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ENVIRONMENTAL SUSTAINABILITY AND POLLUTION PREVENTION

Effect of cadmium exposure on essential omega-3 fatty acids in the edible bivalve *Donax trunculus*

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Abstract *Donax trunculus* is the most consumed bivalve by the local population of the Northeast Algeria for its nutritional value. Therefore, the aim of the current study was to determine the effects of cadmium (Cd), a known toxic metal, on the alterations in main essential omega-3 fatty acids, i.e., eicosapentaenoic acid (EPA; C20:5*n*-3) and docosahexaenoic acid (DHA; C22:6*n*-3), in male and female gonads of *D. trunculus* during the reproduction period at spring (before spawning). Additionally, this work seeks to describe the relation between EPA and DHA with non-methylene-interrupted dienoic (NMID) fatty acids, and explores their possible contribution of to protect against Cd stress. The samples were collected at El Battah, a relatively clean sea shore, and reared in the laboratory. Physico-chemical parameters such as temperature, pH, salinity, and dissolved oxygen were measured. Cd was added to the rearing water at two sublethal concentrations (LC_{10} and LC_{25}-96h, as determined previously). A two-way ANOVA analysis indicated significant effects of concentrations and genders for both fatty acids. Our results showed a significant reduction in EPA and DHA concentrations in the both genders, with a strong effect in females. There was also a negative correlation between NMID fatty acids and the two essential omega-3 fatty acids for each gender.

Keywords *Donax trunculus* · Cadmium · Omega-3 fatty acids · EPA · DHA

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Introduction

Previous studies have reported markedly increased levels of pollution in the city of Annaba (Northeast Algeria), particularly by metal contamination (Beldi et al. 2006; Larba and Soltani 2014). Heavy metals constitute a core group of aquatic pollutants due to their bioaccumulative and non-biodegradable properties (Velma and Tchounwou 2010). Furthermore, Cd has been detected in *Donax trunculus* (Bivalvia, Donacidae), an edible species from the gulf of Annaba, with a variation in capturing site and seasons (Beldi et al. 2006). Recent studies have demonstrated that bivalves are successfully used as bioindicator species to monitor pollution in coastal areas (Amira et al. 2011; Soltani et al. 2012; Sifi et al. 2013; Hamza-Chaffai 2014; Karray et al. 2015). These species are chosen due to their ability to accumulate contaminants usually from water and food, reflecting the bio-available fraction (Chandurvelan et al. 2015). In addition, their relative immobility, wide distribution among different aquatic habitats, abundance, persistence, and ease of collection make them good long-term indicators of environmental contamination (Hamza-Chaffai 2014). Moreover, exposure of bivalves to certain metals may lead to changes in biochemical processes that might be potential biomarkers of the exposure as well as early warning signals of adverse effects of the metal accumulation within the bivalves (Le et al. 2016). Recent reports clearly demonstrate that some of heavy metals exert adverse effects on edible bivalves (Ali Abdel-Salam 2013; Borković-Mitić et al. 2013; Fokina et al. 2013; Nardi et al. 2017), by altering the levels of various minerals, vitamins, essential and non essential amino acids, protein, carbohydrate, and lipids. Cd is involved in growth retardation (Geret et al. 2002), endocrine disruption (Ketata et al. 2007), and interferes with reproduction (Smaoui-Damak et al. 2006; Yeung et al. 2016). In fact, metallic pollution could affect lipid metabolism during reproductive period (Hamdani and Soltani-Mazouni 2011; Sifi et al. 2013; Bensouda-Talbi and Soltani-Mazouni 2014; Rocha et al. 2016). Fatty acids (FA) being essential for cell membrane permeability, constitute the main components of lipids to be used as fuel during metabolic processes. Therefore, these agents play an important role in the modulation of biochemical and physiological responses (Neves et al. 2015). In recent years, there has been an upsurge of research on the beneficial effects of omega-3 fatty acids in health and disease (Swanson et al. 2012; De Camargo Talon et al. 2015; Drudi et al. 2017). Hence, highly polyunsaturated fatty acids (PUFA), e.g., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), play a key role in the modulation of physiological system of a number of species of animal kingdom including zoo planktons, fish, and bivalve. Interestingly, it is noteworthy that humans cannot synthesize de novo these fatty acids (Saito and Aono 2014; Gonçalves et al. 2012). However, very few studies are available on the possible effects of pollutants on these important nutrients. Seafood represents the primary source of two biologically active dietary omega-3 fatty acids. Recent investigations have demonstrated that the bivalve *D. trunculus* is an excellent source of omega-3 fatty acids (EPA and DHA) (Boussoufa et al. 2011). Therefore, the present study focused on the impact of sublethal exposure of cadmium (96h-LC_{10} and 96h-LC_{25}) on the composition of the most important PUFA omega-3 (EPA and DHA), given their role on human health, and maintenance of essential functions such reproduction, in an edible mollusk species *D. trunculus* (in two genders) following an acute exposure (96 h) during the reproductive period, and correlate our findings with particular FA know as NMID (C22:2 NMID1 and C22:2 NMID2), which are distinguished by the presence of more than one methyl group between each double bond (Ojea et al. 2004), which makes them more resistant to oxidative stress (Barnathan 2009).

