

The histopathological effect of methylmercury on the brain in orange spotted grouper (*Epinephelus coioides*) in Zangi Creek and laboratory

Savari Sh.^{1*}; Safahieh A.¹; Archangi B.¹; Savari A.¹; Abdi R.¹

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Abstract

Histopathological studies reflect the overall health of a community in an ecosystem and can be used as a biomarker of pollutants, distinguishing the presence and amount of the pollutant within the organism as well as assessing the risks the organism is facing. These studies can also provide a range for the pollutant concentration to indicate healthy and polluted environments. In order to assess the defects brought by industrial pollutants the histopathological defects of Methylmercury (MeHg) were studied. Methylmercury, is the organic derivative of mercury, possessing more dangerous and harmful effects than mercury. Methylmercury is highly hydrophobic and due to its lipophilicity, it penetrates through the blood brain barrier and targets the brain more than the other tissues. Therefore the effect of MeHg on *Epinephelus coioides* brain in the laboratory and in Zangi Creek were studied. Fish were adapted to the tanks and exposed to 10, 20, 40 and 80 $\mu\text{g L}^{-1}$ of methylmercury chloride in the Fisheries Center of Zangi Creek. The brains were collected on days 7, 14 and 30 of exposure and the depuration studies were performed for 7 and 14 days. The brains were dissected and sectioned for haematoxylin and eosin staining and monitored under a microscope. The defects pertained hyperanemia and hemorrhage, karyolysis and necrosis, nuclear dust, endothelium hypertrophy, hydropic degeneration, vacuolation, cloudy swelling and edema. These defects were seen in different parts of the brain such as olfactory bulb, cereberum, optic lobe, cerebellum, diencephalon and medulla. The most defected part of the brain in response to MeHg seems to be the cerebellum. Depuration studies were performed for 7 and 14 days and mainly showed edema, cloudy swelling and hydropic degeneration in most parts (no rescue of phenotype seen). Fish were studied both in exposure to MeHg and preyed from field to compare the defects and assess the health and safety of the creek. The Histopathological Alteration Index (HAI) was assessed to show the amount of defects brought by the pollutant and revealed severe and irreversible defects in higher concentrations of exposure. Zangi Creek's HAI fell between the control group and the lowest MeHg concentration and is still considered as a safe site below the threat range.

Keywords: Histopathology, Methylmercury, *Epinephelus coioides*, Brain, Zangi Creek.

1-Marine Biology Department, Faculty of Marine Science and Oceanography, Khorramshahr Marine Science University, Khorramshahr, Iran.

*Corresponding author's Email: srsavari@yahoo.com

Introduction

The Orange-spotted grouper (*Epinephelus coioides*), also known as Estuary cod, is a eurythermal and euryhaline (Hamilton, 1822; Heemstra and Randall, 1986) species of fish belonging to the Serranidae family. It is found in the western Pacific, the Indian Ocean, and the Red Sea. It is also found in the Persian Gulf. Its natural habitats are subtropical or tropical mangrove forests, open seas, shallow seas, subtidal aquatic beds, coral reefs, estuarine waters, intertidal flats, and coastal saline lagoons (Cornish and Harmelin-Vivien, 2004). Juveniles are mainly seen in the shallow waters of estuaries over sand, mud and gravel and among mangroves, feeding on small fish, shrimp, and crab. Orange spotted grouper is a benthopelagic fish which lives at the bottom and close to the bottom of the sea (around 100 m). Since this fish is in proximity with the sediments and is affected by the pollution brought by the sea water and sediment, it is important to study the effect of pollutants in the tissue of this fish. Moreover this fish is a carnivore which feeds on small fish and crustaceans which are the major cause of pollutant accumulation in this fish.

Histopathological studies reflect the overall health of a community in an ecosystem. Various anthropogenic pollutants harm the tissues and introduce histopathological disorders into the healthy tissues and organs. These pollutants should be studied and their harmful effect should be characterized. Once the effect of each pollutant is known this defect can serve

as a biomarker of that pollutant, therefore being able to distinguish the presence and perhaps the amount of such pollutant in the environment (Hinton *et al.*, 1985).

