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Impact of Selected Environmental Pollutants on the Ultrastructure of the Gills in *Pinctada radiata* from Coastal Zones, Egypt

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Abstract

There has been an increasing interest in marine oysters (order: Petridae) in recent years because their numbers are declining in many parts of the world and also because they are used as monitors of pollution. The present study describes the microscopic structure of gills as viewed by light and electron microscopy in two locations selected in Alexandria coast, Eastern Harbor (E.H.) and El Asafra. The specimens in the E.H. represent the presence of extracellular mineralized granules.

Keywords

Gill, Bivalve, Transmission Electron Microscope, *Pinctada radiata*

1. Introduction

There are over 6500 species of marine bivalves in the phylum Mollusca [1], including oysters. Marine bivalves are known to be natural unique accumulators of contaminants [2]. The environment had become increasingly aware of the importance environmental risk management in the economic development, health and quality of life [3]. [4] and [5] reported that *Pinctada radiata* could be used as indicator species for heavy metals accumulation studies. The sensitive aquatic environment is suffering of pollution that affects both quantity and quality of

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benthic invertebrate biodiversity [6].

Bivalves are used in monitoring programmes in the marine environment due to their ability to concentrate pollutants to several orders of magnitude above ambient levels in seawater [7]-[9]. [10] and [11] reported that bivalves had been used as successful biomonitors of aquatic metallic pollutant levels internationally prominent examples including the United States National Oceanic and Atmospheric Administration Mussel Watch Programmes and the Australian Oyster Watch Programmes. Cadmium levels in hepatopancreas were lower than those in gills [12]. Shellfish plays an important role in the ecology of aquatic pathogens [13], and [14]. Histopathology is a tool for monitoring anthropogenic contamination [15] [16].

2. Materials and Methods

2.1. Sampling

2.1.1. Water Samples

Coastal water samples were collected from the three selected locations on the coast of the Alexandria at 3 - 5 m depth for the determination of copper, and cadmium. Seawater samples were filtered through 0.45 µm millipore filters to remove any debris particles then stored at −20˚C until analysis. All concentrations are reported as µg/l for seawater. All the precautions recommended by [17] to minimize risks of sample contamination were followed during collection and treatment of samples.

2.1.2. Mollusk Samples

The Bivalve were collected in sterile plastic bag (no. 250/location/season, replicant, 5 time) and were cleaned from attached organisms and then rinsed with seawater from their sampling locations and transported to the laboratory within 4 - 6 hrs.

2.1.3. Analytical Methods

1) Heavy metals in seawater

The concentration of heavy metals were determined in the collected seawater samples using Graphite Furnace Atomic Absorption Spectroscopy (Perkin-Elmer model 2380) under the recommended conditions and the detection limits in the manual for each metal [18].

2) Physicochemical analysis of seawater

Surface water samples were collected five times; bi-week from three sites representing the coastal area in front of Alexandria city at each location, water samples were collected using polyethylene bottles (2-liters capacity). The polyethylene bottles were previously cleaned with detergent rinsed several times with distilled water, soaked in 1 N HCL for several days and finally rinsed with re-distilled water. At each site a 150 ml dissolved oxygen bottle was firstly filled and immediately fixed, using manganous sulphate and alkaline potassium iodide solution [19]. Some parameters were totally or partially measured in the field *i.e*. as soon as the sample was collected. These steps of the methods would be explained by the term "*in situ*" in the text. Temperature measurements: *In situ* at each station, air and water temperatures were measured at the time of water sampling using an ordinary thermometer. Salinity (S‰): Salinity was determined by measuring the electrical conductivity using an inductive Salinometer (Beckman; model RS. 10). Hydrogen-ion concentration [pH]: The pH-value of water sample was measured in the laboratory immediately after collection using Bench type (JENWAY, 3410 Electrochemistry Analyzer pH-meter). Dissolved oxygen (BOD: It was determined by a modified Winkler's method [20].