Experimental

Collection and animal rearing conditions

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 (Fig. 1). The experiments were carried out in March, 2015. *D. trunculus* adults (shell length 25 ± 2 mm) were sampled at El-Battah beach (36° 50′ N–7° 50′ E), a coastal site far from any source of anthropogenic activities and subjected to an important hydrodynamic exposure. Animals were transported in cold boxes to the laboratory, and the genders were identified by macroscopic examination according to the color of gonads, i.e., dark blue in females and yellow-white in males (Manca Zeichen et al. 2002). The rearing was conducted in aquaria, containing sandy bottom and sea water, taken from the sampling area, and equipped with air pumps (Nirox X5). *D. trunculus* is preferentially distributed between 0 and 2 m of depth in the Mediterranean Sea (Gaspar et al. 2002). This species is a filter-feeding species and according to Mouëza and Chessel (1976), it absorbs the finest suspended particles (suspensivore) or those deposited on the sediment via to its elongated siphon (depositivore). The animals were kept unfed in an aquarium for 48 h to acclimatize prior to the start of the experiment of exposure to Cd (Belabed and Soltani 2013). No mortality was observed during the acclimatization. During all the experiments, the water was constantly aerated (dissolved oxygen 7.6–8.3 mg/L; pH 8.1–8.3; salinity 33.1–33.5 g/L; temperature 16 °C) and a 12 h light/dark cycle was maintained. After acclimatization, bivalves were fed daily with a commercial food mixture (Marine Invertebrate Diet. Carolina Ltd., NC, USA), and were exposed to Cd for 4 days while another set of bivalves was kept under control conditions. The metal was added to the seawater as cadmium chloride ($CdCl_2$), according to 96h-LC_{10} and 96h-LC_{25} (lethal concentration inducing 10 and 25% mortality) of Cd treatment in the mollusk *D. trunculus* (Table 1) (Merad and Soltani 2015).

Fatty acid extraction and gas chromatography analysis

Individual gonads of four bivalves from control and treated series were used. Lipids were extracted according to the method of Bligh and Dyer (1959), in the presence of 100 μg of tripentadecanoic acid triglyceride (TG (15:0/15:0/15:0)) as internal standards, then transmethylated by BF_3/methanol after saponification. Fatty acid methyl esters were analyzed by gas liquid chromatography using a Clarus 500 gas chromatograph (PerkinElmer, Waltham, MA, USA), equipped with a flame ionization detector and an Agilent VF-23ms capillary column (30 m × 0.32 mm) (Agilent, Santa Clara, CA, USA). The analysis conditions were as follows: oven temperature was 85 °C/1 min, increased to 150 °C at 30 °C/min, then increased at 3 °C/min to 215 °C. Helium was used as carrier gas, with a flow rate of 3 ml/min. Identification of different fatty acids was performed by comparison of relative retention times with those of commercial standards.

