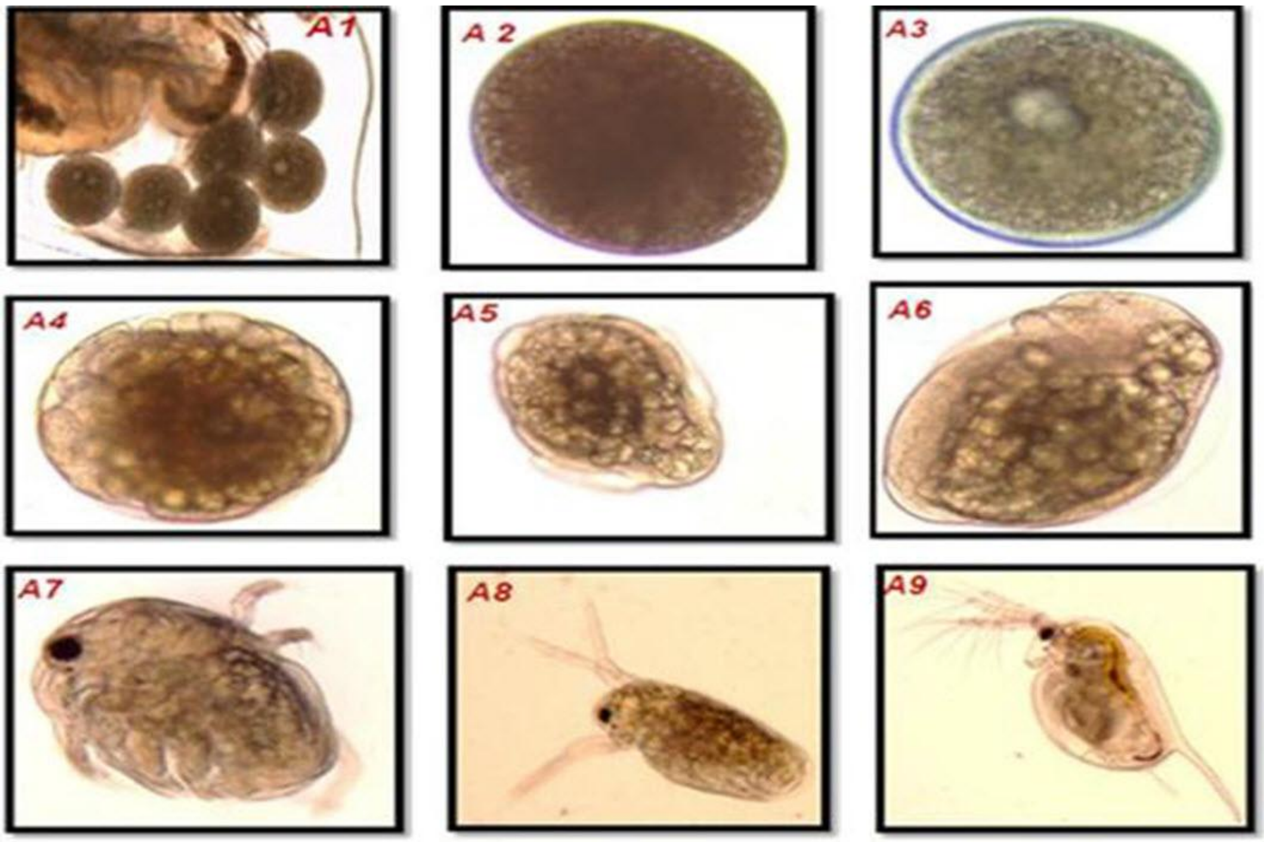
Although *D. magna* is a key species in many freshwater ecosystems and one of the most commonly used test species in ecotoxicology [35].

Eight stages of embryonic development were identified and described [4, 36]. During the stage1, the egg is homogeneous; it is dark brown and round.

Stage 1 generally follows the first 15 hours after the establishment of the embryo in the brood chamber. During stage 2, the egg's periphery becomes clearer because of the yolk mass shrinkage and the membrane becomes visible (Fig1; A3). Stage 3 shows the formation of body parts (head and embryo body). During stage 4, the embryos can discern a capsule, which is considered as the end of the fourth stage and the beginning of the fifth.

The lateral projections corresponding to antennas become visible and two pink eyes appear in the cephalic region (Fig1; A5 and A6). These events occur after the 30th hour. Then, we have recorded the development of antennas and eyes that are well defined during stage 6. The antennas are well developed, the eyes become dark but pigments are not yet evident (Fig1; A7). After 48 h, neonates leave the brood chamber. In stage 7, the secondary antennae become free and finally the two black eyes fuse to form one black eye.

The seta are well developed on the second antennas (Fig1; A8), and neonates become able to swim freely after 65-70 h (Fig1; A9). This event, considered as the end of stage 8, and labels the end of embryonic development.