Histopathological biomarkers are closely related to stress biomarkers, since they act similarly but in a different manner. In the case of stress biomarkers, pollutants have to undergo metabolic activation in order to induce cellular changes in the effected organism. For example the effect of xenobiotics in many cells leads to the production of many enzymes altering the cellular metabolism thus resulting in cell death. In the case of histopathological biomarker, the effect of the pollutant appears as necrosis and other disorders in the cell and tissues. Therefore, similar to stress biomarkers, histopathological biomarkers can be good indicators of the level of a specific xenobiotic in the environment (Velkova-Jordanoska, 2002; Roganovic-Zafirova *et al.*, 2003).

The neuroanatomy of *Epinephelus coioides* has been studied through transverse sectioning (Nagarajan *et al.*, 2013; Savari *et al.*, 2013), but the histology of the brain and the effect of pollutants on this tissue have not been studied. Some studies have previously showed the effect of MeHg on the histology of other fish brain (Oliveira Ribeiro *et al.*, 2002; Cambier *et al.*, 2012). However, no studies have indicated the effect of MeHg on orange spotted grouper brain and progressive gradient concentrations of MeHg on histopathology of fish brain have not been previously studied. This study

aims to show the effect of MeHg on the histology of *E. coioides* brain as a biomarker of MeHg. Mercury is a hazardous waste coming from petrochemical, industrial and agricultural organizations as well as portal communications. Methylmercury, its organic derivative, is even more dangerous and harmful than mercury itself. Methylmercury is highly hydrophobic and due to its lipophilicity, it penetrates through the blood brain barrier and targets the brain more than the other tissues. In addition the accumulation of methylmercury in the brain is more than in other tissues, therefore studying its effect on this tissue is of high importance.

In Iran *E. coioides* lives in the Persian Gulf and is also cultivated in Bandar Emam Khomeini fisheries center. The fish that were studied were cultivated fish from Zangi Creek.

Materials and methods

The fish were cultivated in Emam Khomeini Fisheries Center beside the Zangi Creek. For the experiments, 2 year old fishes were placed in fiberglass tanks filled with water from Zangi Creek (pH: 8.8 and salinity: 48 ± 0.5 ppt). The water was exchanged on even days and the fish were fed with fish meal pellet before water exchange. The fish were given a one week period to adapt to the new condition before the exposure experiments. Each tank contained 9 fish. One fish from each tank was taken for histopathological experiments on days 7, 14 and 30 and the rest were dissected for other experiments. Tanks were exposed to 0,

10, 20, 40 and $80 \mu\text{g L}^{-1}$ of methylmercury chloride (5 different concentrations). Three repeats of each concentration were designed (5 concentrations \times 3 repeats = 15 tanks). The concentration of the stock solution was 0.1 g ml^{-1} and the working solution was 1 mg ml^{-1} . In addition depuration studies were carried out after exposure and one fish from each tank was sampled after 7 and 14 days of depuration (3 repeats of each concentration). The brains were collected from all the 15 tanks and were placed in a fixative. They were fixed in Bouin's solution comprising 75 ml saturated water with picric acid, 25 ml formaldehyde and 5 ml glycolic acetic acid. After 24-48 h they were stored in 70% ethanol until the actual experiment.

The dehydration step consisted of placing the brains in a tissue processor, TISSUE TEK II Rotary model RX-11B, starting from 70% ethanol (two times), 80% ethanol, 90% ethanol, 100% ethanol (2 times) for 2 h each, alcohol-xylene for 30', xylene (2 times) for 2 h each, paraffin (2 times) for 3 h each. After dehydration, the tissues were pertained for sectioning using a microtome, LEICA model VRM 2245, to obtain sections of 4-5 microns. The sections were placed in a 50°C water bath to avoid wrinkling of the sections. The sections were put on the slides for Haematoxylin- Eosin staining. The staining procedure consisted of placement of the slides in xylene for 30 min, xylene 15 min, 100% ethanol for 2 min, 90%, 80% and 70 % ethanol each for 1min,

haematoxylin for 15 min, running water for 10 min, acid alcohol for 1sec, running water for 5min, Eosin plus a few drops of glycial acetic acid for 5 min proceeded by washing the slides with ddH₂O. Afterwards the rehydration step was followed using 70% ethanol for 1 min, 80% and 90% ethanol for 1 min each, 100% ethanol (twice) for 2 min each, xylene for 10 min and another xylene step for 30 min. Finally, the cover slips were placed onto the slides using the glue Canada Balsam.