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

Identification of different fatty acids was performed by comparison of relative retention times with those of commercial standards. Fatty acids were quantified in reference to internal standard (C15:0) and were normalized to the weight of each tissue and expressed as µg/mg of dry weight of tissue.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using Prism version 6.01 for Windows (GraphPad software, La Jolla, CA, USA, www Graphpad.com). The homogeneity of variances was checked by Bartlett's and Brown-Forsythe tests. One-way analysis of variance (ANOVA) with a Tukey post-hoc analysis HSD test was used to evaluate statistically significant differences in omega-3 fatty acid concentrations in control tissues (0 h) for each gender, to assess which sex is the richest in omega-3. Statistical differences among the means of control and Cd-exposed series (96 h) were determined using Dunnett's test. To identify concentrations/genders relationships, a two-way analysis of variance (ANOVA) was performed. Statistical significance was set at $p < 0.05$ level.

Table 1 Acute sublethal concentrations of Cd (96 h) against *D. trunculus*: lethal concentration (LC_{10} and LC_{25}, mg/L) with 95% confidence intervals (CI)

Treatment	Reproduction period	
	Males	Females
LC_{10}	1.15 (0.89–1.47)	0.94 (0.74–1.18)
LC_{25}	2.02 (1.57–2.58)	1.60 (1.26–2.01)

Results

EPA and DHA levels recorded in gonads on untreated individuals at the beginning of the experiment (day 0) were summarized in Table 2. Results show that male controls presented significant higher values of EPA and DHA compared to females ($p = 0.000$ for EPA and $p = 0.029$ for DHA).

Effect of sublethal concentrations of cadmium on eicosapentaenoic acid amounts in the gonad of *D. trunculus*

The impact of sublethal concentrations of Cd on the levels of eicosapentaenoic acid (EPA) varied as function treatment and gender (Table 3). After 96 h of exposure, a significant ($p \leq 0.01$) decrease (37.28%) in EPA was observed in the treated series with the LC_{25} in males as compared with control males (Fig. 2). In females, a significant ($p < 0.05$) decrease in their EPA levels was observed with the two sublethal Cd concentrations (LC_{10}: 31.58%; LC_{25}: 37.60%) (Fig. 3). A two-way ANOVA indicated significant effects of concentrations ($F_{2, 23} = 9.79$; $p = 0.001$) and genders ($F_{1, 23} = 6.79$; $p = 0.018$).

Table 2 EPA and DHA levels (µg/mg of dry weight) in gonads of untreated (0 h) individuals of *D. trunculus* (mean ± SD; $n = 4$; for the same omega-3 PUFA, values followed by different letters are significantly different at $p > 0.05$ between genders)

Sex	Reproduction period	
	EPA	DHA
Males	1.93 ± 0.13A	3.35 ± 0.29A
Females	1.24 ± 0.08B	2.85 ± 0.18B

Table 3 Levels of EPA and DHA (μg/mg of dry weight) in gonads from *D. trunculus* after exposure to Cd-96h (mean ± SD; *n* = 4; for the same omega-3 PUFA, and the same sex values followed by different letters are significantly different at $p > 0.05$ between treatments)

Fatty acid				
Treatment	EPA		DHA	
	Males	Females	Males	Females
Controls	1.69 ± 0.40A	1.33 ± 0.25A	3.73 ± 0.44A	2.67 ± 0.43A
LC_{10}	1.19 ± 0.25A	0.91 ± 0.16B	3.04 ± 0.60A	1.93 ± 0.29B
LC_{25}	1.06 ± 0.27B	0.83 ± 0.21B	2.43 ± 0.40B	1.82 ± 0.43B

However, there was no significant effect of the interaction between concentrations and genders ($F_{2, 23} = 0.12$; $p = 0.884$).