3) Heavy metals in tissue

The preparation of samples to determine concentration of heavy metals was carried out animals were separated from the shells; weighed and digested using conc. HNO_3 in Taflon digestion vessels. Wet digested samples were diluted with deionized distilled water and analyzed by Ion-selective electrod AVL. The obtained data were expressed as µg/g wet weight [21]. The analytical method was checked by (5 replicate) measurements for the studied metals in a sample of marine.

4) Histological studies on gills of (Bivalve, Mollusca)

a) Light microscopical technique: In order to establish the histological state, twenty individuals from each sampling site/month, were processed for light microscopical study [22]. The shells were removed gently then the soft specimen was quickly dissected.

b) Electron microscopy: Specimens were fixed in 2.5% glutaraldehyde solution (pH 7.2, buffered 0.1 M phosphate buffer) for 2 - 4 hrs at 4˚C and rinsed in 0.1 M phosphate buffer and then post-fixed in 1% osmium tetroxide (OsO_4) solution for 2 hrs at 4˚C. After fixation, the specimens were washed with 0.1 M phosphate buffer 4 times for 2 hrs and dehydrated with ascending grades of ethanol. Specimens for transmission electron microscope (TEM) were embedded in Epon 812, cut at ultrathin sections (70 nm in thickness) and placed on copper grids (200 mesh) in order to double-stain with uranyl acetate and lead citrate. Specimens were examined using a TEM (JEM-1200EXII, JEOL, Japan).

Semithin sections (0.5 - 1.0 µm) were cut using LK Bill ultra-microtome. In order to stain the resin embedded sections, they were rinsed for 1 - 2 minutes in about 1% toluidine blue solution in 1% borax. They were then washed in tap water, dried on hot plate (60C) and mounted in Canada balsam. Toluidin blue-stained sections were examined and photographed using Diallux 20EB Leitz research microscope provided with Canon camera. Ultrathin sections were cut from the resin blocks at a thickness of 10 nm using glass or diamond knives. Sections were mounted on coated grids (1% partodion in amyl acetate) and stained in solution of aqueus or alcoholic uranyl acetate for about 15 - 20 minutes. After drying, they were examined.

2.2. Statistical Analysis

Statistical analysis was performed using two-way ANOVA using SPSS computer program (version 14.0) to check for significant difference between metal concentrations in different localities.

3. Results

The ecological investigations of water were restricted to two locations; El Asafra and the Eastern Harbor (E.H.). All parameters were measured monthly and they are shown in **Figure 1(a)**, **Figure 1(b)**.

The gills consists of two plates at each side of animal, the gill plate is comprised of parallel filaments, connected by cillialry discs. Each gill filament is divided into abfrontal, intermediates and frontal zone (**Figure 2(a)**, **Figure 2(b)**). In the center of the filaments, haemocytes circulate through the haemolymph vessel. The frontal surface of a gill filament bears frontal cilia, latero frontal and lateral cilia (**Figure 3(a)**, **Figure 3(b)**). The wall of the gill filament is lined with ciliated columnar epithelial cells with ovoid nuclei, between them there are a number of mucous secretory cells with circular nuclei.

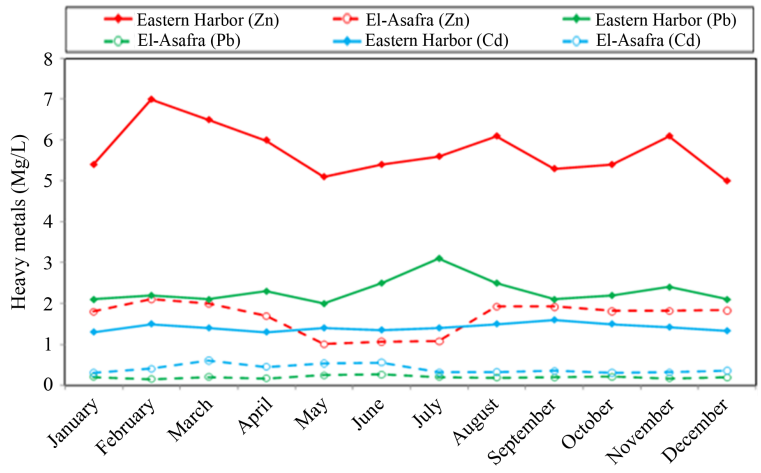
Three types of cells had been reported by transmission electron micrographs. Cells containing several large mitochondria, flattened epithelial cells covered the first type and with elongated microvilli, mucous cells enclosed between the first one. There were no obvious surface morphological abnormalities of gill filament in animals collected from E.H. or El Asafra. Little increase in the mucous secretion and lateral cilia increased in length and number in oyster gill collected from the E.H. (**Figure 4(a)**, **Figure 4(b)**).

