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Toxicity of fluoride to *Paratapes textilis* (Gmelin, 1791) clam: alterations in shell colour, growth, accumulation, digestive gland and feet (Histology) and its potential risk to human health

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ABSTRACT

The toxicity of fluoride to one of the economic clam species in Egypt; *Paratapes textilis* (Gmelin, 1791) was monitored in the present investigation. After one month of exposure of the bivalve to a fluoride ascending concentration of 2.5, 5, 10, 20 and 40 ppm, many biological aspects were affected. For the first time, fluorosis or whitening of clamshells was clearly observed in all tested fluoride concentrations. Moreover, growth of *Paratapes textilis* as represented by total length, width and total wet weight showed significant delay compared to the control specimens. A negative decreased percentage change especially in width was recorded for treated samples with fluoride compared to the control ones. Accumulation of fluoride in species organs showed different patterns. In the digestive gland and shell, as the concentration of fluoride increased there was a significant gradual increase in fluoride accumulation. Conversely, in feet, there was an increased accumulation of fluoride up to 5 ppm followed by a gradual decrease. Histology sections showed impairment of the digestive gland tubules structure, pyknosis, vacuolization, hypertrophy, and hyperplasia, especially at low fluoride concentrations. Feet musculature also showed torn and disintegrated muscle fibers in addition to pyknotic nuclei. Partial recovery was observed in sections of clams' digestive glands and feet subjected to high fluoride concentrations, especially at 40 ppm, which could be attributed to the self-defense mechanism activated by the clam. Amongst the fluoride-treated samples, 2.5 and 5 ppm showed high fluoride bioaccumulation. Assessment of human health risks of fluoride resulting from fluoride treated *Paratapes textilis* consumption indicated a potential risk.

INTRODUCTION

The assessment of invertebrates fishing in the Egyptian Coast of the Mediterranean Sea, in general, is important due to their nutritional importance and economic values. All over the world many countries have used mussel seafood and set up mussel farms since they are a good source of n-3 polyunsaturated fatty acids, necessary minerals, high essential amino acids, high-quality protein, carbohydrates and vitamins. In Egypt, seafood is used as a source of protein **(El Sayes and Sallam, 2010; Chakraborty *et al*., 2020)**. They are also a desirable source to supplement the deficiencies of amino

acids in cereals. In addition, bivalves are reported to repair oxidative damage by reactive oxygen species (ROS) along with increased expression of antioxidant molecules **(Santovito *et al.*, 2005)**. Their meat is recommended in dietary regimens for the reduction of coronary heart disease and the support of cognitive development and vision **(Chiesa *et al.*, 2018)**.

It is widely known that environmental pollution affects economically shrimps and molluscs mostly because they spend most of their life span in the upper reaches of the estuaries where they become closer to the discharged water containing sewage, industrial, and agricultural wastes **(Liteplo *et al.*, 2002)**. As a result, bivalves especially mussels and clams are vastly used as indicators of pollution because of their remarkable ability to concentrate many environmental contaminants **(Yap *et al.*, 2021)**.

Like other pollutants, fluoride accumulates in water and sediments in ecosystems and thereafter travels to marine living organisms including algae, fish, and molluscs. Eventually, it accumulates and settles permanently in the aquatic food web **(Chai *et al.*, 2016)**. World Health Organization (WHO) classified fluorides in water as an inorganic chemical pollutant of natural origin, which is capable of causing adverse effects on human health when present over certain concentrations **(WHO, 2017)**. Fluoride levels in marine biota are higher in areas of anthropogenic origin **(El-Sikaily and El-Said, 2010)**. In aquatic animals, fluoride tends to accumulate in the exoskeleton and bone tissue **(Camargo, 2003)**. It interferes with the phosphorylation of phosphoproteins in cellular membranes, enzyme activities, photosynthetic pigments synthesis and other metabolic processes **(Kumar *et al.*, 2018).**

In recent years, environmental and safety concerns have emerged about the potential consequences of pollutants on the marine environment, and thus human exposure due to the exponential increase in agricultural, industrial, pharmaceutical and domestic operations **(Vighi *et al.*, 2015)**. In Egypt, many studies were conducted on the effect of fluoride on human health resulting from the consumption of various marine organisms (**El-Said *et al.*, 2015; El-Said *et al.*, 2021; El Zokm *et al.*, 2021)**. Therefore, the present work aimed to experimentally document some fluoride effects on the biology of one economic species in Egypt namely; *Paratapes textilis* which is an important bivalves species that lives in the waters of the Egyptian Mediterranean from Abu Qir to Western Harbour along the coast of Alexandria, and in Port Said and Lake Timsah **(El Sayes and Sallam, 2010)**. For one month the studied species was subjected to escalating concentrations of fluoride. Many biological aspects were investigated such as clamshell colour for the first time, growth, fluoride accumulation in shell, digestive gland and feet and the histological structure of the latters. Moreover, the bioaccumulation factor for fluoride and its risks to human health as a result of mussel meat consumption were evaluated.

MATERIALS AND METHODS

1. Collection of *Paratapes textilis*

The land catch bivalves *Paratapes textilis* (Gmelin, 1791) were collected from The Eastern Harbour, Alexandria by using a hand net with a mesh size of 0.2 cm during September 2019. They were kept at a constant temperature (12-17 °C) in an aerated plastic chamber containing fresh seawater during their transportation to the laboratory.

2. Experiments Setup

Six glass duplicate aquaria of dimensions 40 X 40 X 80 cm were used, containing 10 litres of unfiltered seawater collected from The Eastern Harbour. Series of different fluoride concentrations of 2.5, 5, 10, 20 and 40 ppm were added to the five first aquaria duplicates, respectively. The sixth aquarium was filled with un-fluoridated seawater (for the control state). 17 individual bivalves were placed in each aquarium. Bivalves were not fed any artificial meals, but the seawater in each aquarium was changed daily as it was the only source of food. Each aquarium was aerated continuously to obtain an adequate oxygen concentration.

3. Growth of *Paratapes textilis*

To study the effect of fluoride exposure on the growth of *Paratapes textilis*, the total length of shell; T.L. (which is the distance between the two tips measured in cm), total width; W_i. (Which is the distance between the two lateral ends measured in cm) and total wet weight (T.W.W.) were registered at the beginning of the experiment for specimens subjected to different concentrations of fluoride, in addition to the control specimens. During one month, weekly registration of the same parameters was carried out. By the end of that month, the average of total wet weight, total length and width, in addition to the respective percentage change of each parameter were all calculated, statistically treated and compared. The same procedures were adopted by **Nobles and Zhang (2015)**.

4. Sectioning, Preparation, and Digestion of Different Tissues of *Paratapes textilis*

Bivalves were dissected into tissues of feet, liver and shell. Triple composite samples were made from each tissue section. About 0.5 g (triplicates) of each homogenously wet samples were neatly placed into Teflon pots followed by the addition of 3 mL of concentrated HNO_3 (Merck) at room temperature with appropriate closed Teflon cups. Teflon cups were well sealed until sample digestion was completed at room temperature. Samples were then mixed with deionized water to 25 mL and the digested dilute solutions were left in PVC closed bottles until fluoride concentration was determined **(El-Sikaily and El-Said, 2010)**.

