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Sublethal effects of cadmium on energy reserves in the edible Mollusk *Donax trunculus*

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Abstract
Cadmium (Cd) is one of the most toxic metals for marine organisms. The current experiment aimed to test two sublethal concentrations (LC_{10} and LC_{25}-96h) of Cd on the main gonad components (carbohydrates, lipids, proteins) of an edible Mollusk *Donax trunculus* L. (Bivalvia, Donacidae). The samples were collected at El Battah a relatively clean site, during the reproduction period of the bivalves, and reared under laboratory conditions. Physico-chemical parameters of water such as temperature, pH, salinity, and dissolved oxygen were measured during the exposure period. The amount of biochemical components was determined at different exposure times (0, 24, 48, 72 and 96h) for both sexes. Our results revealed that female gonads are richer in energy reserves than males, and Cd caused a significant decrease in proteins and carbohydrates levels in both sex. Moreover, Cd reduced the lipid levels only in females. In fact, the effects of Cd are more pronounced in females as compared to males. A three-way ANOVA indicated significant effects of concentration, time and sex for each biochemical component.

Keywords: *Donax trunculus,* cadmium, acute toxicity, carbohydrates, proteins, lipids

1. Introduction
Heavy metals are considered as the most important form of pollution in the aquatic environment because of their toxicity and accumulation by marine organisms [1]. They are defined as metallic chemical elements that have a relatively high density and are toxic or poisonous at even low levels [2]. The gulf of Annaba is the most important touristic and economic coastal zone in eastern Algeria. It is continuously affected by various contaminants from urban, agricultural, harbor and industrial activities [3-5]. Previous studies showed that cadmium (Cd), a nonessential element, was the most abundant heavy metal in this region [4], it enter into cells through simple diffusion, membrane carriers or ion channels [6,7], and produce wide ranges of biochemical and physiological dysfunctions in humans and laboratory animals [8-10]. Cd can induce the formation of reactive oxygen species (ROS), and generates oxidative stress in aquatic organisms [11]. Unlike organic compounds, Cd is not biodegradable and has a very long biological half-life [12]. When it contaminates the aquatic ecosystem, it can enter the aquatic food chain through direct consumption of water or biota, and through non-dietary routes such as absorption through epithelia [13].
Bivalves can accumulate a wide range of contaminants and play an important ecological role in aquatic environments. They are also widespread commercial products [14, 15]. *Donax trunculus* L. (Bivalvia, Donacidae) has been widely used as a sentinel species for the assessment of marine pollution in the gulf of Annaba through the direct measurement of several biomarkers [16-19]. Studies on the growth and reproductive cycle of *D. trunculus* have been conducted in this region, and physiological parameters such as indicators of the energetic status or the condition of animals have been used as biomarkers to monitor environmental stress on this Mollusk [20]. Indeed, carbohydrates, lipids and proteins are important in marine organisms because the energy storage and its utilization are often closely linked to environmental conditions. Energy reserves also provide complementary information regarding the health status of target organisms [21]. Therefore, the present study was conducted to assess the sub-lethal effects of cadmium (LC_{10} and LC_{25}-96h), the most abundant heavy metal in the gulf of Annaba, on the main biochemical components (carbohydrates, lipids, proteins) of *D. trunculus* gonads of both sexes during the reproduction period.

2. Materials and Methods

2. 1 Sampling site and cadmium exposure

The gulf of Annaba is located in the east of Algeria. It is limited by the Cap Rosa (8° 15' E and 36° 38' N) in the East, and by the Cap Garde (7° 16' E and 36° 68' N) in the West. The experiments were carried out in March, 2014. *D. trunculus* adults (shell length 25 ± 2 mm) were sampled at El Battah beach (36° 50' N - 7° 50' E) a site far from any source of anthropogenic activities and subjected to an important hydrodynamic exposure (Fig. 1). Animals were transported in cold boxes to the laboratory, and sex was separated by macroscopic inspection according to the color of gonads: dark blue in females and yellow-white in males (Fig. 2) [22]. The rearing was conducted in aquariums, containing sandy bottom and sea water that comes from the sampling area, and equipped with air pumps (Nirox X5). The bivalves were acclimatized for at least 48h before an exposure to Cd [23]. The physico-chemical parameters of seawater were measured with a WTW Multi 340i/set measurement (Germany), and were as follow: temperature 14.60 °C, salinity 32.6 g/L, pH 8.10, dissolved oxygen 1.20 mg/L. After an acclimation period (48 H) [23], Cd was added according to preliminary bioassay using CdCl2 (Sigma, USA) on *D. trunculus* [24]. The LC_{10} and LC_{25}-96h were found to be 1.15 and 2.02 mg/L for males, and 0.94 and 1.60 mg/L for females, respectively.