The histo pathological changes directed to the arrangement of regularity of gill lamellae and occasional areas avoid of microvilli appeared on some frontal and lateral surfaces. Black granules were detected throughout the cells and in some cases the mitochondrial membrane started to decay. There were some areas where abfrontal surface were lacking of cilia, in some cases frontal and abfrontal cells appear necrotic, their internal organelles getting out into the extracellular space.

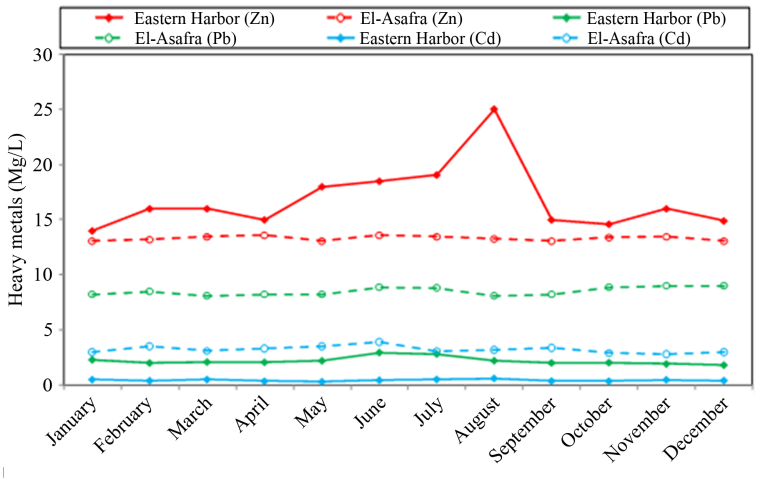
There were some alteration in filaments morphology with increase in vacuoles and decrease in mitochondria number which sometimes completely disappeared and several gill filaments were dilated. In the present study, histology showed haemocyte infiltration (**Figure 6**).

In Electron microscopic sections, the central zone of the gills, consists of a sheet of tissue with outer and inner epithelia separated by loose interstitial tissue containing haemolymph spaces, haemocytes, muscles and variable numbers of large vesicular cells (**Figures 2-4**). In the central zone of the gills of *Pinctada radiata*, there were generally loosely packed vesicular cells and large extracellular spaces, often traversed by thin muscle fibers. In some specie men the interstitial tissue was with numerous clusters of granules among the vesicular cells. In some specimens, the tissue was much denser, as shown in (**Figure 5(a)**, **Figure 5(b)** and **Figure 6(b)**). Larger granules were also often found scattered through the tissue.

The vesicular cells (**Figure 5(a)** and **Figure 6(a)**) were large, with a central region filled with fine granular storage material and a thin peripheral layer of cytoplasm containing the nucleus. Muscle cells contained thin filaments and dense bodies, with no cross striations. Mitochondria lay peripherally, often in large lateral cytoplasmic projections. Lateral projections often contained large amounts of granular material. Muscle cells varied in