5. Fluoride Concentration in the Shell, Digestive Gland and Feet of *Paratapes textilis*

Fluoride concentration was measured by applying the colourimetric procedure of Zr-ARS reagent **(Anselm and Robinson, 1951; El-Said and El-Sikaily, 2013)**. The concentration of the unknown sample (mg/L) was obtained using the calibration curve in

which the standard Na F was 10 µg F^-/mL. The concentration of fluoride in seawater and tissues of bivalve and was expressed as µg/mL and mg/g, respectively. The fluoride content of the digested samples was validated by performing three calibration procedures that were checked for three sets of fluoride standards. One set of criteria should have a concentration close to the expected value. Other calibration procedures should belong to a higher and lower concentrations than the expected concentrations of the samples. The working calibration curve was validated on each work shift by measuring one or more calibration criteria. The detection limit and precision of fluoride determination were 0.01–0.04 µg F^-/mL and 3-5 %, based on duplicate blank samples using 1 cm glass cuvettes.

6. Histology of the Digestive Gland and Feet of *Paratapes textilis*

The digestive gland and feet of *Paratapes textilis* were cut into small portions and were fixed in Carnoy's solution for 24 h, then transferred to 70 % alcohol and processed using routine histological techniques. Paraffin sections of 4-5 mm were stained with Haematoxylin and Eosin and were examined by Nikon Eclipse E20D microscope. Histology of the digestive gland and foot tissues of *Paratapes textilis* were also examined in control and in each fluoride concentration.

7. Statistical Analysis

Statistical analysis was carried out with SPSS 22.0 for Windows. For multiple comparisons of the quantitative variables, Kruskai Wallis' and ANOVA tests were used to compare between means of different studied groups. Generally, *P* values were considered statistically significant at a level ≤ 0.05.

8. Human Health Risk Assessment

The non-carcinogenic risk of fluoride due to bivalve ingestion for toddlers and adults was evaluated by the following equation **(Yee, 2010; El-Said *et al.*, 2015)**:

$$EDI = \frac{Conc_{Bivalve} \; x \; Ing_{Bivalve} \; x \; F \; x \; RAF \; x \; ED \; x \; CF}{BW \; xAT}$$

Where, *EDI* is the estimated daily intake (mg/kg/day), $Conc_{Bivalve}$ is the concentration of fluoride ion in bivalve's tissue (mg/kg), $Ing_{Bivalve}$ is the ingestion rate for bivalve muscle (95 g/day for toddlers and 270 g/day for adults, respectively (**Health Canada, 2007)**. *F* is the fraction of F^- absorbed from the site (1; 100% of contaminant assumed to be available from the site). *RAF* is the relative absorption factor (1 for both toddlers and adults) while *EF* is the exposure frequency (365 days/yr; conservative assumption), *ED* is the exposure duration (4.5 yr for toddlers and 60 yr for adults; conservative assumption), *CF* is the conversion factor (1.0×10^{-3} kg/g), *AT* is the averaging time (1642.5 days for toddlers, 21900 for adults; exposure frequency multiplied by exposure duration), *BW* is the body weight (16.5 kg for toddlers and 70.7 kg for adults **(Health Canada, 2007)**. The hazard quotient (*HQ*) calculation includes the ratio of *EDI* (toddlers and adults) and toxicological reference value (*TRV* for fluoride = 0.122 mg/kg/day; **Health Canada (2007)** and is presented as follows:

$$HQ = \frac{EDI}{TRV}$$

If the estimated daily intake (EDI) is less than or equal to the toxicological reference value (TRV), then the HQ value will be less than or equal to 1.0, and no serious health effects are expected **(Yee, 2010)**. HQ values ranging from 1.0 to 10 may reflect some potential risk, and the severity of this risk should be reassessed from the point of view of the degree to which the health risk assessment is maintained. HQ values greater than 10.0 indicate an increased potential for severe health risks. In this case, human risk management should be carried out to reduce the potential risks from consuming bivalves.

9. Bioaccumulation Factor (BAF)

Bioaccumulation Factor (BAF) describes the transferability of fluoride from seawater to the bivalves **(Wang *et al*., 2018)**. It is the most appropriate approach that is expressed as a function of the basic environmental variables and is presented as follows **(El-Said, 2013)**:

$$BAF = \frac{C_{bivalve}}{C_{seawater}}$$

Where, $C_{bivalve}$ and $C_{seawater}$ are the fluoride concentration in *Paratapes textilis* and in the surrounding seawater, respectively. BAF values are not only related to the uptake of marine organisms but also to the biological characterization of the marine organisms **(Wang *et al*., 2018)**. It is stated that BAF value reflects "no potential for bioaccumulation in the organism" when it is less than 1000 **(Mostafiz *et al.*, 2020)**. It indicates "bioaccumulation in an organism" when it is between 1000 and 5000, and is predicted to be "significant accumulation" when it exceeds 5000.

RESULTS AND DISCUSSION

In aquatic ecosystems, fluoride toxicity is well documented for a wide range of aquatic organisms such as algae, aquatic plants, invertebrates and fish **(Camargo, 2003)**. As a result of industrial, agricultural and municipal activities, fluoride concentrations have increased many folds in fresh and salt waters **(Camargo *et al*., 2008)**. There is a direct relationship between increased fluoride concentration and increased fluoride toxicity to aquatic invertebrates. The toxicity of fluoride also increased with increasing exposure time and water temperature and varied among species **(Casellato *et al*., 2012)**. The concentration of fluoride in unpolluted freshwater ranges from 0.1 to 0.3 ppm. In water contaminated with phosphate fertilizers, pesticides, and contamination from brick, ceramic, and glass manufacturing, fluoride concentrations increased up to 100 times **(Camargo, 2003)**.

In this study, *Paratapes textilis* (**Gmelin, 1791**) clam specimens were exposed for a full month to an increasing series of fluoride concentrations of 2.5, 5, 10, 20 and 40 ppm. At the same time, some other bivalve specimens were placed in a control aquarium which contained water from The Eastern Harbour. Characterizations of the unfiltered

seawater are illustrated **(Table 1)**. Fluoride induced changes were detected in some biological features (shell colour, growth, and tissue fluoride accumulation) and histological structure of *Paratapes textilis* tissues. Moreover, the bioaccumulation factor for fluoride and risks to human health from consumption of mussel meat were detected as well.

Table 1. Characterization of unfiltered seawater from The Eastern Harbour

Parameter	Value ± SD
Temperature	18.7 ± 2.5 °C
Salinity	33.55 ± 2.4 psu
pH	8.00 ± 0.11
Dissolved oxygen (DO)	1.61 ± 0.21 ml/L
Silicon (Si)	1.83 ± 0.04 µg/L
Phosphorus (P)	7.02 ± 0.08 µg/L
Carbohydrates	0.20 ± 0.06 mg/L
Ca^{+2}	466.8 ± 30.2 mg/L
Mg^{+2}	1250.2 ± 24.5 mg/L
SO_4^{-2}	1640 ± 70.3 mg/L
F^-	4.7 ± 0.8 mg/L

1. Shell Colour of *Paratapes textilis*

Shells of bivalves are unique morphological features associated with multifunctional roles including mechanical toughness and individual support **(Chakrabotry *et al*., 2020)**. They consist mainly of two calcareous valves connected by a calcified leather hinge **(Graf, 2013)**. Mussel shells vary greatly in shape, size and colour **(Checa *et al*., 2006)**. Colours, in general, are known to have an essential part in the survival of many species as they help in mate attraction, signalling, camouflage, thermoregulation, immunity, and strengthening **(Williams *et al*. 2016)**. In mussels, the colour pattern is genetically controlled and is strongly influenced by ambient environmental conditions such as pH and salinity **(Geist *et al*., 2005)**.