Data analysis

The Histopathological Alteration Index (HAI) was assessed through the following formula:

$$\text{HAI} = (1.\Sigma\text{I}) + (10.\Sigma\text{II}) + (100.\Sigma\text{III})$$

In this formula, I, II and III show the stages of histological alteration and Σ shows the amount of histological alteration of each stage. The amounts of 0-10 show the normal functioning of this organ, 11-20 show little damage brought to the organ, 21-50 show average damage brought by the pollutant to the organ, 50-100 show high damage occurrence within the organ and amounts over 100 show severe and irreversible damage of the organ due to the pollutant (Poleksic and

Mitrovic-Tutundzic, 1994; Viana *et al.*, 2013).

The stages are defined as follows (in the light of Bernet *et al.*, 1999):

Stage I of damage: No reversible damage was seen at this stage.

Stage II of damage: Karyolysis, hyperanemia, vacuolation, endothelium hypertrophy, cloudy swelling, hydropic degeneration, granular accumulation and hyper chromatin.

Stage III of damage: Necrosis, nuclear dust, hemorrhage and Edema.

Results

In general all the concentrations showed hyperanemia, some extent of hemorrhage, karyolysis, necrosis, nuclear dust, hyper chromatin, vacuolation, endothelium hypertrophy, cloudy swelling, hydropic degeneration and ectopic granular accumulation.

In the Figs. 1-5, pictures of olfactory bulb, cerebrum, optic lobe, cerebellum, diencephalon and medulla are shown at a magnitude of 750X at concentration of 80 $\mu\text{g L}^{-1}$ on day 30. In addition pictures are organized in tables based on the exposure concentrations and days of sampling at a magnitude of 3000X. The Figs are based on the highest defects seen in specific parts of the brain at a certain concentration and time.

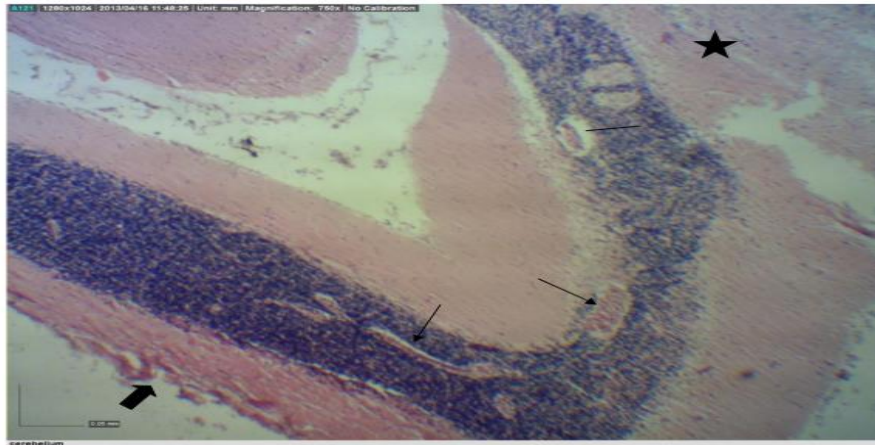


Figure 1: Cerebellum after 30 days of exposure to $80 \mu\text{g L}^{-1}$ of methylmercury exposure. Hemorrhage (Long arrow), endothelium hypertrophy (short arrow), hydropic degeneration (star) and vacuolation (line) are seen in the picture (H&E, X750).

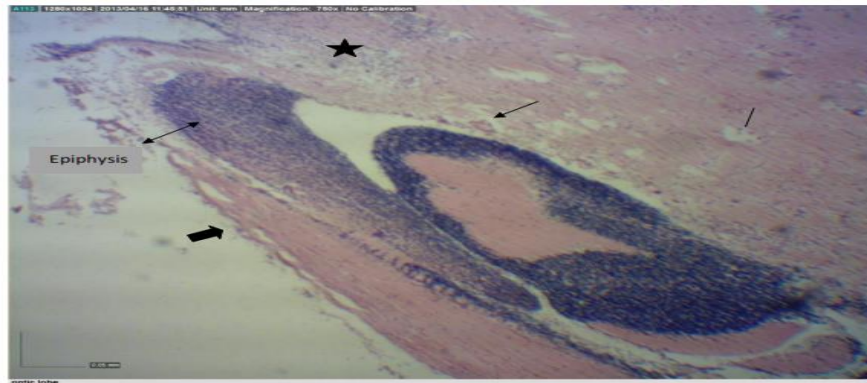


Figure 2: Optic lobe after 30 days of $80 \mu\text{g L}^{-1}$ methylmercury exposure. Vacuolation (line), hyper anemia (long arrow), endothelium hypertrophy (short arrow) and hydropic degeneration (star) are observed in the picture (H&E, X750).