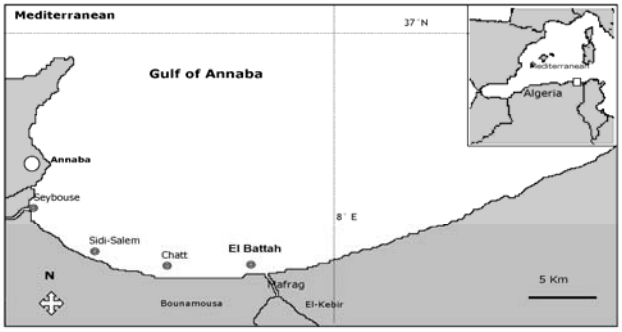

Fig 1: Location of El Battah beach in the Annaba Bay.

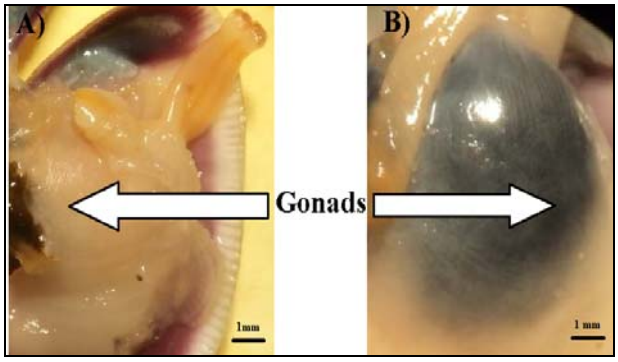

Fig 2: The gonads of *D. trunculus*: male (A) and female (B).

2.2 Analytical methods

Gonad of 4 Bivalves from control and treated series were used (individual analysis). Carbohydrates, lipids and proteins were extracted following the procedure of Shibko *et al.* [25]. In brief, each sample of gonad (weight: 35-45 mg) was homogenized in 1 ml of trichloroacetic acid (20%). After homogenization with ultrasound (Sonifier B-30) and centrifugation (5000 g/min for 10 min), the first supernatant was used for the carbohydrates determination as described by Duchateau and Florkin [26] using anthrone as reagent and glucose (Sigma) as standard. The absorbance was estimated with a spectrophotometer at a wavelength of 620 nm, while the pellet added with a mixture of ether and chloroform (1V/1V) was subjected to a second centrifugation (5000 g for 10 min). The resulted supernatant was used to quantify the lipids based on the vanillin method of Goldsworthy *et al.* [27], and absorbances were measured at 532 nm. Finally, protein quantitation was determined in resulting pellet using the Bradford [28] assay with blue brilliant of coomassie (G 250, Merck) as reagent and bovine serum albumin (Sigma) as standard, The reading of absorbance was performed at a wavelength of 595 nm. Data were expressed in µg/mg of fresh tissue.

2.3 Statistical analysis

All data have been expressed as mean ± standard deviation (SD). Statistical analyses were performed using MINITAB Software (Version 17, Penn State College, PA, USA). One-way analysis of variance (ANOVA) with a Tukey post-hoc analysis HSD test was used to evaluate statistically significant differences in component levels for the same treatment between times of exposure. Statistical differences among the means of control and Cd-exposed series were determined using Student's *t*-test. To identify concentration/time/sex effects a three-way analysis of variance (ANOVA) were performed. Statistical significance was set at $p<0.05$ level.