In the present study, *Paratapes textilis* species were subjected to different concentrations of fluoride (2.5, 5, 10, 20, and 40 ppm). After one month of exposure to fluoride, discolouration or whitening of clamshells was observed, when compared to the control specimens **(Fig. 1)**. Shells of the studied species clearly showed abnormal calcification, pitting and bleaching of colour, especially at 10 and 40 ppm fluoride-treated samples. This abnormal calcification at high fluoride-treated samples seemed to lead to some preservation procedures and hence to a deformation effect of fluoride on shells. Basically, bivalve shells consist of successive layers of precipitated calcium carbonate in addition to open spaces. These layers resulted partly from interference with the organic

matter and partly from crystallization of calcium carbonate **(El-Said *et al*., 2015)**. This pitting could likely be attributed to the formation of the slightly soluble salt calcium fluorite (CaF_2) within the shell layers. This salt has a solubility product of $K_{sp} = 10^{-10.84}$ at elevated fluoride concentrations and in an aquatic medium **(El-Said *et al*., 2015)**. In fact very few studies have investigated the effect of different pollutants on the colour of clamshells, in general, and the effect of fluoride on colour change in particular. Interestingly, the current study could be the first to record this effect. Most studies of the effect of fluoride on colour development have examined the impact of excessive doses of fluoride in drinking water (fluorosis) on the colour of human teeth. Comparably, these studies concluded the presence of white spots on human teeth **(Collivignarelli *et al*., 2020)**, which could partly explain the current finding as human teeth and bivalve shells are composed mainly of calcium carbonate. On animals' level, **Salama *et al*. (2020)** detected a slight change in colour of the date mussel *Lithophaga lithophaga* as a result of heavy metal contamination.

2. Growth of *Paratapes textilis*

Growth provides basic information about the biology and ecology of a species **(Lomovasky *et al*., 2002)**. It is defined as the gradual increase in size or weight over a given period and is a species-dependent process **(Torroglosa and Giménez, 2019)**.
Fluoride ion is essential for the growth and development of humans and animals **(Shi *et al*., 2013)**. However, an increased concentration of fluoride can have adverse toxic effects on plants and animals such as skeletal fluorosis in animals **(Dhar and Bhatnagar, 2009)**.

In this study, *Paratapes textilis* growth was determined by comparing the mean total length (T.L.), width (Wi) **(Fig. 2a)** and total wet weight (T.W.W.) **(Fig. 2b)** of control specimens and specimens treated with different concentrations of fluoride after one month. Our results showed a significant decrease in total length, width and the total weight of fluoride treated *Paratapes textilis* samples (2.5. 5, 10 and 20 ppm) when compared to the control ones.

However, at 40 ppm, while width showed a significant decrease compared to control samples, total length showed no significant difference. Additionally, total wet weight showed a significant increase when compared to control samples **(Table 2)**.

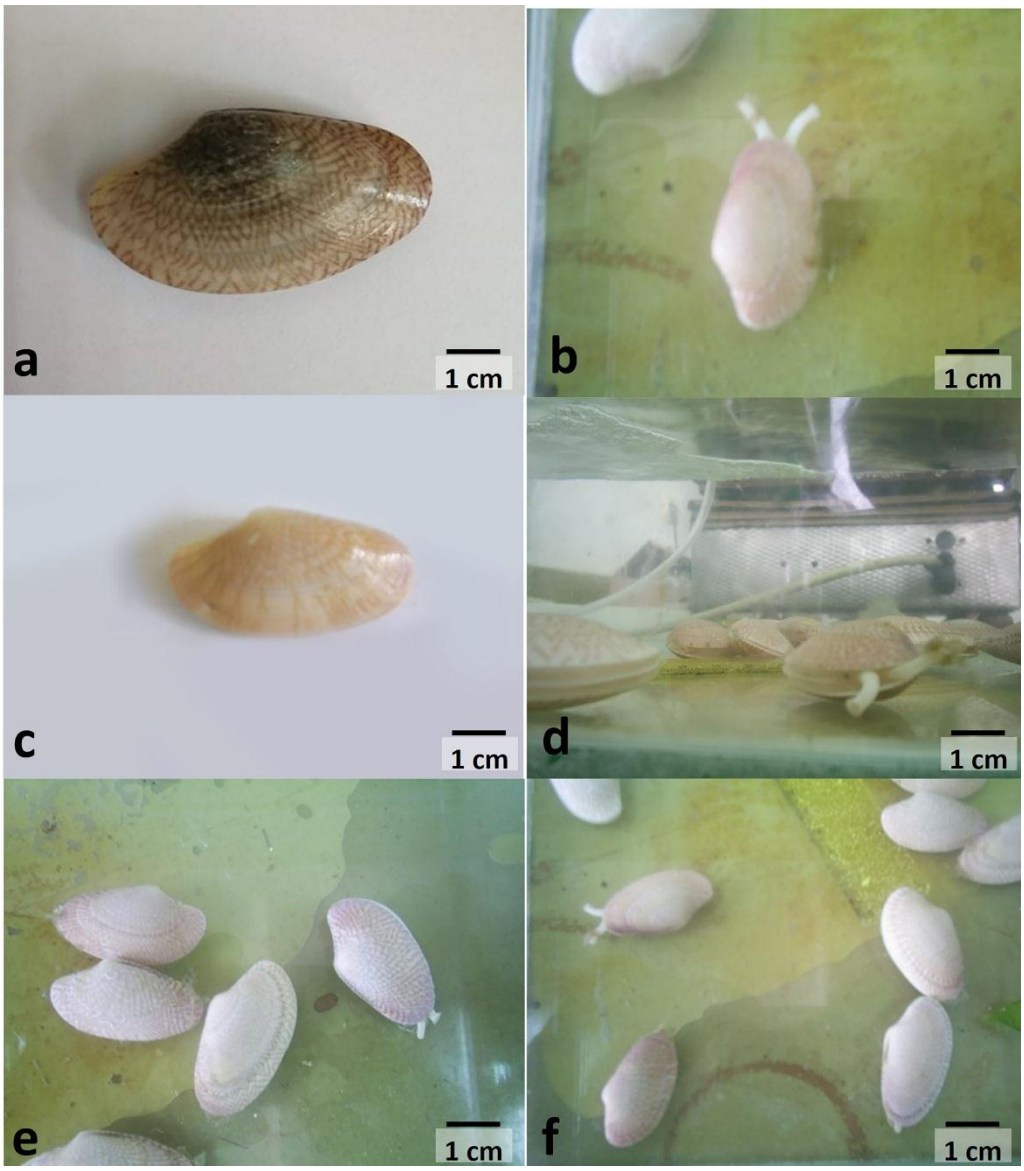

Fig. (1): Changes in shell colour of *Paratapes textilis* (a) control specimens and (b), (c), (d), (e) and (f) treated specimens with 2.5, 5, 10, 20 and 40 ppm fluoride for one month, respectively

