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Mantle and its protective role of the Slipper-Shaped Oyster (*Crassostrea iredalei*) in response to crude oil

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Abstract— The mantle plays important role in the mechanism of oyster protection caused by environmental pollutants. This study aims to analyze the effect of water accommodated fraction of crude oil on the mantle of Slipper-Shaped Oyster (*Crassostrea iredalei*) at different doses and time exposure. The ventral and posterior segments of the mantle were fixed, and tissue sections were stained with hematoxylin-eosin, PAS-Periodic acid–Schiff, and TEM-transmission electron microscopy techniques. HE-hematoxylin and eosin, PAS-alcian, and TEM-transmission electron microscopy were used to characterize the different mucosubstances and to describe the ultrastructure-related response on a certain part of the mantle after exposure. The tissues of epithelium, connective tissue, mucus cells, pigmented cells, numerous hemolymph sinuses, shell formation, and blood sinus were recognized under a light microscope. The mucous cell was excreted in all the concentrations (control, 12.5, 25, 50, and 100% Water Acomodate Fraction) and also in the time exposure (24, 48, 72, and 96 hours). A large number of mucous cells was produced in the inner mantle cavity (IMC) and outer mantle cavity (OMC). Mucous cells increased in number with increasing WAF concentration as well as the length of exposure time. The highest number of mucus cells was observed at 100% Water Accommodate Fraction (WAF) concentration and 96 hours of exposure. The structure and function of the mantle, the shell formation, the edge of the mantle, mucous cell, muscle bundles, nerve fibers, and epithelium layer of the Slipper-Shaped Oyster (*Crassostrea iredalei*) were documented in this study.

Keywords— Ultrastructure, Mantle, Secretory cells, WAF crude oil

I. Introduction

Oysters belong to the family Ostreidae, order Ostreoida, class Bivalvia, and have been recognized as species of commercial and recreational importance [1]. They have a high economic value as edible mollusks [2,3,4]. In tropical countries, the oyster is one of the attractive species for aquaculture candidates [5,6]. Among indigenous oysters in South East Asia, *Crassostrea iredalei*, is an economically important species widely cultured in Thailand [7], the Philippines [8], Malaysia [9] and Fiji [10].

To grow optimally, farmed aquatic animals need an optimal living environment. Water quality can affect the growth and development of oysters [11,12]. If the environmental quality drops below the required threshold, aquatic animals may experience stress and be more susceptible to disease which can subsequently lead to death [13]. Seawater pollution can affect the physiology, behavior and genetics of oysters. Research conducted on the Pacific oyster, *Crassostrea gigas* (Thunberg) showed that oxidative stress by pollutants and water temperature affects physiological changes related to metabolism and cell protection. Another study on the same species showed that pollution by groups of xenobiotics (PAHs, PCBs, and pesticides) affects the modulation of hemocyte activities. Pollutants also decreased phagocytotic activity [14]. Pollution from pesticides might have a small direct effect Pacific oyster via altered gene expressions that can have long-term effects [15].. This is supported by research on another species (Japanese oyster) which shows that pollutant derived from pesticides (diuron) triggers genetic damage in both the hemocytes and spermatozoa. This damage can affect the development and survival of offspring (low hatching rate, high larval abnormalities, and metamorphosis delay) [16].

In general, oysters include animals that are filter feeders and even non-selective filter feeders (eat by filtering without being picky) [17]. Oysters eat particles and organic matter, as well as

living things suspended in water. Intake of sea water moves to the gills and lips where in that part it will go through several filtering processes by cilia around the mouth. Small food particles will escape into the mouth, while the large ones will be expelled back through the mouth pumping device (exhalant siphon) in the form of pseudofeces [18].

As filter feeders, oysters may accumulate heavy metals, pathogens and other pollutants from the surrounding waters, and this can alter physiological and morphological responses [8, 20]. Parts of organ tissue such as the gills, mantle, gonads, intestines and stomach of oysters can absorb pollutants and show tissue damage when the content is higher than the standard. The mantle or pallial lobe is a thin layer of tissue that covers the internal organs of all oysters. The two lobes of the mantle join at the posterior edge to form a cap or hood that covers the mouth and labial palps. The pallial lobes are separated anteriorly, ventrally, and posteriorly but unite dorsally below the hinge line of the shell [21].