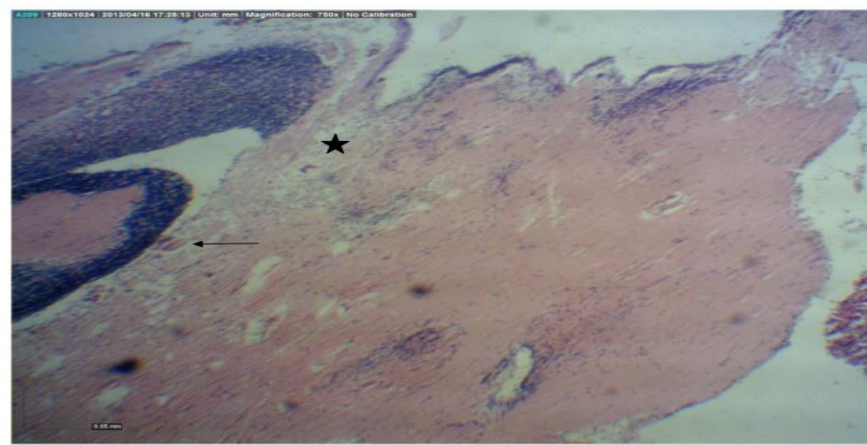


Figure 3: Diencephalon after 30 days of exposure to $80 \mu\text{g L}^{-1}$ of methylmercury. Hyper anemia (arrow) and hydropic degeneration (star) are seen (H&E, X750).

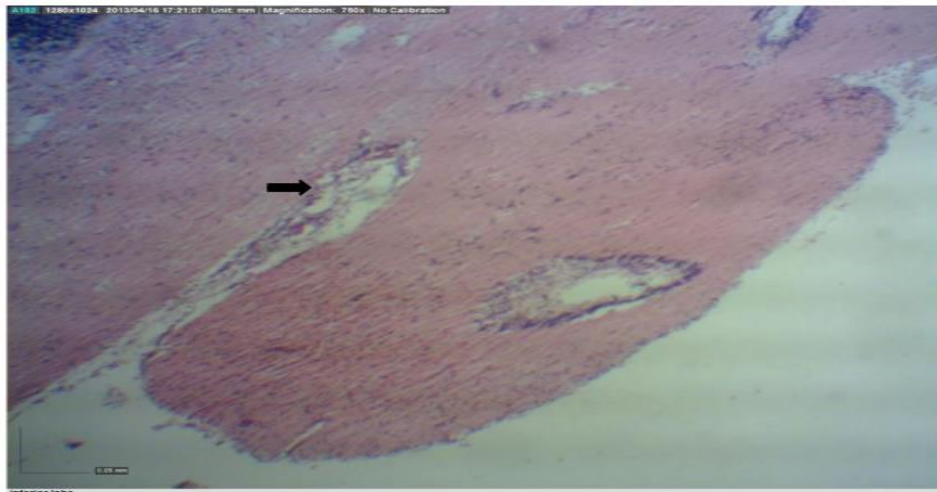


Figure 4: Endothelium hypertrophy of inferior lobe (short arrow) after 30 days of $80 \mu\text{g L}^{-1}$ methylmercury exposure (H&E, X750).