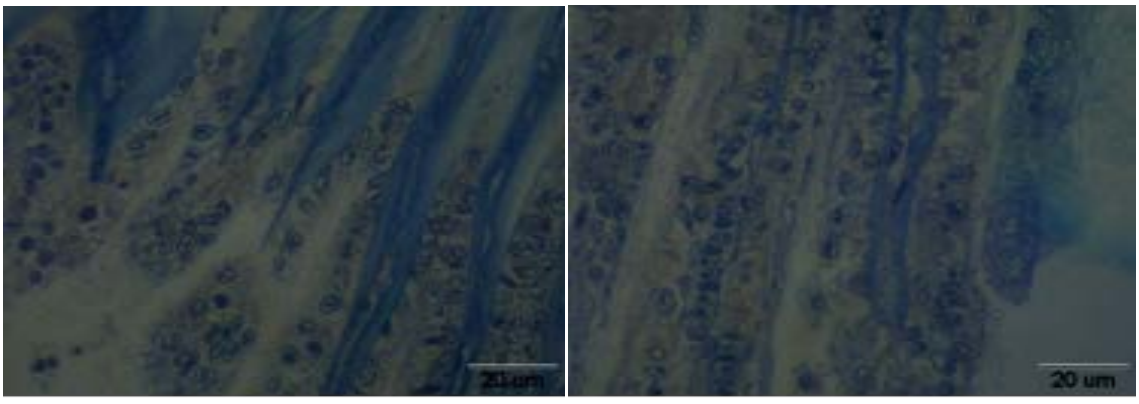


(a)



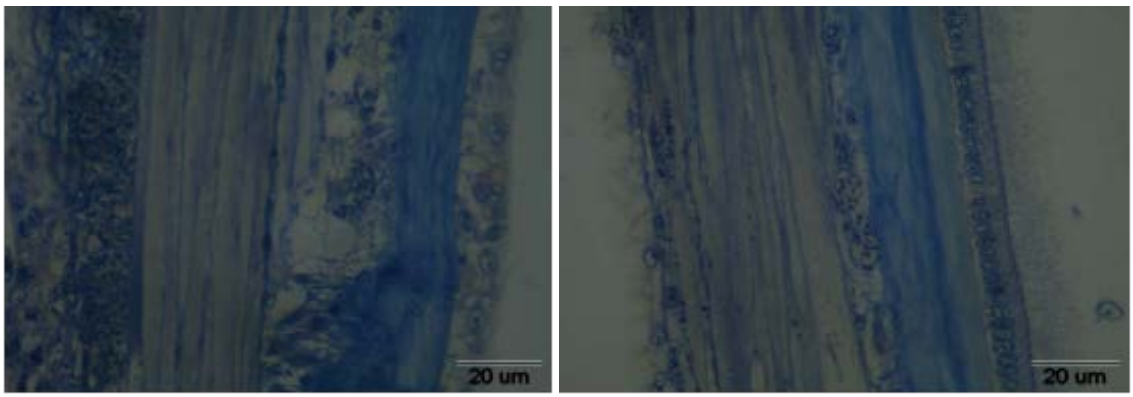
(b)

Figure 1. (a) Comparison between E.H. and El Asafra in the sea sample according to heavy metals. (b) Comparison between Eastern Harbor and El Asafra in gills of *Pinctada radiata* (µg/g) according to heavy metals.



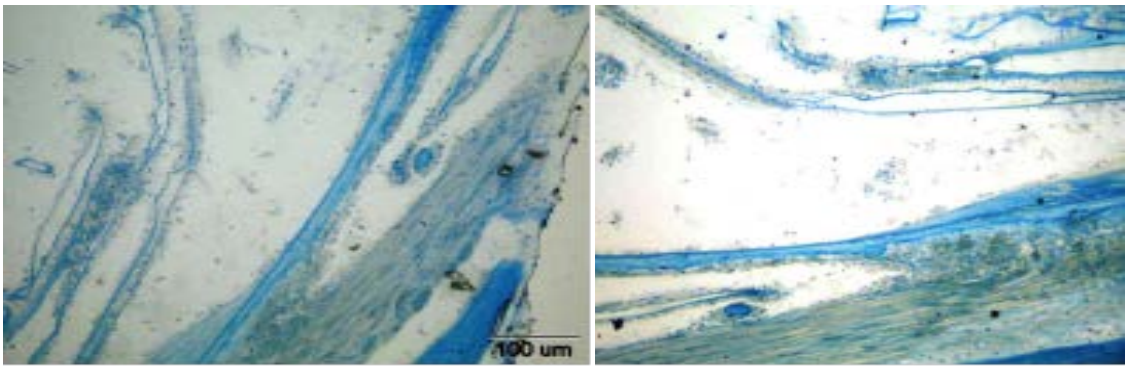
(a) (b)

Figure 2. Photomicrograph, semithin, of gill of *Pinctada radiata*, collected from El Asafra showing gill filaments (G.F.), inter filamentary junction (IFJ), frontal (FS) and lateral (LS) and inter lateral surfaces (ILs) of gill filaments.