Several studies have looked at the structure and ultrastructure of the mantle function in oysters [20,22,23], transport and rejection mechanisms, pigmentation [24], regeneration [25], and mantle cilia [26]. Other studies demonstrated the molecular regulation of the shell and the anatomical differentiation of the mantle [27,28]; physiological and histologic responses [29-31]; TEM transmission electron microscopy and SEM scanning electron microscopy of the mantle [32,33]; mechanisms in the process of shell formation and biomineralization [34]; shell secretion [22]; ATP or ABC proteins [30, 35]; as well as early larval development of C. iredalei [2]. However, studies on the role of mantle ultrastructure and its protective mechanisms against marine pollutants, particularly in C. iredalei oysters, are still few.

The Southeast Asian Sea, which includes the Gulf of Thailand, the Indonesian Sea, and the Sulu-Celebes Sea and South China Sea (SCS), hosts 1390 offshore platforms for the production of fuel oil. This ocean area is semi-enclosed and shared among several coastal States, increasing the risk of transboundary pollution [36]. (Lyons, 2016). The Gulf of Thailand, especially in Rayong Province, often experiences sea water pollution in 2013. Rayong oil spill causes damage to the coastal and maritime environment, as well as undermines trust in the overall safety system. This study aims to analyze the effect of water accommodated fraction of crude oil on the mantle and mucus cell of Slipper-Shaped Oyster (*Crassostrea iredalei*).

II. Materials and Methods

A. Experimental oyster

Adult oysters were collected from Angsila farming area, Chonburi Province, and acclimated for 5 days at a marine hatchery, Faculty of Science, Burapha University. A total of 250 oysters with sizes ranging from four to seven cm were placed in a 200 L conical tank. Those oysters were transferred to the laboratory. During acclimatization, oysters were aerated and fed with plankton Nannochloropsis sp. The room temperature ranged from 24°C to 27°C. the oyster was randomly distributed to 40 L volume of aquarium size, then exposed to 10 L of water accommodated fractions (WAF) concentration.

B. Chemicals

Crude oil was obtained from the PTT Global Chemical Public Company Limited. In this study, only the water-soluble element of crude oil was considered. Therefore, it was used WAF for experimental exposure. The stock solution of WAF of crude oil was freshly prepared according to Singer method [37]. Briefly, 300 ml of crude oil was mixed with 2,700 ml of seawater to attain a ratio of 1: 9. Mixing was performed in a 4-L mixing chamber by using a 3.5 cm magnetic stirrer bar on a stirrer for 20 hours at room temperature ($25^{o}C \pm 2$) and then stopped. The mixing solution was left to stand for 6 hours for a complete phase (oil/water) separation. The lower phase was collected and stored in a refrigerator until experimental exposure. This fraction is a 100% stock solution of crude oil WAF.

C. Exposure design

After acclimatization, 12 oysters were assigned to each aquarium. The WAF solution was diluted to varying concentrations as follows: 0 (control), 12.5, 25, 50, and 100%. The oysters were exposed to 10 L of each WAF concentration for 24, 48, 72 and 96 hours. All treatments with 180 total oysters were carried out in three replicates. Until the end of time exposure (96 hours), every 24 hours, 3 oysters were randomly collected from each replicate to examination. Soft part tissues were dissected and fixed in Bouin's solution for histopathological examination.

D. Histopathological examination

Fixed tissues were washed in alcohol 70% and gradually dehydrated by passing the tissue through increasing concentrations of ethyl alcohol 80, 90, and 99% (absolute ethanol), one hour each step. Tissues were then placed in Dioxane overnight and embedded in warm paraffin wax. Embedded tissues were sectioned with a microtome to ensure the thickness of 6 um and stained with Hematoxylin and Eosin. After staining, gill structure and tissue alterations were observed under a light microscope Olympus BX51. Additionally, histochemical staining with Alcian Blue was performed to detect the presence of mucous cells in the mantle.

For transmission electron microscopy (TEM), 60-nm semi-thin sections were cut using a Reichert Ultracut E ultramicrotome. Sections were floated on water and stained with uranyl acetate and lead citrate and viewed with a JEOL JEM-1011 transmission electron microscope at 80 kV. Assessments were focused on the mantle and the parameter of organ response are mucus cells/400 μm, hemocytes, blood sinus, connective tissue, and the cilia.