<u>Figure 1</u>:The different stages of embryonic development of *Daphnia magna* witnesses:
(A1) shows several shell Daphnia eggs stage 1 (× 4), **(A2)** Step 1 of the egg, round and covered by a jacket (× 10), **(A3)** in stage 2 egg periphery becomes lighter and the inner and outer membranes are clearly visible (× 10), **(A4)** stage 3 shows the regions of the head and body (× 4) , **(A5)** embryo at stage 4 (× 10), **(A6)** stade5 the embryo well differentiated, two pink eyes appear in the head region (arrow), **(A7)** stage 6 shows that the two antennas become free from the shell and dark eyes; **(A8)** shows the stade7 development of antennae and secondary antennae; **(A9)** stage 8 a neonate swimming freely with the caudal spine and with a black eye (× 4).

3.2. Treated:

Malformations were recorded following the contact of eggs with various concentrations of Cd. (Figure 1). Indeed, the exposure to $60\mu gl^{-1}$ of Cd induced a retraction of the yolk mass (B1), and the cells's arrangement seems to be completely disrupted (B2). For 80mgl^{-1} of Cd, some treated showed severe embryos developmental defects (B3, B4). We also noted the appearance of a caudal spine malformation following the exposure to Cd (B5, B6, B9, B10, and B11).

Eggs exposed to 80 µg of Cd for 48 h showed a dark aspect without organogenesis (B7). Some embryos have not well developed neither secondary antennae, no setae, with the disappearance of the eyes (B8). In (B12), abnormal daphnia having different shapes, with poorly developed carapace, and without antennas are observed (B12). These defects characterize the end of the toxicity step. We have recorded the death of several abnormal embryos at different developmental stages that are dose dependent.

After this modest research work, the results have underlined the negative impact seriousness of water contamination by heavy metals on Daphnia, namely cadmium.

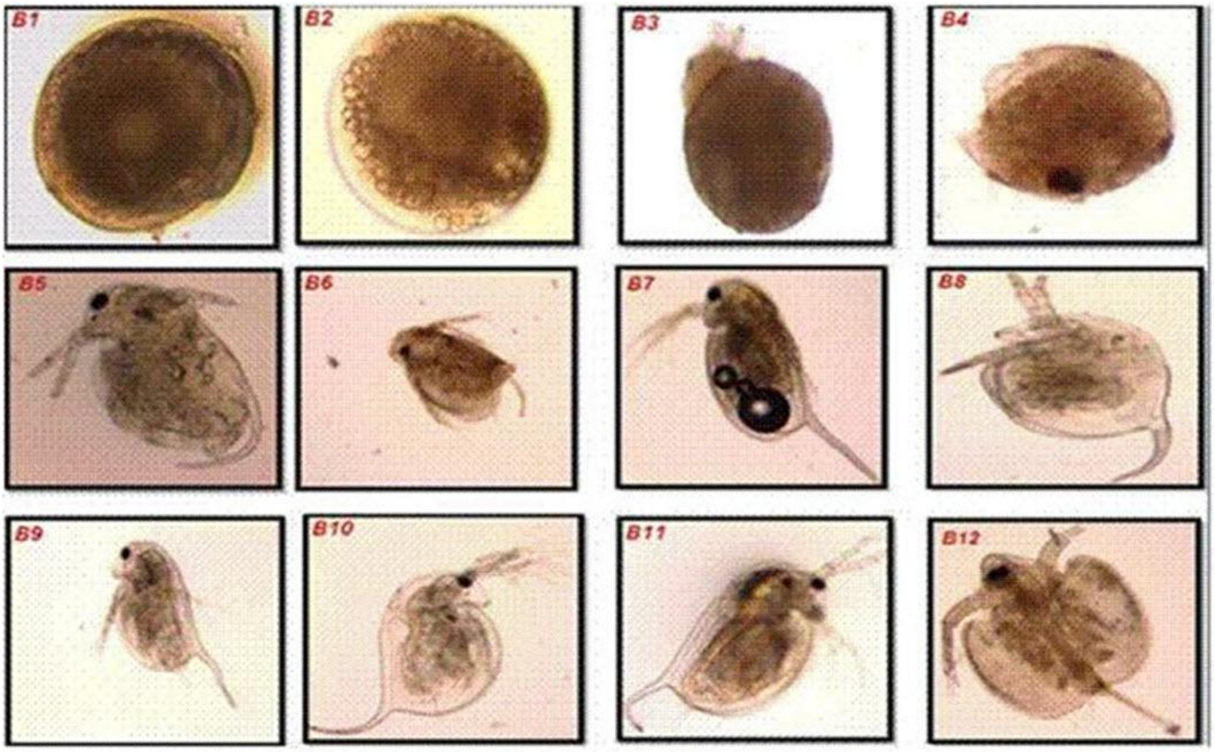


Figure 2: Developmental stages *of Daphnia magna* treated after exposure to cadmium.

Daphnia are important aquatic system organisms and they are among the most favorable organisms for aquatic toxicology tests. The many benefits of Daphnia are sensitivity to toxic substances, and parthenogenetic reproduction with short reproduction and life cycle [36].