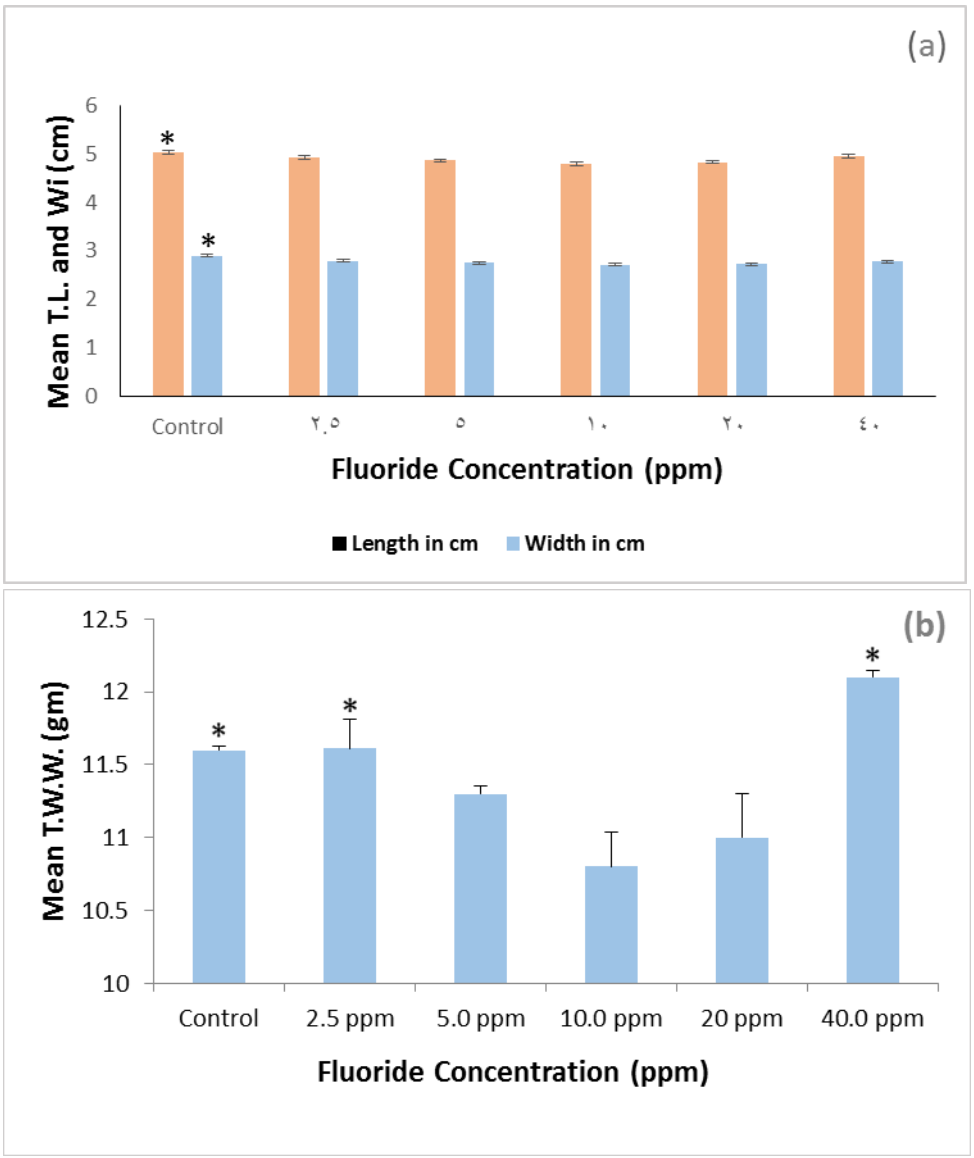

Fig.(2): Mean values ± Standard Error (S.E.) of (a) total length (T.L.) and width (Wi), (b) total wet weight (T.W.W.) of *Paratapes textilis* in control and treated specimens with 2.5, 5, 10, 20 and 40 ppm fluoride for one month (* significant value)

Table 2. Mean ± Standard Error (S.E.) and *P* value of total length (T.L.), width (Wi), total wet weight (T.W.W.) of *Paratapes textilis* in control and treated specimens with 2.5, 5, 10, 20 and 40 ppm fluoride for one month. *P* value was calculated using KrusKai Wallis' test and considered significant at the level of ≤ 0.05

F^- (ppm)	T.W.W. (gm)		Wi (cm)		T.L. (cm)	
	P value	Mean ± S.E.	*P* value	Mean ± S.E.	*P* value	Mean ± S.E.
Control	-	11.58 ± 0.03	-	2.90 ± 0.02	-	5.03 ± 0.07
2.5	-0.01*	11.61 ± 0.20	-0.046*	2.79 ± 0.06	-0.002*	4.93 ± 0.03
5	-0.0001*	11.31 ± 0.05	-0.018*	2.75 ± 0.03	-0.004*	4.86 ± 0.01
10	-0.001*	10.76 ± 0.24	-0.015*	2.71 ± 0.04	-0.006*	4.80 ± 0.03
20	-0.002*	11.04 ± 0.30	0.019*-	2.73 ± 0.04	-0.036*	4.83 ± 0.03
40	0.009*	12.05 ± 0.05	-0.027*	2.78 ± 0.03	0.599	4.95 ± 0.01

*: Significant increase (-): Significant decrease

Percentage change calculated for the three parameters (particularly for width) confirmed a negative growth decrease in fluoride-treated samples, while the control samples showed a positive increase **(Table 3)**. This result could highlight the importance of the width as a significant parameter for evaluating the growth process of bivalves in general and *Paratapes textilis* in particular. Generally, the growth of aquatic organisms is an important process that can be affected by environmental conditions such as atmospheric exposure of the mussel *Brachidontes rodriguezii* **(Torroglosa and Giménez, 2019)**. It can be particularly affected by stress conditions such as pollution. Several studies concluded a reduced growth of bivalves exposed to different environmental stress conditions, which coincided with the current recorded observations. **Gobler *et al*. (2014)** also evaluated a reduced growth in early stages of bay scallops *Argopecten irradians*, and hard clams *Mercenaria mercenaria* due to the synergetic effect of hypoxia and water acidification.

Table 3. Percentage change in total length (T.L.), width (Wi), total wet weight (T.W.W.) of *Paratapes textilis* in control and treated specimens with 2.5, 5, 10, 20 and 40 ppm fluoride for one month

F^- (ppm)	Percentage change (%)		
	T.L. (cm)	Wi (cm)	T.W.W. (gm)
Control	4.31	4.95	1.13
2.5	0.61	-7.99	-9.15
5	0.62	-3.57	2.22
10	-41	-5.36	-11.24
20	-1.64	-3.87	1.51
40	-1.2	-3.85	-1.39

(+): Increased percentage change (-): Decreased percentage change

In addition, **Nobles and Zhang (2015)** noted the negative impact of municipal wastewater effluents of some Texas streams, U.S.A. on the growth of *Amblema plicata* and *Corbicula fluminea*. Fluoride compounds present in aquatic ecosystems had adverse effects on embryonic and larval development and the growth of *Rana chensinensis* as explained by **Chai *et al*. (2016)**.

Since growth from the energy view point is the difference between energy gained and energy expended and from a physiological view point it is the net difference between absorption and metabolic rate **(Prieto *et al*., 2020)**. Therefore, any damage to one component of the equation-particularly in metabolic organs such as the digestive gland, growth process can be affected as a result. In fact, according to the present results, significant damage to the digestive gland structure of *Paratapes textilis* was observed as a result of one-month exposure to fluoride. **Pellerin-Massicotte *et al*. (1989)** discussed and proved the negative impact of water pollution on the metabolism of blue mussel *Mytilus edulis*.