E. Data analyzes

The observed data on the effect of WAF crude oil concentration and its time exposure were analyzed using the general linear model ANOVA. Furthermore, the post hoc test was carried out using the LSD method. The results of the analysis were considered significant if $p < 0.05$. Statistical analysis was carried out using SPSS version 20 software. The results of the histopathological examination were carried out descriptively.

III. Results and Discussion

A. Histology

Both sides of the mantle are covered by cylindrical epithelial cells set on an elastic basal membrane (figs 1 and 2). large goblet cells which secrete mucus and cells containing eosinophile granules are abundant on both sides of the mantle. The cells on the inner pallial cavity are long and ciliated, whereas the outer pallial cavity is much shorter, has no cilia, and almost cubical.

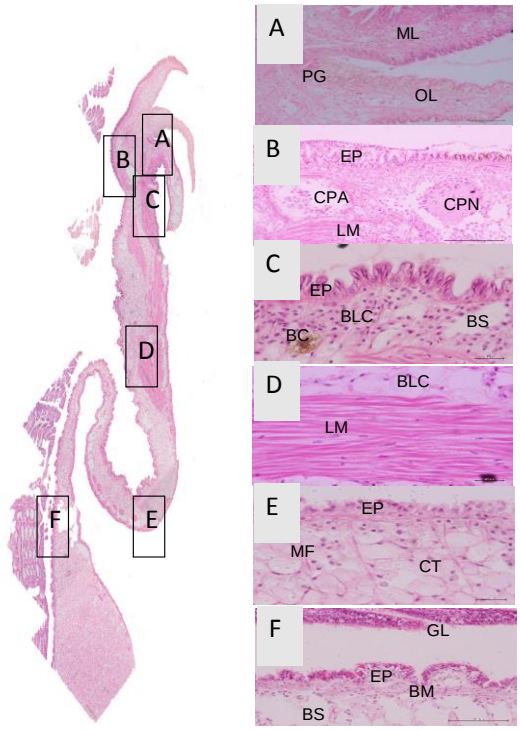

Figure 1. HE stained mantle pallial cavity. (A). Transverse the edge of the mantle, ML = middle lob, PG = pariastracal groove, OL = outer lobe. (B) Transverse the inner marginal of the edge mantle. EP = epithelium, CPA = circum pallial artery, CPN = circum pallial nerve, LM = longitudinal muscle. (C). Transverse outer marginal of the edge mantle. EP = epithelium. BC = brown cell, BLC = blood cell. BS = blood sinus. (D). Transverse the center pallial cavity of the mantle. BLC = blood cell, LM = longitudinal muscle. (E). Transverse the outer mantle cavity. EP = epithelium, MF = muscle fibre, CT = connective tissue. (F). Transverse the inner mantle cavity. GL = gill, EP = epithelium, BM = basement membrane, BS = blood sinus.

The edge of the mantle is usually provided with a three-lobe, which may be designated as the outer (shell) fold, the middle fold, and the inner fold (Fig. 2A). Both the inner surface and the shell surface of the mantle are covered by a layer of epithelial cells, columnar, cylindrical, or elliptical (Fig 2,3).

The mantle was a small variation in the size of tentacles and their pigmentation almost occurred in the part of all the mantle. The three mantle lobes are not different in length, with the outer lobes (OL) being the longest (fig 2A, B), separated from the middle lobe (ML) and inner lobes by the periostracal groove (PG). The longitudinal muscle of tentacles (LM) is associated with the middle lobe. The circumpallial nerve (CPN) and circumpallial artery (CPA) are visible as are small blood sinuses and nerves.