Effect of sublethal concentrations of cadmium on docosahexaenoic acid amounts in the gonad of *D. trunculus*

Table 3 shows the docosahexaenoic acid (DHA) levels in gonads after an acute exposure (96 h) to Cd. In males, a comparison between controls and treated series revealed a significant ($p < 0.001$) reduction (34. 86%) in this PUFA only with the highest concentration (LC_{25}) of Cd (Fig. 4). As regards females, a significant reduction of in DHA levels was observed with the two tested concentrations (LC_{10}: 27.72%, $p < 0.05$; LC_{25}: 31.84%; $p < 0.05$) (Fig. 5). A two-way ANOVA indicated significant effects of concentrations ($F_{2, 23} = 12.07$; $p = 0.001$) and genders ($F_{1, 23} = 25.54$; $p = 0.001$), and no significant ($F_{2, 23} = 0.77$; $p = 0.476$) effect of interaction (concentrations × genders).

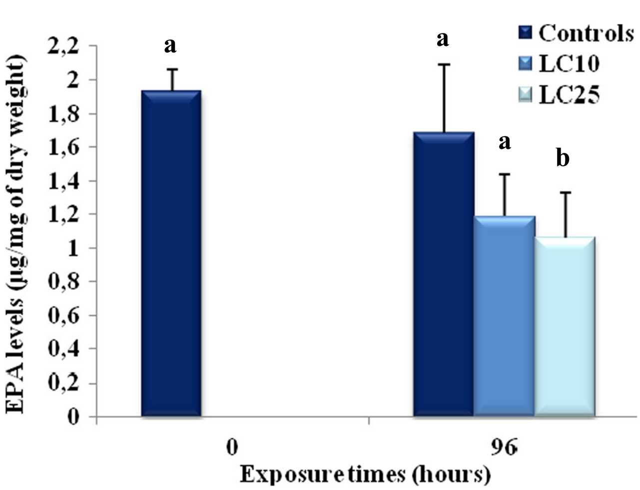

Fig. 2 Effect of Cd on EPA (μg/mg of dry weight) in gonads from *D. trunculus* males (m ± SD, *n* = 4). Mean values followed by the same letter are not significantly different ($p > 0.05$, LSD test)

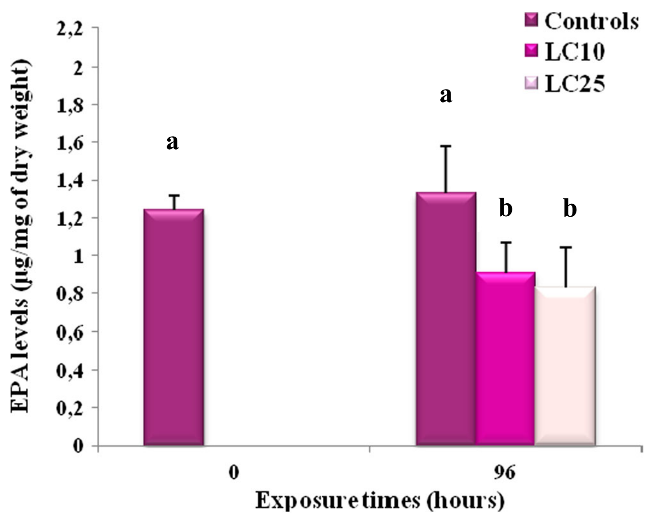

Fig. 3 Effect of Cd on EPA (μg/mg of dry weight) in gonads from *D. trunculus* females (m ± SD, *n* = 4). Mean values followed by the same letter are not significantly different ($p > 0.05$, LSD test)

Effect of sublethal concentrations of cadmium on non-methylene-interrupted dienoic amounts in the gonad of *D. trunculus*

The two C22 dienoic (C22:2 NMID1 and C22:2 NMID2) acids appeared successively between the C22:1n- 9 and C22:5n 6. They were present at low levels: between 0.45 and 1.13 (μg/mg of dry weight) in all samples. These FA increase in all treated individuals, except in male bivalves exposed to the lowest concentration of Cd (CL_{10}). The increase in non-methylene-interrupted dienoic (NMID) levels coincided with a decrease in EPA and DHA in *D. trunculus* (Table 4).