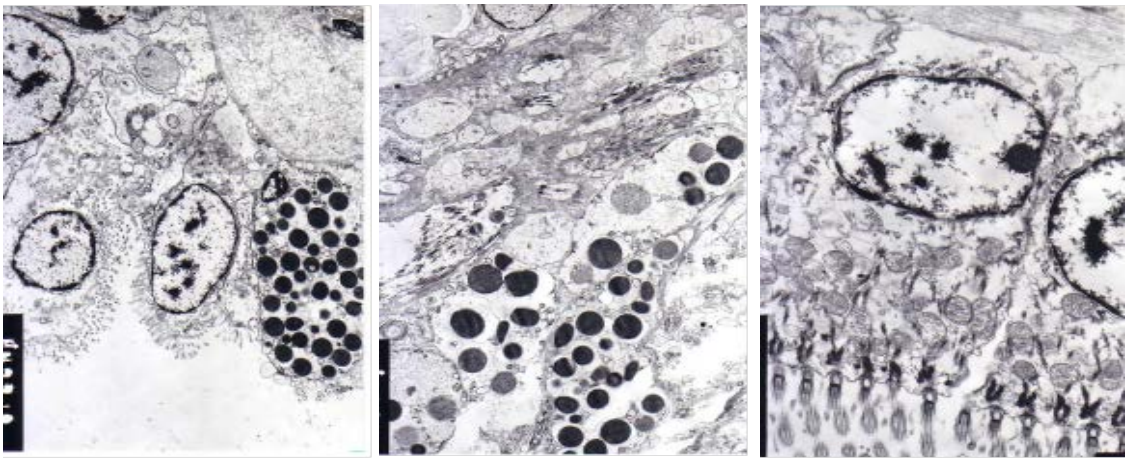
(a) (b)

Figure 3. Photomicrograph, semithin, of gill of *Pinctada radiata* collected from El Asafra showing; mucocytes (Mu), frontalsurface (FS), haemocoel (H), lateral cilia (LFC), lateral cilia (LC), mucocyte (Mu) and nucleus (N).



(a) (b)

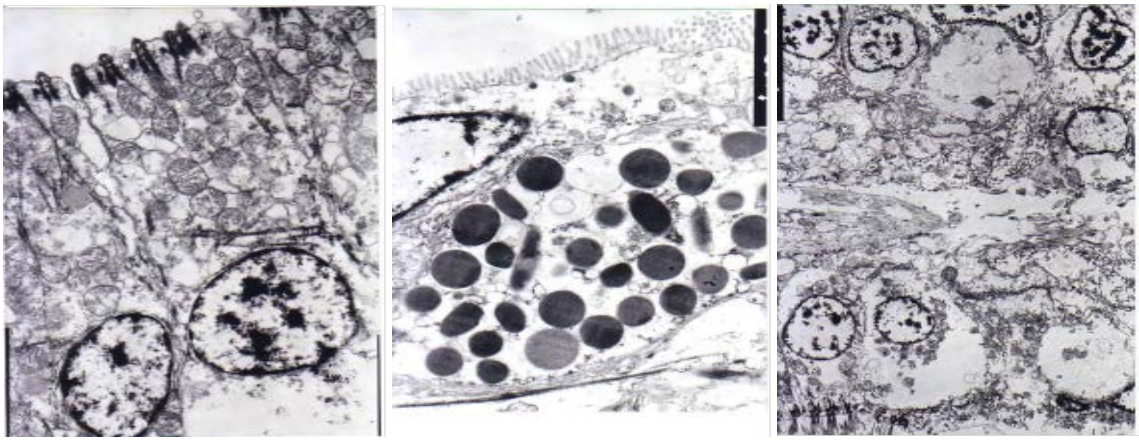
Figure 4. Photomicrograph, semithin, of *Pinctada radiata* collected from E.H. showing abnormal gill showing abnormal shape and irregular arrangement of gill filaments (G.F.), haemocoel (H.) and decay of frontal surface.