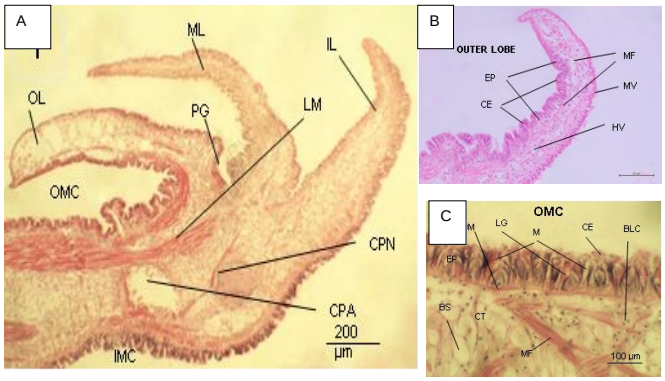

Figure 2. Transverse section of HE stained mantle. (A). transverse the edge mantle. OL = outer lobe, ML = middle lobe, IL = inner lobe, PG = periostacal groove (conchiolin), CPN = circum pallial nerve, CPA = circum pallial arteri, LM = longitudinal muscle, OMC = outer mantle cavity, IMC = inner mantle cavity. (B), transverse section of the outer mantle lobe. EP = epithelium, CE = ciliated epithelium, MV = microvilli, HV = Haemolymph vessel, MF = Muscle fiber. (C) transverse section of outer mantle cavity. CE = ciliated epithelium, CT = connective tissue, LG = cells with large secretory granules, M = mucous cell, MF = muscle fiber, BM = basement membrane, BS = blood sinus, BLC = blood cell, EP = epithelium

B. Histochemistry (Periodic acid–Schiff)

The different types of secretory cells in the mantle, such as mucous cells, large granular cells, and empty cells, have been recognized in the part of the mantle, outer mantle cavity, outer lobe, periostacal groove, middle lobe, inner lobe, inner mantle cavity, and Isthmus. The epithelial cells of the surface inner mantle cavity zone are remarkably pigmented (Fig. 3). The secretory cells were found throughout the mantle region and are almost present in epithelia (Fig 3). The secretory cells are differentiated into three groups by HE, PAS Alcian blue stain, and TEM, namely, mucous cells, 1. cells with large secretory granules, 2. cells carrying mucous and large secretory granules, and 3. cells containing granules (Fig 6).

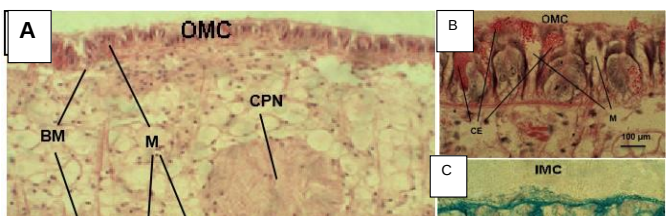

Figure 3. Transverse section of mantle epithelia showing the distribution of secretory cells from the oyster exposed to 12.5 % WAF during 24 hours (A) HE stained of the transverse section of the mantle cavity; OMC =outer mantle cavity, IMC = inner mantle cavity. (B). HE stained of transverse section of epithelia; CE = ciliated epithelium, CT = connective tissue, LG = cells with large secretory granules, M = mucous cell, MF = muscle fiber, BM = basement membrane, BS = blood sinus, BLC = blood cell, EP = epithelium. (C). PAS Alcian blue stained of mantel epithelia; PE = pigmented epithelium, M = mucous cell, LM = longitudinal muscle, LG = a cell with large secretory granules, ML = cells with mucous and large secretory granules, BG = cells with brown granules, ME = empty mucous cells, TM = transverse muscle, LM = longitudinal muscle, CON = connective tissue.

These secretory cells were generally few in the part of the mantle, while they are abundant in the fold. Secretory cells were more numerous and larger on the pallial side of the mantle than on the shell-facing side (Fig 4). The inner portion of the mantle consists of connective tissue being dark when treated with HE stain.

Periostacal groove conchiolin is located between the outer lobe and middle lobe (Fig 4), the organic matrix (conchiolin) and foliated layers of calcite are needed for increasing the thickness of the valves, on the other hand, mucous are secreted by the epithelium covering the entire outer surface of the mantle and close contact with the inner surface of the valve. The function of the periostracal gland is to supply large quantities of the material required for new shell growth at the edge of the valves (Fig 4C-D)