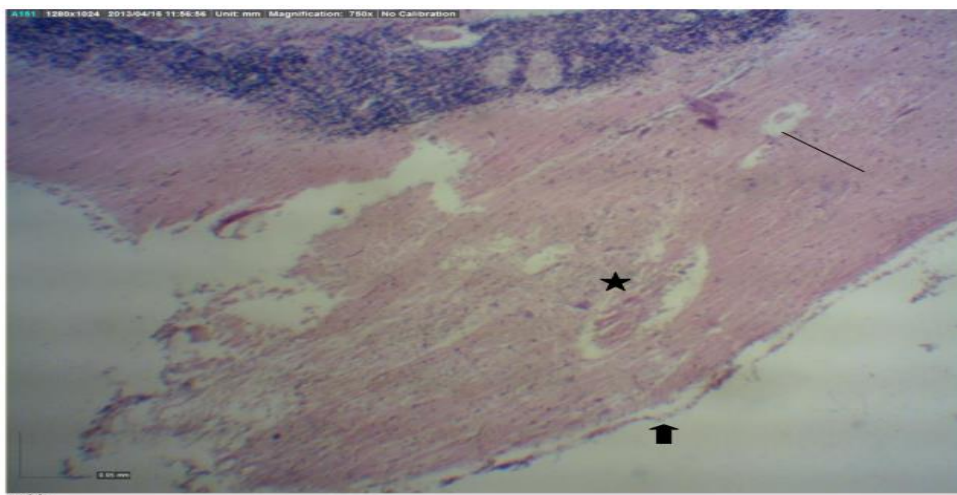


Figure 5: Medulla after 30 days of $80 \mu\text{g L}^{-1}$ methylmercury exposure. Vacuolation (line), hydropic degeneration (star) and endothelium hypertrophy are observed (short arrow) (H&E, X750).

In olfactory bulb, necrosis in the stage of karyolysis is obvious in all concentrations and days but more in higher concentrations and later stages of sampling (Fig. 6). Hyperanemia is seen in all the pictures and hemorrhage is severe on depuration day 7 especially at $20 \mu\text{g L}^{-1}$ in these pictures. Endothelium hypertrophy is seen in concentration $80 \mu\text{g L}^{-1}$ shown on day 7. The depuration experiments display

huge amounts of edema, cloudy swelling and hydropic degeneration (concentration $80 \mu\text{g L}^{-1}$ on day Dep7). There was no rescue of phenotype 7 and 14 days after depuration, but vacuolation and edema (at concentration of $80 \mu\text{g L}^{-1}$ on day Dep14) is obvious in all parts of brain at these days.

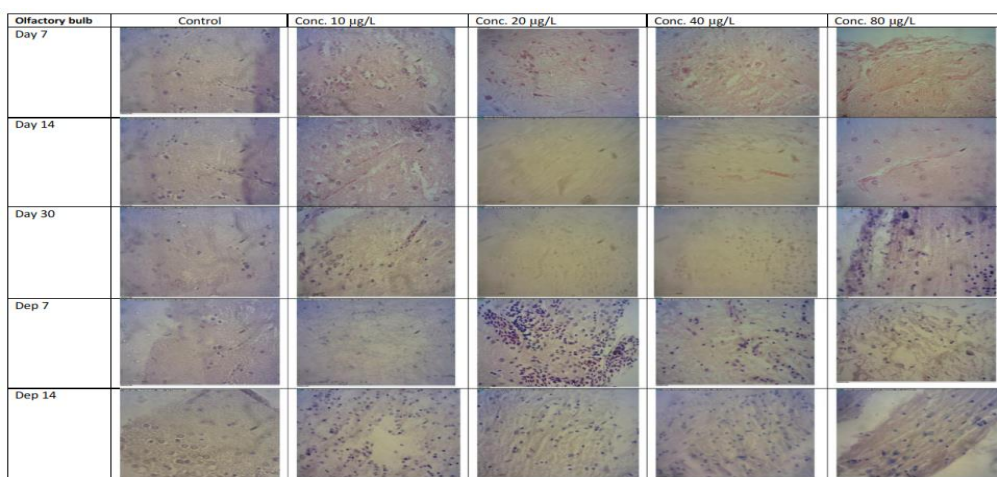


Figure 6: Olfactory bulb of *Epinephelus coioides* in response to 0, 10, 20, 40 and 80 $\mu\text{g L}^{-1}$ of methylmercury exposure after 7, 14 and 30 days and depuration of 7 and 14 days (H&E, X3000). (Conc: concentration, Dep: depuration).

Fig. 7 shows cerebrum at different concentrations of methylmercury at different days of exposure. Severe hemorrhage is seen in all the figures as well as karyolysis and necrosis. Cloudy swelling is seen in concentration of 80 $\mu\text{g L}^{-1}$ on day 7 and concentration of 40 $\mu\text{g L}^{-1}$ on day 14. Depuration after 7 and 14 days shows edema and swelling in all concentrations. Ectopic granule

accumulation is seen specifically at concentration of 40 $\mu\text{g L}^{-1}$ 7 days after depuration. Nuclear dust and hyperchromatin is also seen in the cerebrum. Again as previously seen, depuration studies shows more defect in terms of edema and hemorrhage perhaps due to more settlement of the pollutant intake.