(a) (b) (c)

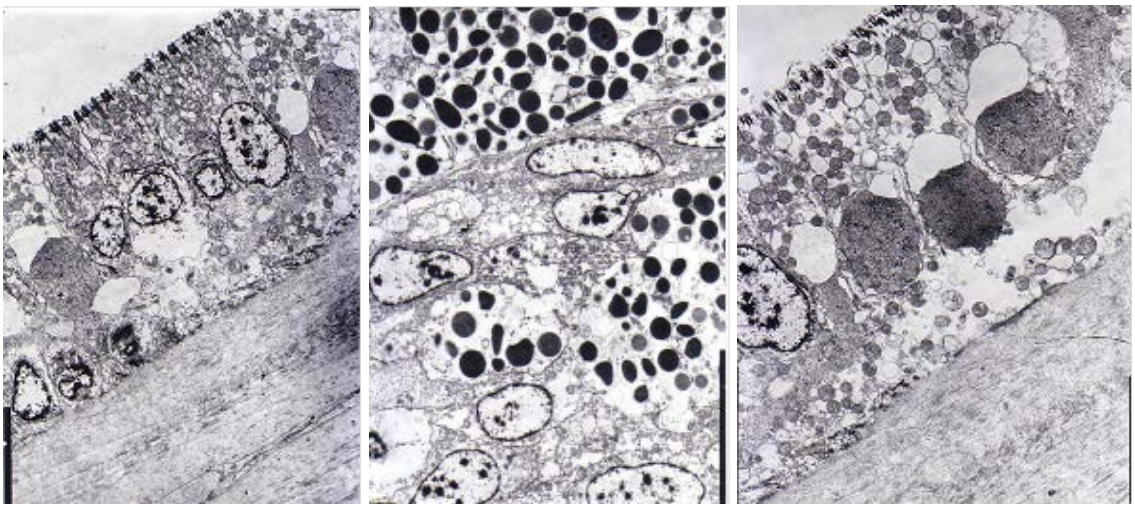
Figure 5. (a) (b) TEM micrograph of normal gill filament in the frontal zone of gill filament of oyster collected from El Asafra, showing the thin cells with microvilli (MV) cover the apical surface of cells. (c) TEM micrograph of normal gill filament from El Asafra showing the mucous cell (Mu), cells with many mitochondria (M) and nucleus (N).

size, from large thick cells in the muscle bands traversing the distal margin of the gills, to thin fibers laying under the epithelia (**Figures 7(a)-(c)**).



(a) (b) (c)

Figure 6. (a) (b) TEM micrograph of normal gill filament in the frontal zone of gill filament of oyster collected from El Asafra, showing the thin cells with microvilli (MV) cover the apical surface of cells. (c) TEM micrograph of normal gill filament from El Asafra showing the mucous cell (Mu), cells with many mitochondria (M) and nucleus (N).



(a) (b) (c)

Figure 7. (a) (b) (c) TEM micrograph of gill illustrating the decayed mitochondria (M) and mitochondrial membrane; gill oyster collected from E.H.

The most common type of haemocyte observed in the oysters was a large granulocyte with vesicles containing amorphous material (granules), in descriptions of haemocytes because of their granular appearance by light microscopy. There were also smaller numbers of haemocytes without large vesicles. These had variable nucleus, cytoplasm ratios and variable numbers. Gills contained varying quantities of granules. Most granules were extracellular, Granules were also scattered in the interstitial tissue. In some specimens, granules were generally very numerous, the majority of granules occurred as large clusters of small granules.