3. Accumulation of Fluoride in Shell, Digestive Gland and Feet of *Paratapes textilis*

Fluorides are known to accumulate in vertebrate bones, soft tissues of barnacles and mussels and in the exoskeleton of arthropods **(Ballarin *et al*., 2014)**. In bivalves such as clam, the digestive gland is the main organ responsible for the detoxification of organic and inorganic xenobiotic compounds **(Krautrachue *et al*., 2011)**. The results of the present study showed the pattern in which fluoride accumulation occurred in *Paratapes textilis* clam when exposed to increasing fluoride concentrations. In the control samples, fluoride accumulation was highest in the digestive gland, followed by shell and feet. However, during the progressively escalated exposure to fluoride, the order of accumulation was as follows: feet > digestive gland> shell **(Fig. 3)**. The accumulation of fluoride in the digestive gland and the shell was gradually increasing from one concentration to another. Conversely, in feet, there was a gradual increase in fluoride accumulation that reached its maximum at 5 ppm, followed by a gradual decrease in fluoride accumulation. In other words, during the gradual exposure to an increased concentration of fluoride, feet played an important role in the fluoride detoxification process. Feet of bivalves are known to have important biochemical activities in addition to the ionic and osmotic regulation function that can be involved in fluoride detoxification **(Chandrudu and Radhakrishnaiah, 2008)**.

Statistically, there was a significant increased accumulation values of treated fluoride samples over control fluoride values in all tested organs **(Table 4)**. However, this significant increase was not reached by each organ with the same fluoride concentration. At 2.5 ppm fluoride concentration in the shell of *Paratapes textilis* was the only significant increase. At 5 ppm all organs showed a significant increase. Finally, at 10, 20 and 40 ppm, significance was only shown by shell and feet. *Paratapes textilis* clam appeared not to rely primarily on the digestive gland and its shell as the only organ in the fluoride detoxification process, but feet were also heavily involved in the process,

especially at 40 ppm exposure. *Paratapes textilis* feet tended to accumulate up to 5 ppm of fluoride, however, any further increase in fluoride concentrations was accompanied by the elimination of fluoride from the organ.

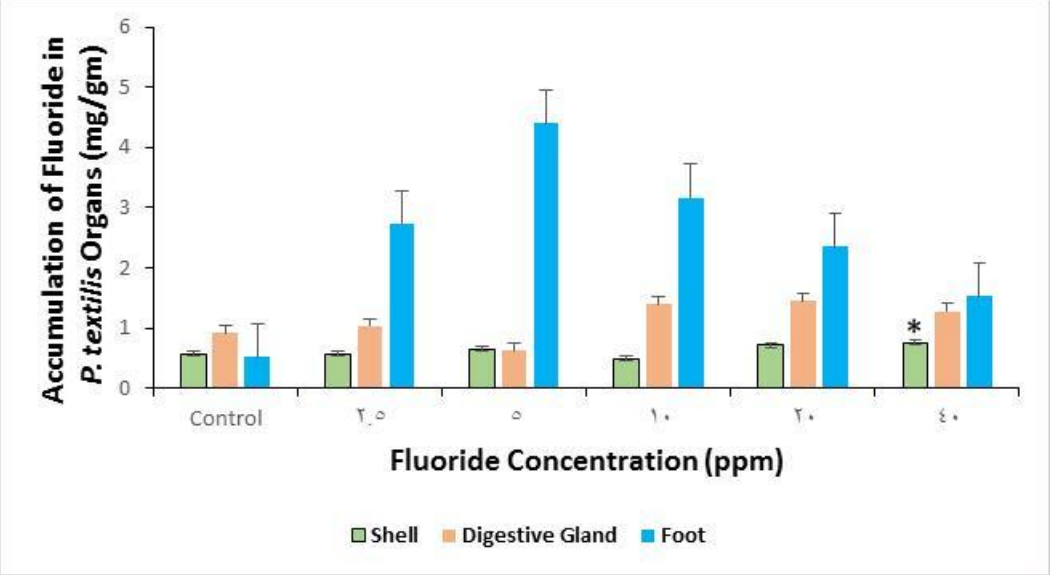

Fig. 3. Mean values ± Standard Error (S.E.) of fluoride accumulation in shell, digestive gland and feet of *Paratapes textilis* in control and treated specimens with 2.5, 5, 10, 20, and 40 ppm fluoride for one month (*significant value)

Table 4. Mean ± Standard Error (S.E.) and *P* value of fluoride concentrations (F^-) in shell, digestive gland and feet of *Paratapes textilis* in control and treated specimens with 2.5, 5, 10, 20 and 40 ppm fluoride for one month. *P* value was calculated using KrusKai Wallis' test and considered significant at the level of ≤ 0.05

F^- (ppm)	Shell		Digestive gland		Feet	
	P value	Mean ± S.E.	*P* value	Mean ± S.E.	*P* value	Mean ± S.E.
Control	-	0.57 ± 0.04	-	0.92 ± 0.06	-	0.51 ± 0.06
2.5	0.032*	0.57 ± 0.04	0.098	1.03 ± 0.32	0.075	2.74 ± 0.93
5	0.047*	0.65 ± 0.09	0.039*-	0.62 ± 0.20	0.048*	4.41 ± 2.21
10	0.018*	0.49 ± 0.08	0.122	1.39 ± 0.32	0.034*	3.17 ± 1.51
20	0.043*	0.71 ± 0.03	0.062	1.45 ± 0.23	0.017*	2.53 ± 0.54
40	0.037*	0.76 ± 0.05	0.176	1.28 ± 0.28	0.019*	1.53 ± 0.41

(*) Significant increase (-): Significant decrease

4. Histological Effect of Fluoride on Digestive Gland and Feet *of Paratapes textilis*

Associated histopathological features in the digestive gland and *Paratapes textilis* feet exposed to an increasing series of fluoride concentrations were as follows:

4.1. Digestive Gland

As was previously mentioned, the digestive gland in bivalves functions mainly in the basic processes of endocellular digestion. It also functions in the accumulation and detoxification of various organic and inorganic toxic substances **(Usheva *et al*., 2006)**. The control histological samples of *Paratapes textilis* digestive gland (**Fig. 4a**), showed the normal appearance of digestive tubules (dt) which was almost oval in shape and lined with a single layer of ciliated (ci) epithelial digestive cells (dc). The digestive tubules were resting on a prominent basement membrane (bm). Another type of cell lining digestive tubules were also observed namely; basophil cells (bc) that have a known secretory function. **Kruatrachue *et al*. (2011)** emphasized that basophil cells of *Nerita saxtilis* and golden apple snail *Pomacea canaliculata,* Lamarck (1822), respectively, occurred singly or in groups and contained basophilic granules of different sizes. These cells were involved in intracellular absorption and digestion. Generally, the digestive tubule tissue of the control samples showed an intact cells structure. Similar results were found by **Usheva *et al*. (2006)** during the study of the digestive gland of *Crenomytilus grayanus* bivalve taken from a clean area of Troitsa Bay in the Sea of Japan.

On the other hand in fluoride-treated specimens, the histological structure of the epithelial cell layer of digestive tubules revealed significant structural impairment (**Fig. 4b-f**). Histological, cytological and histochemical studies were performed on the epithelial cells of bivalves. They proved to be sensitive targets to the damaging effect of many polluted marine habitats **(Usheva *et al*., 2006; Agwuocha *et al*., 2011)**.