The results of this study suggest that the test on Daphnia eggs could be a good biological test and an alternative to conventional test of reproduction for 21 days. The heavy metals effects on the life cycleof various cladocerans species were also evaluated, especially on reproduction [1, 37]. Some preliminary investigations studying the viability of eggs (number of brood / number of eggs) that are exposed to different heavy metals showed that embryonic development is a very sensitive stage in the different species of Daphnia.

For example, embryos of *D. magna* were inhibited at the beginning of the steps when the concentrations of Cu and Cd were increased [38]. In *D. magna*, the early embryonic stages are known to be more sensitive than juvenile stages to heavy metals and other toxic substances according to EC50 values [35]. Development steps of *D. magna*'s parthenogenetic eggs have been successfully used as potential parameters of heavy metals toxicity tests and other toxic substances [33]. Embryonic structures at different stages of development are an easy way to monitor the toxicity process continuity using an optical microscope [1]. Under controlled conditions, at 20 ± 2 ° C daphnia put a new clutch every 3 days. During this period, the eggs inside the brood pouch go through different stages of development, which can be easily distinguished when morphological alterations appear.

Some studies has been devoted to investigate the embryotoxicity of hazardous pollutant in Daphnia [3, 4, 8, 34], but little has been done on the embryotoxicity of heavy metals. According to our results, Cd with different tested concentrations is toxic for daphnia embryos. According to Mu and LeBlanc [40, 41], the increase of embryonic developmental abnormalities could be related to decreased levels of ecdysteroids in embryos, because ecdysteroids are essential for normal embryonic development. They act as hormones of arthropods' moult, but they are also produced by other invertebrates, where they can play different roles. Similar observations have been recorded by Zhang et al., [2] on *D.magna* after 96 h exposure to 4-nonylphenol at 281 μgl^{-1} with an increase of embryos's lethality and neonates malformations (eggs development inhibition, absence or deformity of the caudal spine, the absence of secondary antennas). Khangarot and Ray [42] found that in vitro sensitivity of *D. carinata* eggs development after exposure to mercury was higher than *D. magna* juveniles and the mortality rate at $32\mu g\ l^{-1}$ was about 100%. The results indicated that Cd is responsible for malformations in different parts of the body. The appearance of abnormal neonates of different shapes and sizes with poorly developed carapace and the disappearance of antennas, confirm that Cd has a high mutagenic activity [43] that, according to Waisberg et al, interferes with DNA repair rather than causing damage directly [44]. We share the assumption of Jemec et al., [45], who evaluated the effect of Cr and Cd on biochemical biomarkers (protein level, the activity of cholinesterase (ChE), catalase (CAT) and glutathione S-transferase). According to these authors, the disruption of these parameters is closely linked to the survival and the reproduction of Daphnia. They found that Cd with a concentration ranging between 0.656 μgl^{-1} and $2.62\mu gl^{-1}$ affects the reproduction of Daphnia.

(B1) embryos exposed to cadmium ($60\mu gl^{-1}$), shows retraction of the yolk mass; **(B2)** stage 3 of the egg; **(B3)** stade5, the embryo has a developmental delay after 48 hours of exposure ($80\mu gl^{-1}$), **(B4)** spherical embryo with has undergone developmental arrest during early stages development of the embryo (gastrulation), we note the presence of the eye (arrow) characteristic of stage 4, **(B5)** embryo with malformation at the caudal spine which is curved (arrow) **(B6)** abnormal embryo exposed to $80\mu g\ l^{-1}$ Cd; **(B7)** eggs embryos exposed to 60 μgl^{-1}), Cd treatment for 48 hours showing no differentiation of organogenesis, **(B8)** daphnia with multiple abnormalities : disappearing of under antennae, silks and the eyes; **(B9, B10, B11)** daphnia showing several malformations of caudal spine; **(B12)** embryos exposed to Cd at 100 $\mu g\ l^{-1}$ showing carapace destruction.

Conclusion

The results of this study show that Cd affects the embryotoxicity of *D. magna*. The results of this study demonstrate that the ecotoxicological tests, covering the entire life cycle of these alternative models, could be an useful tool to determine the mechanism of action, by which pollutants cause changes in *D. magna* population. This study has clearly shown that the development stages of parthenogenetic eggs can become an important research tool for evaluating the lethal and sublethal toxicity and for testing environmental contaminants teratogenicity. Various experimental scenarios can be used to facilitate the elucidation of the toxicity mechanism with respect to direct exposure of embryos or the maternal organism contamination. Development parameters that were affected by Cd include development inhibition at various stages of organogenesis and the release of live neonates with developmental abnormalities of the secondary antennae, carapace, eyes and caudal spine. Further studies can be planned to determine the specific mechanism responsible for the embryo toxicity of mercury compounds and other heavy metals. These studies attempt to provide an overview on the objectives of the toxicity tests development that are unique for crustaceans. This bioassay provides useful information to evaluate acute and chronic toxicity of chemicals in the environment and the differences in sensitivity of the development stages. On the basis of these data, we suggest that Cd has a negative environmental impact on aquatic invertebrates.

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