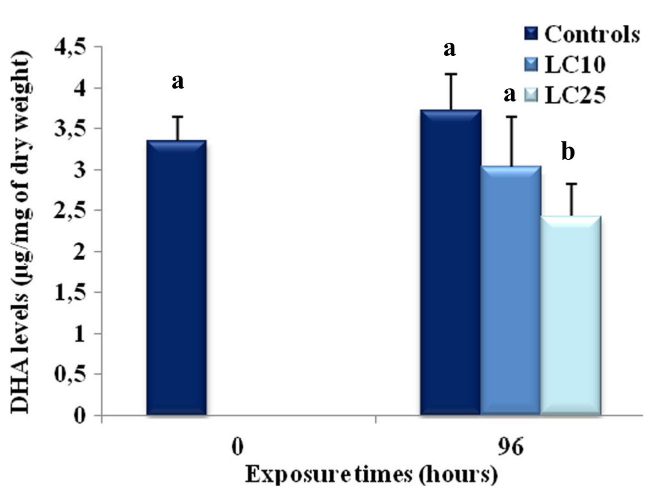

Fig. 4 Effect of Cd on DHA (μg/mg of dry weight) in gonads from *D. trunculus* males (m ± SD, *n* = 4). Mean values followed by the same letter are not significantly different ($p > 0.05$, LSD test)

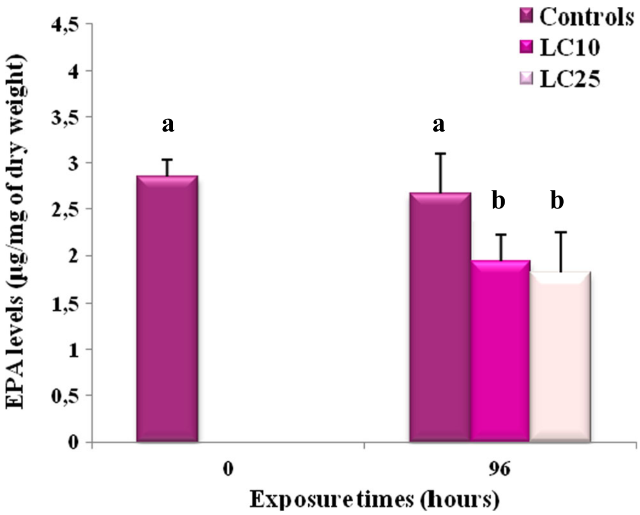

Fig. 5 Effect of Cd on DHA (µg/mg of dry weight) in gonads from *D. trunculus* females (m ± SD, $n = 4$). Mean values followed by the same letter are not significantly different ($p > 0.05$, LSD test)

Discussion

Cadmium have no physiological function for cells, and can cause toxic effects at sublethal concentrations in many bivalves (Fang et al. 2010; Company R et al. 2010; Yeung et al. 2016; Piló et al. 2017), due to the bioavailability of its free ionic form $Cd2^{+}$ (Part et al. 1985; Wang and Rainbow 2006). Cd has the ability to break the balance between pro- and antioxidative systems, to cause oxidative stress in tissues, and the accumulation of lipid peroxidation products in bivalves (Dovzhenko et al. 2005; Franco et al. 2006; Regoli 2012). Indeed, the dysregulation of lipid metabolism is recognized among the most significant biochemical responses (Parrish 2013). Many studies have demonstrated that fatty acid composition can be altered in the aquatic environment pollution by anthropogenic activities (Rocchetta et al. 2006; Kainz et al. 2008; Penha-Lopes et al. 2009; Cheung et al. 2010; Perrat et al. 2013).