At 2.5 and 20 ppm (**Fig. 4b and 4e**), respectively, histological sections showed alterations that were comparable to each other. Clear lysis of the digestive tubules in specific parts and fusion in others were observed. The digestive cells had lost their normal organization and became desquamated. Many pyknotic nuclei (pn) began to appear and spread throughout the entire tissue. Remarkably, both concentration sections were characterized by vacuolization. Several vacuoles (v) were observed within all digestive tubules. Though, it was established earlier that vacuoles of digestive cells were normally involved in a system of heterophagosomes and lysosomes performing intracellular digestion **(Owen, 1974)**. Later, researchers attributed abnormal vacuoles in the digestive cells of bivalves to a drastic increase in the number of lysosomes as influenced by pollutants **(Usheva *et al*., 2006)**. Frequent emptying of digestive gland cells have been recorded, usually occurring in bivalves, as a result of organic pollution **(Zupan and Kalafatic, 2003)**, inorganic pollution **(Wedderburn *et al*., 2000)**, and xenobiotic ones including fluoride as well **(Usheva *et al*., 2006)**. Secretory granules (sg) or basophil cell products especially in specimens subjected to 20 ppm, proliferated in most parts of the digestive tubules (**Fig. 4e**). Desquamation of epithelial cells was observed by **Sheir**

(2020) in treated freshwater clam *Caelatura nilotica* (Cailliaud, 1827), which coincided with our present observations.

Significantly at fluoride concentration of 5 ppm, large dark granules or lysosomal granules (lg) of variable sizes in many dilated digestive tubules comprising their greater area was rampant (**Fig. 4c**). These dark granules could be large residual bodies of lysosomes in which toxic substances had accumulated **(Hamed *et al.*, 2007)**. Similar dark granules have been recorded by several researchers in the digestive gland of the bivalve *Mytilus galloprovincialis* **(Domouhsidou and Dimitriadis, 2000)**, and the golden apple snail *Pomacea canaliculata*, Lamarck (1822) **(Kruatrachue *et al.*, 2011)**. Sections at 5 ppm also revealed thickened, torn and eroded sites in the connective tissue (ct) separating one digestive tubule from another. A thickened epithelial basement membrane has been documented by **Sheir (2020)** in freshwater clam *Caelatura nilotica* (Cailliaud, 1827), exposed to various mixed pollutants. We also detected the appearance of some interspersed granulocytomes (gc) in the surrounding connective tissue of different sizes (**Fig. 4c**). Granulocytomes were often a recorded pathological change in bivalves living in conditions of anthropogenic pollution and also in bivalves experimentally exposed to various pollutants, such as oil, chlorinated pesticides, and heavy metals, respectively **(Lowe and Moore, 1979; Wolfe, 1992; Wedderburn *et al.*, 2000)**. At 10 and 40 ppm (**Fig. 4d and 4f**), cells of the digestive tubules showed enlarged and dilated borders or hypertrophy (h) and hyperplasia. **Kumar *et al.* (2011)** while working on freshwater mussels showed the lining of the digestive tubule to be enlarged in height after 96 h of exposure to dimethoate leading to the disruption of the apical portion of the digestive tubules. These adverse effects recorded in *Paratapes textilis* could cause damage at a higher level of biological regulation such as pyknosis related to DNA fragmentation **(Kumar *et al.*, 2011; Casellato *et al.*, 2012)**.

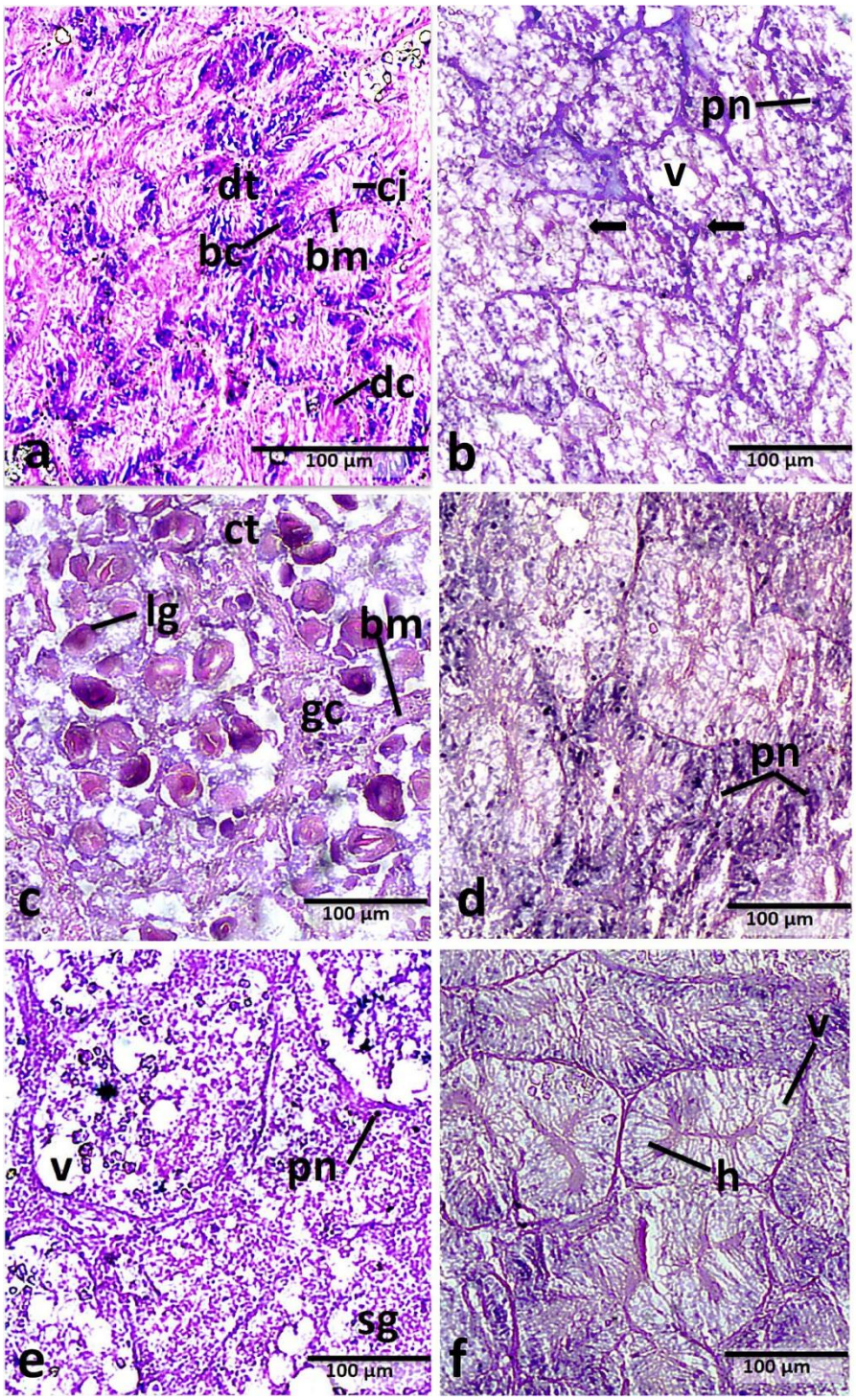

Fig. 4. T.S. in the digestive gland of *Paratapes textilis* (a) control specimens and (b), (c), (d), (e) and (f) treated specimens with 2.5, 5, 10, 20 and 40 ppm fluoride for one month, respectively. Abbreviations; basement membrane (bm); basophil cells (bc); cilia (ci); connective tissue (ct); digestive cells (dc); digestive tubules (dt); granulocytomes (gc); hypertrophic cells (h); lysosome granules (lg); pyknotic nuclei (pn); secretory granules (sg); vacuoles (v). Torn and fused digestive tubules (black arrows)

In general, the present alterations were similar to many of the histopathological changes in the digestive gland recorded by previous researches on bivalves subjected to a number of different contaminants **(Usheva *et al.*, 2006; Agwuocha *et al.*, 2011)**. However, at 40 ppm specimens changes were less severe than those recorded for lower fluoride concentrations. Seemingly a partial recovery due to the animal defence process occurred.