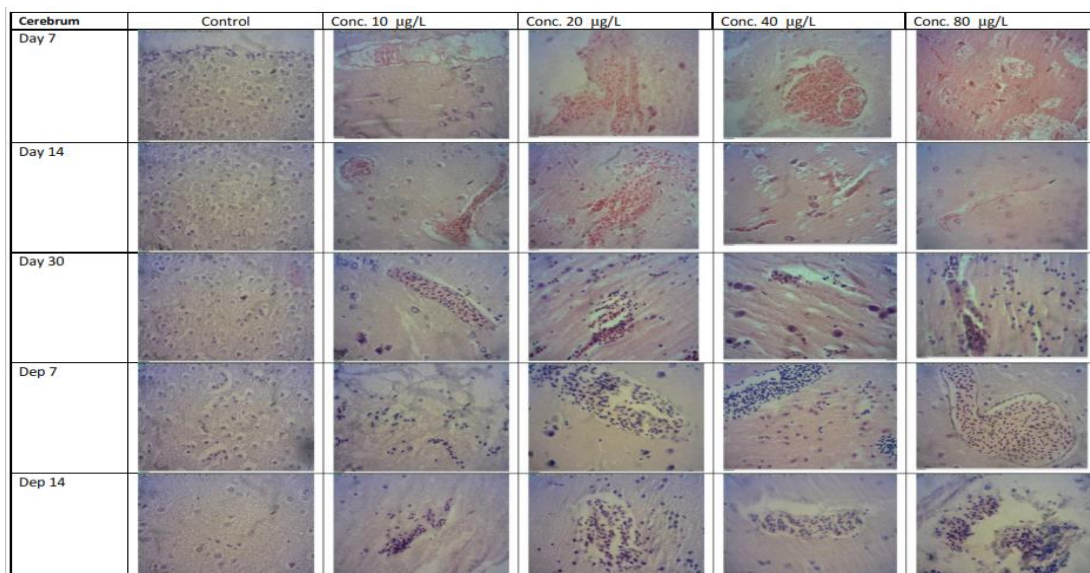


Figure 7: Cerebrum of *Epinephelus coioides* in response to 0, 10, 20, 40 and 80 $\mu\text{g L}^{-1}$ of methylmercury chloride exposure after 7, 14 and 30 days of exposure and depuration of 7 and 14 days (H&E, X3000). (Dep: depuration, Conc: concentration).

Fig. 8 shows mesencephalon and different parts of optic lobe: optic tectum and tegmentum. In the optic tectum hyperanemia, hemorrhage, karyolysis and necrosis is quite

obvious. In tegmentum severe necrosis and vacuolation appeared. In depuration experiments hydropic degeneration and swelling like other parts of the brain occurred.

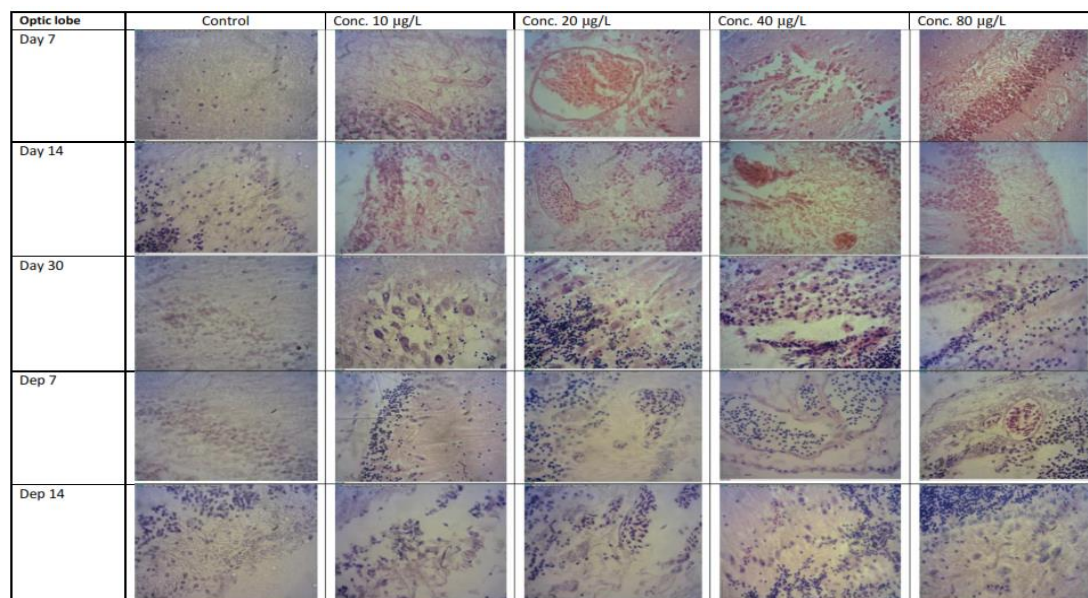


Figure 8: Optic lobe from orange spotted grouper exposed to 0,10,20,40 and 80 $\mu\text{g L}^{-1}$ of methylmercury chloride after 7, 14 and 30 days and depuration of 7 and 14 days (H&E, X3000). (Dep: depuration, Conc: concentration).