Table 4 Levels of non-methylene-interrupted dienoic (C22:2 NMID1 and C22:2 NMID2) (µg/mg of dry weight) in gonads from *D. trunculus* after exposure to Cd-96h (mean ± SD; $n = 4$; for the same NMID FA, and the same sex values followed by different letters are significantly different at $p > 0.05$ between treatments)

Fatty acid				
Treatment	C22:2 (NMID1)		C22:2 (NMID2)	
	Males	Females	Males	Females
Controls	0.49 ± 0.04A	0.42 ± 0.05A	0.73 ± 0.08A	0.59 ± 0.06A
LC_{10}	0.71 ± 0.06B	0.54 ± 0.04B	0.78 ± 0.07A	0.79 ± 0.06B
LC_{25}	0.96 ± 0.07B	0.56 ± 0.05B	1.03 ± 0.16B	0.98 ± 0.13B

EPA and DHA are excellent energy sources, and involved in the maintenance of membrane structure and functions (Costa et al. 2015). The (n-3) PUFA have been reported to be essential for optimal growth for at least some species of juvenile bivalves (Bergé and Barnathan 2005). These omega-3 PUFA serve as precursors of eicosanoids (prostaglandins, thromboxanes, leukotrienes, etc.), which have a wide range of physiological actions in immune system, inflammatory response, neural function, reproduction, and enhancing the organisms adaptation to environmental stress (Vance and Vance 2002; Merzouk et al. 2008; Nani et al. 2015). Omega-3 fatty acids reduce cholesterol levels and the incidence of heart disease, stroke, and preterm delivery (Daviglus et al. 2002; Patterson 2002). According to the recent researches, DHA should be considered as a potential antiangiogenic candidate for future clinical trials (Siddiqui et al. 2011; Berger et al. 2013). For this reasons, the recommended intake of omega-3 fatty acids for healthy adults is 0.3–0.5 g/day of EPA + DHA and approximately 1 g/day for people with or at high risk for coronary heart disease (Kris-Etherton et al. 2002).

It has been reported that the EPA and DHA concentrations are generally decreased with increasing contamination levels and are often associated to peroxidation or decreasing membrane permeability (Filimonova et al. 2016; Signa et al. 2015). Our study showed that Cd can reduce levels of omega-3 PUFA significatively at sublethal concentrations following an acute exposure (Table 3) confirming previous reports. For instance, the bivalves *Mytilus edulis* exposed to 50 µg/L of copper after 24 and 72 h (Fokina et al. 2013), and *Mizuhopecren yessoensis* exposed to 0.25 ppm $CdCl_2$ (Chelomin and Belcheva 1991) showed lower value of EPA and DHA, compared with controls. *Mytilus galloprovincialis* transplanted from a reference site to an impacted site, to assess the biochemical response of caged mussels to high trace element and polycyclic aromatic hydrocarbon (PAH) contamination, presented reduction in both EPA and DHA contents (Signa et al. 2015). Indeed, for the bivalve *Scrobicularia plana*, EPA percentages were particularly low in specimens from sites contaminated by PAH and pollutants with estrogenic activity (Perrat et al. 2013). Recently, Primextra ®Gold TZ, a herbicide, was found to cause a significant reduction in the amount of the omega-3 fatty acids in two marines bivalves, namely *Cerastoderma edule* and *S. plana* (Gonçalves et al. 2016). The same effect was observed in fish's species like *Lates calcarifer* treated with nickel and mercury during 96 h (Senthamilselvan et al. 2016), and *Oreochromis niloticus* collected from contaminated sites by human activities, in particular with heavy metals (Muinde et al. 2013).

The reduction in $n-3$ PUFA observed in our study can be due to the activation of the lipid peroxidation mechanism: since PUFA are primary targets of ROS, when the process of lipid radical formation begins, higher lipid saturation and high oxygen concentrations cause the increase in the velocity of lipid radical chain reactions (Anacleto et al. 2014). NMID

FA are seemingly ubiquitous lipid mollusk components (Ojea et al. 2004), and can be synthesized de novo by bivalves and used to replace the more sensitive FA (Barnathan 2009). Decrease observed for PUFA, especially for DHA coupled with increase in NMID FA, was a compensatory mechanism reported in bivalves to lower susceptibility to lipid peroxidation (Munro and Blier 2012; Signa et al. 2015) while maintaining proper membrane fluidity. NMID FA can be used to replace the more sensitive PUFA (EPA and DHA), because of an unusual structural property (i.e., isolated double bonds) which makes them more resistant to the oxidative stress (Barnathan 2009). The higher proportion of these PUFA reduces the susceptibility to lipid peroxidation. Indeed, NMID FA are distributed in higher quantities in the organs that are more exposed to the external environment, such as gills, mantle, and foot (Berge and Barnathan 2005).