4.2. Feet

Feet are locomotory organs used mainly for burrowing. They have a wedge shape that is adapted to advance in the mud or sand where the animal lives. Histologically, the bivalve muscular feet have distinct tissues from the rest of the body. In the present work, the control sections had a normal structure of the feet musculature with normal vertically oriented muscle fibres (vmf) and horizontally oriented muscle fibres (hmf) interspersed with connective tissue layers (ct) **(Fig. 5a**). **Park *et al.* (2012)** revealed similar observations in feet musculature of equilateral bivalves *Gomphina Veneriformis*. The muscle fibres as a whole showed integrity and proper structure with no signs of splitting fibres. On the other hand, the muscles of the fluoride-treated samples of *Paratapes textilis* feet showed variable severity of the abnormal build. Feet muscles were examined at various concentrations of 2.5, 5, 10, 20 and 40 ppm (**Fig. 5b-f**). A pronounced splitting and atrophy of muscle fibres, which in addition appeared torn, loose, disorganized and interspaced in different parts. Pyknotic nuclei (pn) were prevalent in all sections. The surrounding connective tissue (ct) was loose and caused a loss of intactness of muscle bundles. Especially at 2.5, 5 and 10 ppm fluoride concentration, the horizontally arranged muscle fibres disintegrated to a large extent and were not evident (**Fig. 5b, c, d**). **Chandrudu and Radhakrishnaiah (2008)** highlighted similar degenerative changes while studying the effect of cadmium on feet muscle of freshwater mussel *Lamellidens marginalis* (Lam.). These alterations could lead to a failure of some biochemical functions of feet such as Osmo and Iono-regulation.

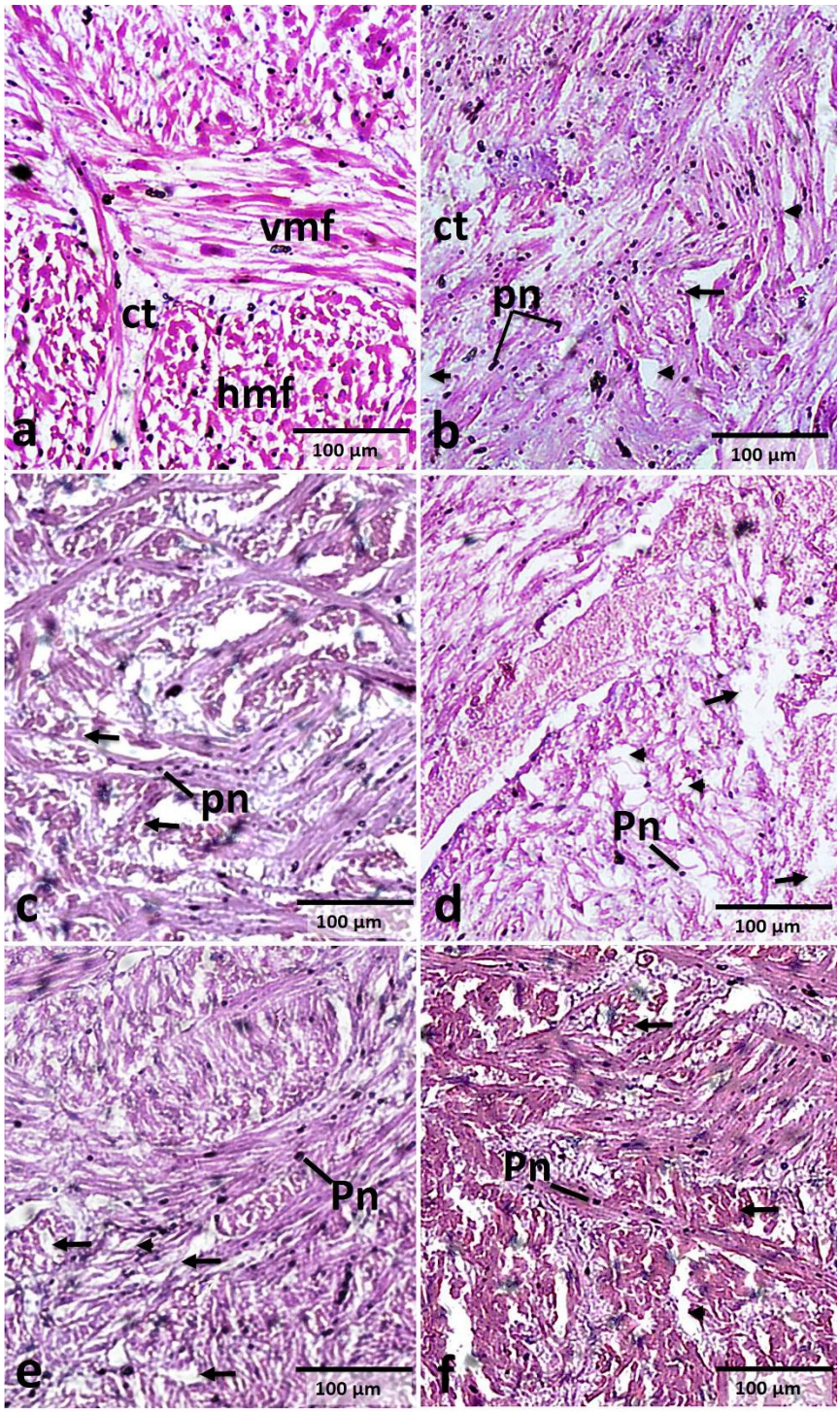

Fig. 5. T.S. in feet of *Paratapes textilis* (a) control specimens and (b), (c), (d), (e) and (f) treated specimens with 2.5, 5, 10, 20 and 40 ppm fluoride for one month, respectively. Abbreviations: connective tissue (ct); horizontally oriented muscle fibres (hmf); pyknotic nuclei (pn); vertically oriented muscle fibres (vmf). Torn and disintegrated tissue (black arrows); spacing (black arrows' heads)

Chetty *et al.* (1988) working on the freshwater mussel *Lamellidens marginalis* exposed to an acute concentration of methyl parathion showed similar results. Spacing and dissociation of muscle fibres were detected at 20 ppm sections (**Fig. 5e**). At a fluoride concentration of 40 ppm, a slight partial recovery in the arrangement and integrity of muscle fibres were observed which could be attributed to clams' struggle against fluoride stress (**Fig. 5f**).

5. Fluoride Bioaccumulation Factor (BAF)

Average *BAF* values in the flesh of *Paratapes textilis* of fluoride-treated samples (2.5, 5, 10, 20 and 40 ppm) ranged from 1511 at 2.5 ppm to 70 at 40 ppm (**Fig. 6a**). Flesh samples of 2.5 and 5 ppm fluoride-treated gave 1511 and 1094, respectively exceeding 1000 and showed a high fluoride bioaccumulation. The other higher fluoride-treated samples were less than 1000. The present study reflected intense bioaccumulation of fluoride in *Paratapes textilis* flesh, especially feet subsection at 2.5 and 5 ppm fluoride-treated samples compared to the other fluoride-treated samples. However, 2.5 and 5 ppm fluoride content were relatively similar to that of some coastal seawater (> 5 ppm; **El-Said *et al.*, 2016)**. Whereas flesh *Paratapes textilis* absorbed fluoride by 10-40 ppm from the fluoride-treated samples as it did in the feet compared to the control group, but to a relatively similar extent it accumulated in the digestive gland (**Figs 3, 6a**). This fact coincided with fluoride bioaccumulation, which appeared to be related to the rate at which fluoride was eliminated from each organ of the species.