4. Discussion

Sea food is considered as an important source of protein for human [23]. In the present study the concentration of heavy metals is considered less than that reported by [24]. It is concluded that the coastal area in Mediterranean sea of Egypt might be considered relatively unpolluted with heavy metals. [25] reported that the coastal area in Mediterranean sea of Egypt might be considered relatively unpolluted with heavy metals. In all cases the average concentrations of trace elements in Egyptian Mediterranean coast are far from the hazardous concentrations [26]. They revealed that the origin of trace elements in the sampled water of the Egyptian Mediterranean

was mainly the waste water discharge. [27] reported that the uptake of metals may take place at the gills of bivalve. Only gills of bivalves can be considered as an adequate target tissue for heavy metals [28]. [29] suggested that the gill in Mussel *Mytilus edulis* appeared to be more suitable organ for biomonitoring heavy metals. Bivalves are frequently used in marine ecotoxicology for the purpose of assessing seawater quality because they are very sensitive to pollutants [30]. Gills are frequent targets of environmental pollutants because they are the main interface between the organisms and their environment [31]. Gills are the target organ in oysters exposed to high concentrations of heavy metals [32].

Histopathological alterations of gills of bivalve tissues have been shown to be responsive and sensitive to wide range of contaminants because they play an important role in respiration and food collection [33]-[35]. The histopathological changes of gills of bivalve in the present study are in agreement with [2] and [36] as they reported irregularity of gill lamellae of the cells, swelling of gill filaments and haemocytes infiltration of bivalve. Epithelial cells of the gills play a crucial role [37]; the damage in the epithelium results in serious dysfunction of tissues consequently leading to deleterious effect on the organization levels [2]. Bivalves possess different measures of defense against environmental hazards e.g. particle rejection and formation of pseudo faces, reduced filtration rate and valve closure [38]. Haemocytes in bivalves possess a variety of functions, including regeneration, digestion and phagocytosis of foreign particles and pathogens [39]. The major environmental problem in the coastal area is directly related to the impact of domestic effluents [40].

[41] reported that metal concentrations recorded in the soft tissues of mussels *Mytilus galloprovincialis* increased without a source of extra metals in water. [26] revealed that the origin of trace elements in the sampled water of the Egyptian Mediterranean was mainly the waste water discharge. They added that the surface east water current and south west winds blowing on the Mediterranean coast of Egypt contributed mostly in spreading the trace elements to wide areas of the coast. [28] found that the degree of environmental contamination was only one among several factors that influenced metal concentrations in animals. Bioavailability or specific sources may be responsible for higher concentrations in apparently less impacted environments. [29] suggested that gills of bivalves appeared to be a more suitable organ for biomonitoring than that of the hepatopancreas. Bivalves are frequently used in marine ecotoxicology for the purpose of assessing seawater quality because they are very sensitive to pollutants [30]. Gills are frequent targets of environmental pollutants because they are the main interface between the organisms and their environment [31] and [32].

Mediterranean is surrounded by 18 countries from three continents (Europe, Africa and Asia) [42]; intense human activities from these countries produce a strong environmental impact in form of marine degradation [43], and cause heavy metal stress on the Mediterranean waters mainly through discharging different sources of pollutants through discharging different sources of pollutants through coastal waters [30]. The Egyptian Mediterranean coast has been influenced by untreated urban and industrial effluents that caused coastline degradation [44] and [45], particularly in Alexandria coast due to the high population growth and rapid development [45]. [46] reported Zn in gills of Mussels in France as 17.8 + 2.1 µg/g. In the present study data the gills of *P. radiata* is considered less than that, as it is reported to be 13.33 ± 0.2 in El Asafra and 16.84 ± 3.04 µg/g in the E.H.

In British Columbia, Canada along an apparent pollution gradient of acid mine drainage, tissue Zn concentrations were likely not high enough to have a direct impact on mussels (*Mytilus edulis*) health [47]. Metal binding induction differs markedly among the gills of the bivalve: mollusks *Mytilus galloprovincialis* and *Ruditapes decussatus*. [48] concluded that gills could preferentially be used in biomonitoring studies in the blue mussels. The gill tissue of *Mytilus galloprovincialis* is responsible for the uptake of metal ions from water [49]. Cd is not an essential element for animals [50]. He added that the occurrence of Cd in the marine environment was rare, therefore the impact of Cd on the environment was considerably small. [51] stated that cadmium was not needed for clams' growth and may be deleterious. Exposure to high levels of Cd does not stimulate Reactive Oxygen Species [52]. Gills are suggested as a possible route for accumulation of Cd as a possible route for Cd excretion [53]. [54] reported that Cd was present in the effluent and had accumulated significantly in mussels' gills. [55] reported that, the concentration of dissolved cadmium in gills of *Pinctada radiata*, collected from coastal zones was fluctuated between 3.03 µg/g wet weight and 0.57 µg/g wet weight. [46] reported; the level of Cd level in the gills of oysters collected from (Loire Atlantique, Bourgneuf, France) as 0.27 + 0.01 µg/g wet weight. They added; the level of Cd level in the gills of mussels collected from (Loire Atlantique, Bourgneuf, France), as 0.1 + 0.01 µg/g wet weight.