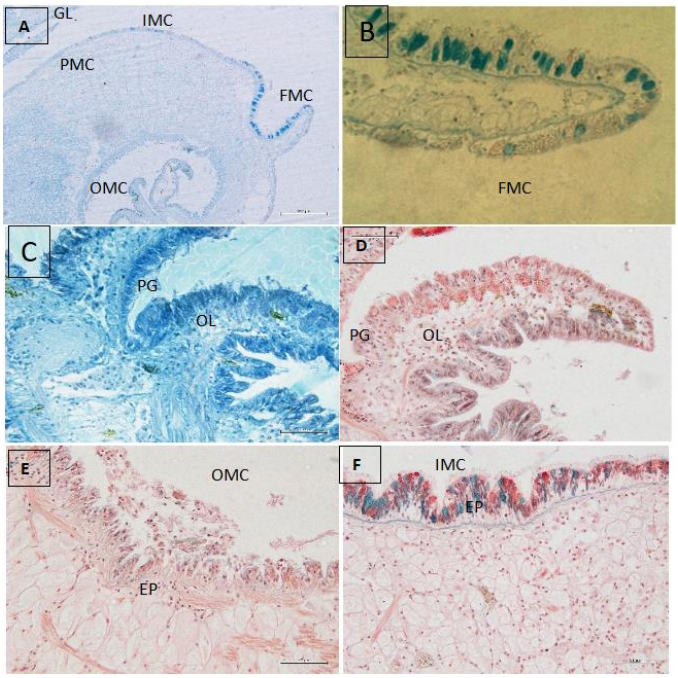

Figure 4. Periodic acid–Schiff /AB. (A), Mantle pallial cavity with the folding of the mantle, FMC = folding mantle cavity, IMC = inner mantle cavity, PMC = pigmented mantle cavity, GL = gill, OMC = outer mantle cavity (B). PAS/AB, Folding of the mantle. (C). Acian blue; PG = periostacal groove conchiolin, OL = outer lobe. (D). PAS, PG = periostacal groove.conchiolin, OL = outer lobe. (E), EP epithelium; OMC = outer mantle cavity. (F). EP, epithelium, IMC = inner mantle cavity.

C. Transmission electron microscopy

TEM micrographs of the pallial epithelium of the mantle of *C. iredalei* demonstrated that the mantle epithelium composes of ciliated cells, microvillous epidermal cells, and secretory cells (Fig 5A). The hemocytes were generally observed within intercellular space of the epithelium. A mucous layer was often observed at the epithelium surface. Subepidermal secretory gland cell was situated under the epithelium (Fig 5B).

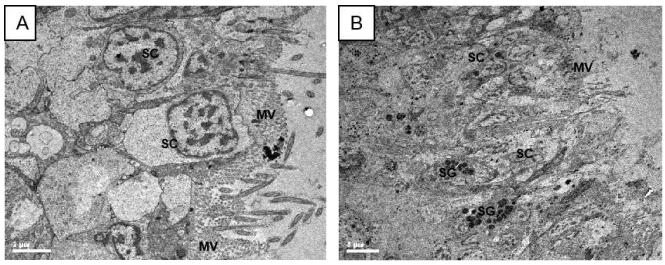

Figure 5. TEM micrographs of the pallial epithelium of mantle of *C. iredalei*. (A) Mantle epithelium composes of microvilli (mv), and secretory cells (sc). (B) A mucous layer is often observed at the epithelium surface. Subepidermal secretory gland cell is situated under the epithelium, sc = secretory cell, sg = secretory granule, mv = microvilli.

D. Exposure to WAF Crude oil

Histological analysis of the mantle C.iredalei revealed damage of MC, PE, mantle edge, connective tissue, increase in hemocytes, and necrosis, correlated with concentration and the time of exposure. The function of the mantle organ may probably be concerned with the detection of mechanical disturbances in the surrounding water (fig 6).

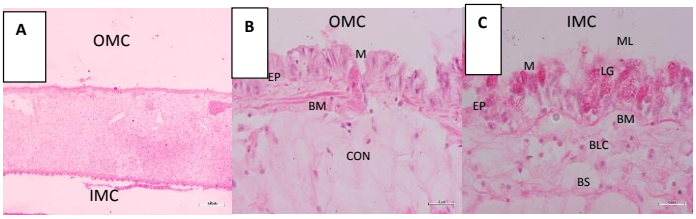

Figure 6. HE stained the transverse section of mantle epithelium with the distribution of secretory cells. (A), transverse section of the mantle cavity, OMC =outer mantle cavity, IMC = inner mantle cavity. (B). epithelial secretory cells in outer mantle cavity, CE = ciliated epithelium, CON = connective tissue, LG = cells with large secretory granules, M = mucous cell, ML = mucous layer, MF = muscle fiber, BM = basement membrane, BS = blood sinus, BLC = blood cells, EP = epithelium. (C). epithelial secretory cells in IMC, EP = epithelium, M = mucous cell