6. Human Health Risk Assessment

Figure (6b) shows the estimated daily intake *EDI* for toddlers (9.9-62.4 mg/kg/day) and adults (6.6-41.4 mg/kg/day) for all fluoride-treated samples that exceeded the LOAEL (lowest-observed-adverse-effect level; 0.25 mg/kg/day for skeletal effects **(Li *et al.*, 2001)**. Our results revealed that the hazard quotient calculated for toddlers (HQ_T; 80.9-511.1) and adults (HQ_A; 53.7-339.0) exceeded 10, indicating the severe health risks from consumption of *Paratapes textilis.*

However, fluoride was absorbed in an acidic media (gastric solution) then converted into HF. According to **Barbier *et al.* (2010**) about 40% of ingested fluoride could be absorbed from the stomach. The HF molecule appeared to penetrate the cell membrane more easily than the separate fluoride ion **(Barbier *et al.*, 2010)**. The reported bioavailability of fluoride from various foodstuffs varied from 2 to 79 % **(Viswanathan *et al.*, 2010; El-Said and El-Sadaawy, 2013)**. Fluoride affected not only hard tissues but also soft tissues including renal, endothelial, gonadal, and nervous cells **(Barbier *et al.*, 2010, El-Said *et al.*, 2015)**. In infants, about 80–90% of the absorbed fluoride was retained, but in adults, this level dropped to about 60% **(ATSDR, 2003)**.

Since bivalves are known to be filter feeder organisms that can uptake and excrete organic matter containing fluoride such as faeces, droppings and soil particles. Thus, as previously reported by **El-Said *et al.* (2015)**, it seems desirable to keep live *Paratapes*

textilis in clean water for some time before eating or cooking, in order to overcome the condensed fluoride in their soft tissue.

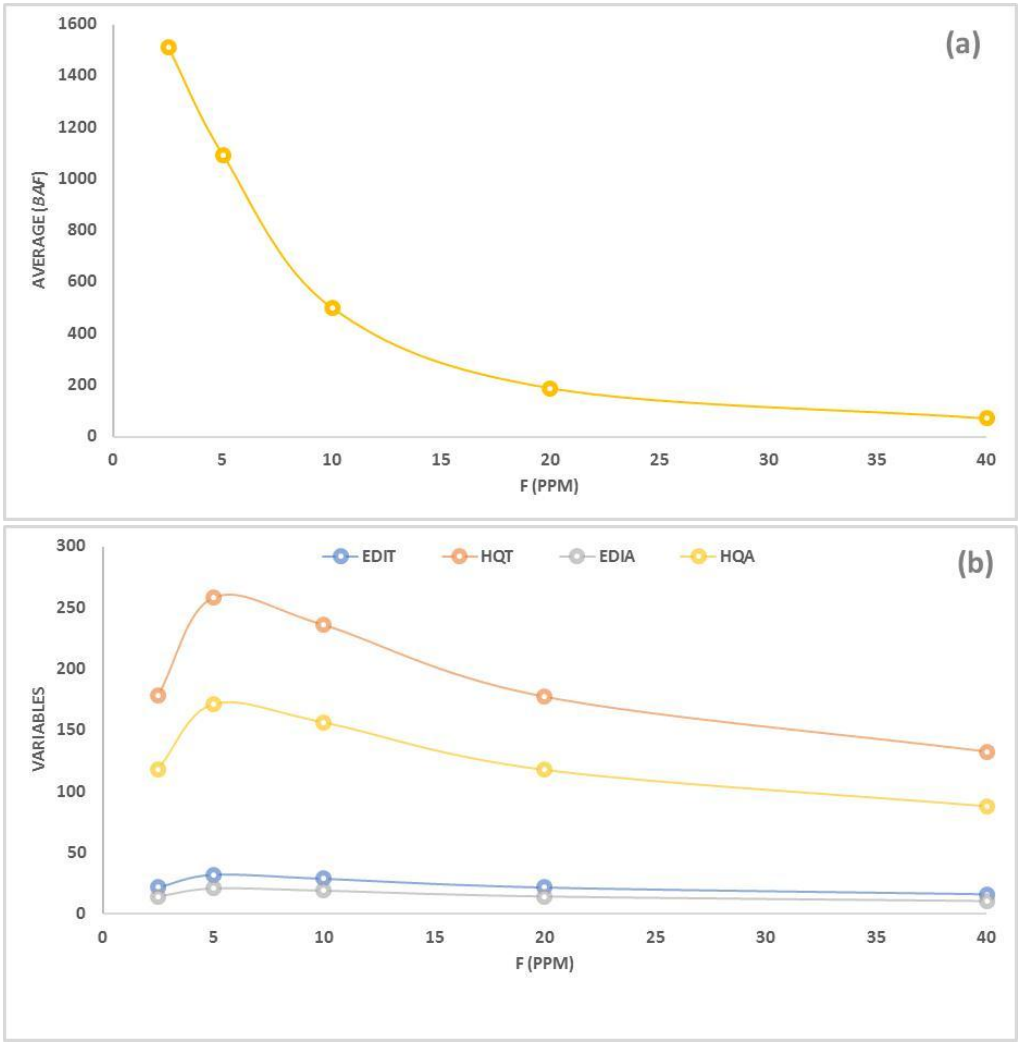

Fig. 6. Variables (a) Average BAF and (b) Estimation daily intake (EDI_T and EDI_A) and hazard quotient (HQ_T and HQ_A) of toddler and adult, respectively, of *Paratapes textilis* treated with 2.5, 5, 10, 20 and 40 ppm fluoride for one month

CONCLUSION

Fluoride toxicity to *Paratapes textilis* (Gmelin, 1791) was obviously shown in morphometric and physiological levels. For the first time, discolouration or shell whitening due to fluoride exposure (fluorosis) was clearly observed in the present study. This phenomenon could result in the inhibition of many crucial vital physiological operations such as camouflage and mating. The present results also detected slowness in the growth process, which could be ascribed to slower or impeded metabolic rate. Our results highlighted the probable significant role of measuring shell width for the

evaluation of the growth process. Accumulation of fluoride showed a proposed role played by clam feet in the detoxification process of fluoride, especially at 5 ppm.

Histological observations of digestive gland and feet of *Paratapes textilis* subjected to different concentrations of fluoride showed considerable impairment of digestive tubules, desquamation of digestive cells, epithelial disruption, cellular and nuclear hypertrophy, hyperplasia, in addition to neoplastic and pyknotic cells. As a result, damage and disruption of digestive tubules due to fluoride exposure could cause serious dysfunction of the tissue such as delayed growth shown in the present study. Similarly, feet musculature showed a negative effect of fluoride. Vertically and horizontally muscle appeared torn and degenerated, in addition to vacuoles and pyknotic cells that were scattered in feet sections. It is worth mentioning that at 40 ppm, a considerable positive change in growth (significant increase in weight) and in histological sections of the digestive gland and feet were observable. This recovery could be attributed to a developing internal body resistance of *Paratapes textilis* to fluoride.

Not only did fluoride-treated samples had shown adverse effects in the histology of tissues, but also fluoride showed intensive accumulation and consequently high potential human health risk even when ingested in lower fluoride concentrations by *Paratapes textilis*. It is thus higly recommended to keep live *Paratapes textilis clams* in clean water for some time before eating or cooking in order to overcome the condensed fluoride in the soft tissue of the bivalve.

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