Reproduction of intertidal bivalves includes gametogenesis, development and metamorphosis, all of which are energy-consuming processes (Martinez et al. 2000). The success of those processes depends on the physiological condition and especially the pre-spawning condition of the adult (Hendriks 2004). The DHA and EPA concentrations peaked in spring and winter coinciding with gametogenesis in *D. trunculus* (Boussoufa et al. 2011), and in other species of bivalve such as oyster (Dridi et al. 2007). Additionally, a direct and significant relationship was observed between EPA and DHA with the gonadosomatic index and ripe stage (Freites et al. 2010). The latter observation suggests the accumulation of the essential fatty acids in reproductive tissue during gametogenesis and oocyte maturation. EPA is used preferentially during embryonic development as an energy source (Freites et al. 2010; Martínez-Pita et al. 2012), while DHA plays an important role at the structural and functional levels of cell membranes involved in oogenesis and embryogenesis and it influences larval survival (Soudant et al. 1999; Pazos et al. 2003).

The fatty acid profile show differences between the genders in *D. trunculus*. Indeed, males present higher values in EPA and DHA. The content of 20:5n-3 and 22:6n-3 differs among bivalve's species. In *M. galloprovincialis*, the percentage of the PUFA (20:5n-3 and 22:6n-3) were higher in males than in females (Martínez-Pita et al. 2012), and in the testes of *Patella depressa* (Morais et al. 2003) confirming our findings in *D. trunculus*. However, several investigators reported higher values of EPA in female gonads such as *Argopecten purpuratus* and *Pecten maximus* (Caers et al. 1999; Soudant et al. 1996). Similarly, the value of DHA is also more important in the females of *P. maximus* (Soudant et al. 1996). As previously reported, the differences in fatty acid composition between genders could be related to differences in the lipid composition since PUFA are mainly present in phospholipids, whereas neutral lipids such as TG and sterols ester accumulate 14:0 and MUFA (Caers et al. 1999; Ojea et al. 2004; Pazos et al. 2003; Saito 2004; Soudant et al. 1999). In addition, previous studies showed that Cd can strongly affect at low concentrations energy metabolism by suppressing the mitochondrial function and increasing basal energy demand of an organism to cover the energy costs of detoxification and damage repair (Cherkasov et al. 2006; Ivanina et al. 2009; Cannino et al. 2009). In addition, there is a difference in the energy demand of male and female gametes. Male bivalves produce small spermatozoa with few energy reserves by comparison with females which elaborate vitellin reserves for developing oocytes (Beninger and Le Pennec 1997) and have high needs of energy for oogenesis. Moreover, it has been demonstrated that the bioaccumulation of several heavy metals including cadmium depends on gender during the gametogenesis in *M. galloprovincialis*; this bioaccumulation was found higher in females (Richir and Gobert 2014). These findings can explain the difference in the responses observed between the genders in *D. trunculus*, especially the greater sensitivity of females, as they showed decreases in their omega-3 levels with the two test concentrations (CL_{10} and CL_{25}) (Table 3).

Conclusions

Cadmium in both genders decreased omega-3 contents in gonads. The results of the experimental treatments point out the possibility to use EPA and DHA PUFA composition/parameters as biomarkers, reflecting the adverse effects of the heavy metals on bivalve mollusks, including the fact that cadmium can reduce significantly their nutritional value. The sensitivity of females could be correlated to different metabolic requirements during the reproductive period. Further more studies are wanting particularly on the enzymes, like elongases and desaturases which are involved in the synthesis of PUFA, for obtaining more information on their mode of action on reduction of FA.

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