The average of mucus cells increased along with the increase in WAF crude oil levels. The highest mucus cell content was observed in 100% of WAF crude oil (40.1 ± 3.6 cells), while the lowest was in control (17.2 ± 1.9 cells) (Fig 7A). Those also slightly increased with the time exposure. The highest mucus cell content was observed in 96 hours of WAF crude oil exposure (31 ± 11.5 cells), while the lowest was in 24 hours (28.5 ± 9.4 cells) (Fig 7B). Statistical analysis showed that the effect of WAF crude oil concentration was significant ($F = 287.2$; $p < 0.001$), as was the effect of time exposure ($F = 3.8$; $P < 0.05$). However, there was no interaction effect between the two factors, indicating that the level of time effect was consistent for all concentrations.

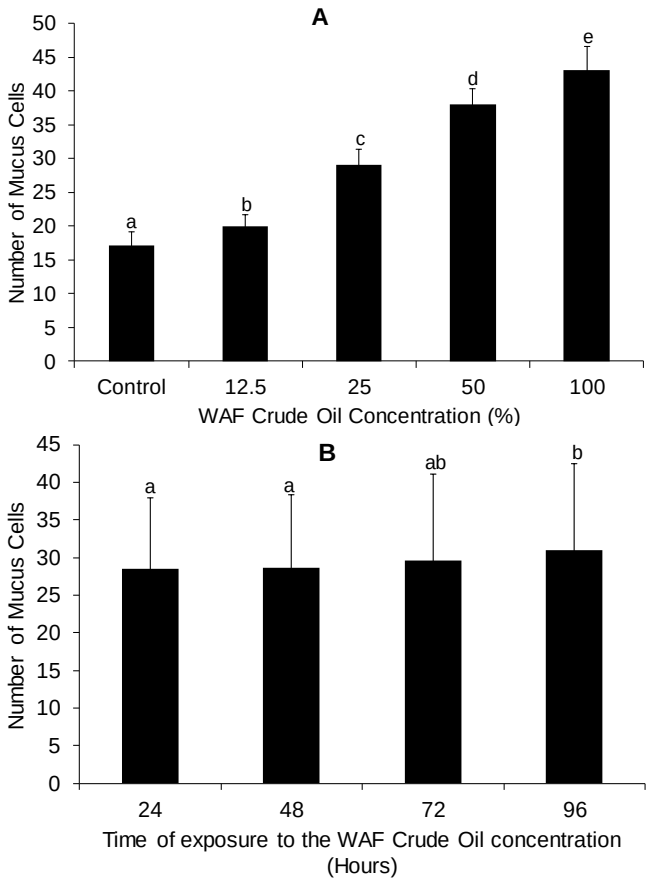

Figure 7. Graphic Concentrate and Time of exposure to the WAF crude oil concentration. (A). graphic concentrate of WAF crude oil. (B) graphic time of exposure to the WAF crude oil concentration.

HE stained the transverse section of mantle epithelium with the distribution of secretory cells and showed that WAF Crude oil affected mantle tissues causing mucus excretion and hemocyte aggregation (Fig 8B). the normal mantle epithelium as the control on 0 % concentration and 24 hr time of exposure to the WAF crude oil concentration (Fig 8A).

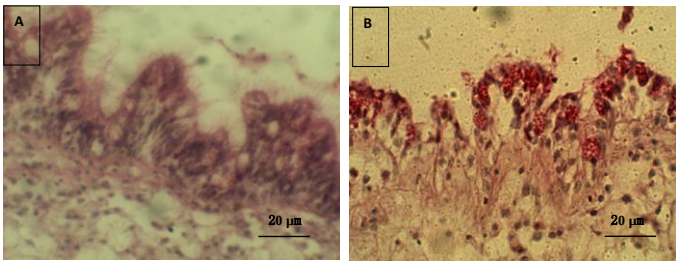

Figure 8. HE stained the transverse section of mantle epithelium with the distribution of secretory cells. A. Control or 0 % concentration and 96 hr time of exposure to the WAF crude oil concentration. B. 100 % Concentrate and 96 hr Time of exposure to the WAF crude oil concentration.