An assessment of potential risks to human health due to consumption of the mussels (*Mytilus edulis*) and (*Perna viridis*) was undertaken for the metals. Metals could pose a health risk to heavy seafood consumers [56].

[54] reported; Cd accumulated significantly in mussels' gills. Uptake of metal in bivalves may take place at the gills and their relative importance is a function of the speciation of the metals in the environment [27]. The surveys of contaminants in shellfish conducted by Agency for Toxic Substances and Disease Registry [17] which reported the mean of Cd level for shellfish as 360 µg/g dry weight. [29] suggested that gill appeared to be more suitable organ for metal biomonitoring more than the hepatopancrease. [49] reported that the gill represented the quick answer of mussels to water concentrations of metals. [41] concluded; in nature metals in *Pyganodon grandis* are bound in the gills.

Very high Cd concentration may result from food chain bioaccumulation of elevated Cd levels brought into the productive surface water by upwelling into the region [40]. Lead is leader member of the toxic metals in the marine environment [50]. He added that metal variations were result of both natural and human activity. Moreover, some mollusk species represent a valuable seafood source. Therefore, high concentration of heavy metals in mollusk species gives dangerous indicator to deteriorate the marine life and pose a health risk to human. Exposure to Pb generally resulted in reduced oyster growth [11]. Reduction in growth has been reported to oyster *Pinctada imbricata* exposed to 270 µg/l Pb [57]. Lead uptake at the gill surface may occur via a number of possible pathways including passive diffusion, active transport [11]. [51] stated that in an environment affected by contaminant oyster tissues have an opportunity to adsorb heavy metals. Surveys of contaminants in shellfish conducted by [17] found that the mean Pb concentration in health tissues of the shellfish should not exceed 250 µg/g, when Pb concentration exceeded 250 µg/g, shellfish became harmful to consumers.

The bioavailability of metals such as lead concentrations in soft tissues of oysters *Pinctada imbricata* is highly dependent on the speciation or physicochemical forms of the metals in seawater [11]. *Ruditapes philippinarium* was exposed to different concentrations: Pb (350 - 700 µg/l) for seven days. The highest concentrations were found in the gills for Pb [28]. They reported that gills of clam *Ruditapes philippinarum* could be considered as an adequate target tissue for heavy metals. Some studies showed that both Mediterranean and red sea seawater are relatively unpolluted with heavy metals as compared to other regions in the world [25]. The selection of histology as indicator of disease and contamination was based on previous studies that showed strong relation between pollution and the histology of gills [12]. Histological examinations showed clearly different pathological changes in the structure of the gills of *Pinctada radiata* (Bivalve) exposed to pollution and collected from Alexandria coast, Egypt [55]. Some of these areas are polluted with different kinds of contaminants caused by the discharges of industrial and municipal effluents containing chemical and biological contaminants such as heavy metals [3]. Untreated sewage and waste waters were discharged annually from large numbers of outlets into Alexandria coastal area through local sewage system and endangers human health [6]. Marine bivalves have been used to monitor environmental health conditions and potential pollution by using the whole animal or specific organs to determine contamination levels and facilitate comparisons over space and time [5]. Several indicators of exposure to stress were reported in bivalves including, histopathological changes mostly confined to organs directly involved in the metabolism and detoxification of pollutants and elevated expression of stress proteins [58]. To protect public health, we have to harvest shellfish from approved waters where water quality standards have been met.

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