3. Results

3.1 Sublethal effects of cadmium on carbohydrate levels

The impact of sub-lethal Cd on the levels of carbohydrates varied as function treatment and sex. As shown in tables 1 and 2, there was no significant difference between the values recorded during the experimental period in control series. In contrast, exposed males and females respectively present a significant decrease in carbohydrate levels as compared to control series. There was a rapid depletion in treated series with the highest concentration of Cd (LC_{25}) in both sexes at 24h (p= 0.010 for males, and p= 0.035 for females), while the LC_{10} induce a significant decrease in carbohydrates amounts at 72 and 96h (p= 0.043, and p= 0.004, respectively) for male, and from 48h (p= 0.010) to 96h (p= 0.006) for females. A three-way ANOVA (concentrations, times and sex) indicated significant effects of concentrations ($F_{2, 119}= 47.87$; $p<0.001$, exposure time ($F_{4, 119}= 41.88$; $p<0.001$), and sex ($F_{1, 119}= 645.20$; $p<0.001$).

Table 1: Effect of Cd on carbohydrate levels (µg/mg of fresh tissue) in control and treated series of *D. trunculus* males (m ± SD; n = 4 repeats; for the same treatment, means followed by the same letter in miniscule are not significantly different by HSD test, while for each time values followed by the same letter in majuscule are not significantly different at $p>0.05$, LSD test).

Treatment	Exposure time (hours)				
	0	24	48	72	96
Control	67.02 ± 1.56a A	66.31±1.58 a A	66.03±1.78 a A	65.57±1.68 a A	64.70±1.86 a A
LC_{10}	67.02 ± 1.56a A	64.69±1.98 ab A	64.33±2.50 ab A	62.42±0.35 bc B	59.66±1.24 c B
LC_{25}	67.02 ± 1.56 a A	61.89±1.77 b B	61.41±1.86 bc B	60.89±1.67 bc B	57.86±1.96c B

Table 2: Effect of Cd on carbohydrate levels (µg/mg of fresh tissue) in control and treated series of *D. trunculus* females (m ± SD; n = 4 repeats; for the same treatment, means followed by the same letter in miniscule are not significantly different by HSD test, while for each time values followed by the same letter in majuscule are not significantly different at $p>0.05$, LSD test).

Treatment	Exposure time (hours)				
	0	24	48	72	96
Control	74.64±1.26 a A	74.05±1.79 a A	73.63±1.71 a A	72.68±1.57 a A	71.99±1.74 a A
LC_{10}	74.64±1.26 a A	73.69±2.42 a A	69.91±1.87 b B	69.88±1.19 b B	67.61±1.15 b B
LC_{25}	74.64±1.26 a A	70.84±1.56 b B	69.68±1.57 bc B	68.68±1.27 bc B	67.30±1.55 c B

3.2 Sublethal effects of cadmium on lipid levels

Cd (LC_{10} and LC_{25}) had no significant effect ($p>0.05$) in lipid amounts in males as compared to control series (Table 3). In females Cd caused a significant (p= 0.009) decrease in lipid amounts with the LC_{10} only at 96h, while the highest concentration (LC_{25}) induced a significant decrease at 48h (p= 0.002) and 96h (p= 0.003) (Table 4). A three-way ANOVA indicated significant effects of concentrations ($F_{2, 119}= 12.68$; $p<0.001$, exposure time ($F_{4, 119}= 34.36$; $p<0.001$), and sex ($F_{1, 119}= 19$; $p<0.001$).

Table 3: Effect of Cd on lipid levels (µg/mg of fresh tissue) in control and treated series of *D. trunculus* males (m ± SD; n = 4 repeats; for the same treatment, means followed by the same letter in miniscule are not significantly different by HSD test, while for each time values followed by the same letter in majuscule are not significantly different at $p>0.05$, LSD test).