E. Discussion

Kebanyakan studies mengkaji keberadaan dan pengaruh parasites terhadap *C. iredalei* [38-41]. Effect of gregarine parasite, Nematopsis sp. to the structure of the mantle and other organs. The study carried out a macroscopic examination and histological examination, and the results showed that the sample oysters in Sabah had a severe level of histopathological intensity. Oyster's mantle has hypertrophy of muscle fiber and hyperplasia of epithelial tissue. In addition, oysters also experienced lesions, accumulation of brown cells and accumulation of hemocytes [38]. In addition to the gregarine parasite, *C. iredalei* is also susceptible to digenean trematodes, cestode parasites, polychaetes, copepods, bacteria and viruses attack [38-40]. Meanwhile, studies on the influence of abiotic environmental factors such as pollutants are still rare, for example, the effect of heavy metals [8,20]. Some of the heavy metal content that accumulates in *C. iredalei* tissues are zinc and copper [8].

The mantle are play important role in regulatory mechanisms and response to environmental pollutants. Several studies have reported about pearls oyster i.e *Pinctada maxima*, *Pinctada margaritifera*, *Pinctada persica*, *Pinctada radiata*, *Pteria penguin*, and *C. iredalei*. The studies observed the effect of environmental factors on the structure and function of the mantle [24-26, 32, 42, 43]. A study on the other oyster species, *Saccostrea cucullata* showed that DDT caused the alteration of mantle tissue, mucocytes in the epithelium and hemocyte aggregation [44] This study observed the structure and morphology of the mantle, especially in the mantle cavity of *C. iredalei*.

The mantle color was opaque and cloudy, the appearance coincide with seasonal cycles in the glycogen content of the connective tissue and with the progressive stages of gonad development. In some specimens, the mantle may be thin and transparent whereas in others it is thick [45].

The secretion of epithelial mucus is used to isolate oysters from their environment and mucus may also serve as an ion regulator. Mucus may also contain specific products, agglutinin and lysozyme have been found in mucus in marine mollusks

Mucus is most diverse in function, mucus is produced from epithelium, acts as a barrier to diffusion, and isolate and counter some condition of the water environment. This study showed that the mantle is the first line of defense against chemical /physical stress and secreting of mucus was to reduce the effect of WAF crude oil exposure.

There are thick and thin epithelial layers in the inner pallial mantle, the thin layer with hemolymph heading to the layer may possible to gas exchange in the area. The thick epithelial layer with the cilia is also close to the gill that supports the gill function.

The other study reported extensive damages to the adductor muscle, mantle and gills of the copper exposed to the Indian edible oyster, *Crassostrea madrasensis* [29]. In another study, the effect of heavy metals was evaluated on oysters (*Crassostrea virginica*). The levels of heavy metals Cd and Pb detected in the oysters were very high, and the oysters were unable to remove the concentrations of these metals during the detoxification stage. Histopathological analysis revealed lesions of the digestive glands, edema, epithelial atrophy in the digestive tubules, presence of brown vesicles, hemocytic reaction, and necrosis [46]. The study of the effect of Exposure to water-accommodated fractions of crude oil on fish showed a serious impact on the heart and the specification and development of the epithelium and outer mesothelium of the swim bladder in zebrafish early-life stages [47].

This study observed the left and right mantle or both pair mantle lobes in the ventral and posterior using TEM and HE Histological techniques. The difference in concentration and the time of exposure to water accommodated fraction of crude oil affected the ultrastructure of the mantle in a certain part of the mantle. The mucus cell appeared in high concentrations and in many treatments of concentration with the massive excreted occurred on 12.5 and 100% of concentration.

The mantle performs several functions including secretion of the shell and formation of inhalant and exhalant currents via the inhalant and exhalant chambers. The mantle play important role in the regulatory mechanism

IV. Conclusion

The mucous cell was excreted in all the concentrations (control, 12.5, 25, 50, and 100% WAF) and also in the time exposure (24, 48, 72, and 96 hours). A large number of mucous cells was produced in the inner mantle cavity (IMC) and outer mantle cavity (OMC). Mucous cells increased in number with increasing WAF concentration as well as the length of exposure time. The highest number of mucus cells was observed at 100% WAF concentration and 96 hours of exposure.

The mantle is the first line of defense against chemical /physical stress and secreting of mucus to reduce the effect of WAF crude oil exposure. The structure and function of the mantle, the edge of the mantle, mucous cell, muscle bundles, nerve fibers, and epithelium layer of the Slipper-Shaped Oyster (*C. iredalei*) were affected by WAF.

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