Treatment	Exposure time (hours)				
	0	24	48	72	96
Control	8.65 ± 0.69a A	7.93±0.71 a A	7.53±0.77 a A	7.48±0.84 a A	7.21±0.38 a A
LC_{10}	8.65 ± 0.69a A	7.78±0.73 ab A	7.29±0.88ab A	6.82±0.67 b A	6.66±0.48 b A
LC_{25}	8.65 ± 0.69a A	7.52±0.56 ab A	6.88±0.56 b A	6.53±0.55 b A	7.07±0.59b A

Table 4: Effect of Cd on lipid levels (µg/mg of fresh tissue) in control and treated series of *D. trunculus* females (m ± SD; n = 4 repeats; for the same treatment, means followed by the same letter in miniscule are not significantly different by HSD test, while for each time values followed by the same letter in majuscule are not significantly different at $p> 0.05$, LSD test).

Treatment	Exposure time (hours)				
	0	24	48	72	96
Control	9.25±0.50 a A	8.91±0.91 a A	8.01±0.50 a A	8.41±0.41 a A	8.23±0.56 a A
LC_{10}	9.25±0.50 a A	8.59±0.93 a A	7.97±0.79 ab A	7.81±0.43 ab A	6.61±0.63 b B
LC_{25}	9.25±0.50 a A	8.14±0.58 ab A	7.18±0.84 bc A	6.85±0.50 bc B	6.04±0.66 c B

3.3 Sublethal effect of cadmium on proteins

As shown in table 5, the values of proteins recorded in males does not change significantly ($p>0.05$) in treated series with the LC_{10} as compared to control series ($p>0.05$), proteins treatment with the LC_{25} decreased significantly the values from 24h ($p<0.001$) to the end of experiment (96h, $p<0.001$). In females, Cd at the two tested concentrations reduced the amounts starting 48h (controls vs LC_{10} series p= 0.045; controls vs LC_{25} series p= 0.014) until the end of exposure (96h) (controls vs LC_{10} series p= 0.028; controls vs LC_{25} series $p<0.001$). These results were confirmed by a three-way ANOVA that revealed significant effects of concentrations ($F_{2, 119}= 72.14$; p=0.000, exposure time ($F_{4, 119}= 51.70$; $p<0.001$), and sex ($F_{1, 119}= 417.84$; $p<0.001$).

Table 5: Effect of Cd on proteins contents (µg/mg of fresh tissue) in control and treated series of *D. trunculus* male (m ± SD; n = 4 repeats; for the same treatment, means followed by the same letter in miniscule are not significantly different by HSD test, while for each time values followed by the same letter in majuscule are not significantly different at $p>0.05$, LSD test).

Treatment	Exposure time (hours)				
	0	24	48	72	96
Control	18.51±1.06 a A	17.92±0.78 a A	17.60±0.70 a A	17.39±0.84 a A	17.15±0.39 a A
LC_{10}	18.51±1.06 a A	16.89±0.96 ab A	16.52±0.87 b A	16.30±0.35 b A	16.12±0.89 b A
LC_{25}	18.51±1.06 a A	15.05±0.24 b B	14.83±0.20 b B	14.58±0.68 b B	13.98±0.12 b B

Table 6: effect of Cd on protein levels (µg/mg of fresh tissue) in control and treated series of *D. trunculus* females (m ± SD; n = 4 repeats; for the same treatment, means followed by the same letter in miniscule are not significantly different by HSD test, while for each time values followed by the same letter in majuscule are not significantly different at $p>0.05$, LSD test).

Treatment	Exposure time (hours)				
	0	24	48	72	96
Control	22.49±0.77 a A	22.09±1.42 a A	21.94±1.32 a A	20.71±1.36 a A	20.49±1.42 a A
LC_{10}	22.49±0.77 a A	21.98±0.80 ab A	20.14±0.52 bc B	18.67±0.90 cd B	17.64±1.38 d B
LC_{25}	22.49±0.77 a A	19.11±1.04 b B	18.80±1.27 b B	17.76±1.22 bc B	15.75±0.47 cB

4. Discussion

Aquatic ecosystems are impacted by the presence of chemical contaminants. Biomarkers have been extensively developed to evaluate the general health condition of species, in particular mollusks bivalves, to assess marine ecosystem quality [29]. A number of sub-lethal endpoints for toxicity testing with bivalve have been proposed, including cytological impairment [30] or DNA damage [31]. Moreover, energy reserves could be a valuable diagnostic tool for assessing health quality of ecosystems [32].

The toxic stresses induced metabolic changes to a depletion of energy reserves [33]. Generally, in contaminated organisms the energy budget was used for detoxification processes to maintain homeostasis to the detriment of growth and

reproduction. The present study supports this hypothesis. In fact, our results revealed that Cd exposure at two sub-lethal concentrations induced a significant decrease in biochemical components and the responses observed varied according to the concentration, time and sex. Carbohydrates form an important biochemical constituent of an animal tissue which acts as building blocks of the cells and they are the primary and immediate source of energy [34]. The depletion in carbohydrate levels observed in our study was also reported in the same specie *D. trunculus* collected during the reproduction period from a contaminant site in the gulf of Annaba [18] and other species exposed to several heavy metals such as *Mytilopsis sallei* [35], *Panulirus homarus homarus* [34], *Anodonta anatina* [36]. Glycogen is the most important carbohydrate in bivalve species and the depletion recorded in carbohydrate levels suggested a high glycogenolysis activity [36]. Glycogen mobilization in Mollusks is known to be regulated by a neurohormonal factor the hyperglycemic factor [37] whose secretion is induced by Cd [38].

The concentrations of the lipids were found to decrease significantly in *D. trunculus* females after treatment with two sublethal cadmium concentration confirming a previous report on *Perna viridis* exposed to Cu (5 µg/L). A depletion in gonad lipids levels was observed only in *D. trunculus* females sampled during the reproduction period from the gulf of Annaba [20]. The decrease in the gonadal lipids from females may be due to the increased activity of lipase, the enzyme responsible for the breakdown of lipids into free fatty acids and glycerol. Lipids constitute the rich alternate energy reserves and their mobilization may be due to high energy demands to counter the toxic stress [38].

Proteins are mainly involved in the architecture of the cell, and play an important role in metabolic pathways and biochemical reactions. Under stress conditions, protein supply energy in metabolic pathways and biochemical reactions [34]. In our study *D. trunculus* need more energy to detoxify the toxicants to minimize the adverse effect of Cd. Since bivalves have a limited amount of carbohydrates, the next alternative source of energy to meet the increased energy demand is proteins. Protein synthesis cost is one of the major components of cellular energy demand [39]. Many bivalves species showed a reduction of proteins to overcome the sustained stress; protein levels tended to decrease in some Mollusk species such as *Mytilopsis sallei* [35], *A. anatine* [36] or *Mytilus edulis* [40]. A proteomic analysis performed in the bivalve *Saccostrea cucullata*, revealed eleven proteins in gonads, whose expression levels were significantly altered by Cd (1 mg/ L) after 48h [41]. The decrease in total protein levels suggests an increased proteolysis and a possible utilization of their degradation products for metabolic purpose. The decreased protein level during exposure to pollutants may be due to increased catabolism and decreased anabolism of proteins as reported in the freshwater bivalve *Parreysia corrugata* [42], and in the crustacean *Panaeus kerathurus* treated with diflubenzuron an insecticide growth regulator [43]

There is a difference in the energy demand between male and female gametes [44]. Indeed, male bivalves produce small spermatozoa with few energy reserves in comparison to females which elaborate vitellin reserves for developing oocytes [45] and have high needs of energy for oogenesis. Cd was found to disrupt vitellogenesis in bivalve [46]. Moreover, several heavy metals were measured in *Mytilus galloprovincialis* and the concentrations were found higher in females as compared to males [47]. In addition, heavy metals can impede steroid levels and alter the estrogen metabolism in the Bivalve *Mya arenaria* [48]. These findings can explain the difference in the responses observed between the sexes in *D. trunculus* during the exposure to sublethal concentration of Cd.

5. Conclusion

The present study gives information on the biochemical composition of an edible Mollusk *Donax trunculus* by the determination of the main constituents (proteins, carbohydrates and lipids). Cd affects the changes in these biochemical levels during the acute exposure (96h). The decrease observed in carbohydrate, lipid and protein levels suggests the fast mobilization of energy resources and their rapid utilization to overcome the stress induced by Cd. The difference in the responses between the sexes during the exposure to sublethal concentrations of Cd could be due to differences in bioaccumulation and needs of energy.

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