Fig. 9 shows metencephalon, the cerebellum. The valvula cerebelli and corpus cerebelli are obvious. The cerebellar cortex consists of three different layers: the granular layer, the molecular layer and ganglionic layer (Savari *et al.*, 2016). The granular layer consists of small, granular cells and numerous golgi cells. The molecular layer constitutes a continuous sheet of neuropil containing a huge amount of oriented dendrites. The intermediate or ganglionic layer contains purkinje cells and numerous smaller cells (Savari *et al.*, 2016). In contrast to the arrangement in most vertebrates, the purkinje cells are placed less regularly between the molecular and granular

layer (Genten *et al.*, 2009). After exposure to methylmercury the valvula cerebelli goes under severe changes, the most defected part among all parts of brain in orange spotted grouper. Cerebellum shows severe hemorrhage of valvula cerebelli as seen in Fig. 9. Nuclear dust of necrosis and karyolysis of purkinje cells and stellate cells are apparent among exposures. Most significantly hyperanemia of granular layer, karyolysis of purkinje cells and vacuolation and cloudy swelling of molecular layer are noticeable in all exposure concentrations. Edema and swelling of depuration durations are seen in this part of the brain.

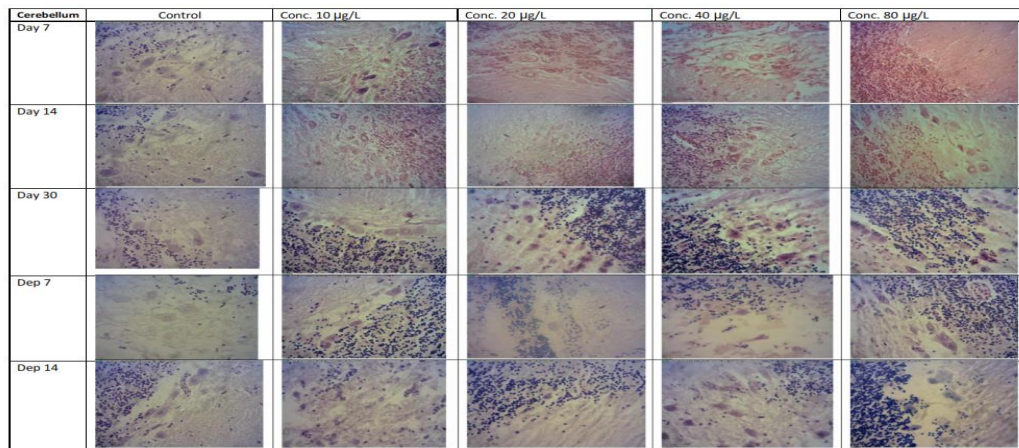


Figure 9: Optic lobe from orange spotted grouper exposed to 0,10,20,40 and 80 µg L⁻¹ of methylmercury chloride after 7, 14 and 30 days and depuration of 7 and 14 days (H&E, X3000). (Dep: depuration, Conc: concentration).

Fig. 10 shows diencephalon of *E. coioides*. The diencephalon or the “between brain” is divided into the dorsal epithalamus comprising the pineal organ and the habenular ganglion, the thalamus itself and the hypothalamus ventrally. The hypothalamus, consisting of the infundibulum and the two inferior lobes, is the major anatomic structure of the diencephalon and functions to regulate the pituitary gland (Genten *et al.*, 2009). Hyperanemia, hemorrhage, necrosis, karyolysis and cloudy

swelling are defects seen in all areas including hypothalamus, the infundibulum region, as well as other parts of diencephalon. Karyolysis and necrosis of pituitary, hydropic degeneration and granule accumulation of infundibulum and diencephalon around the swollen area as well as severe hemorrhage of diencephalon are also obvious in the Figs. During depuration of methylmercury, hydropic degeneration, swelling and edema are apparent.

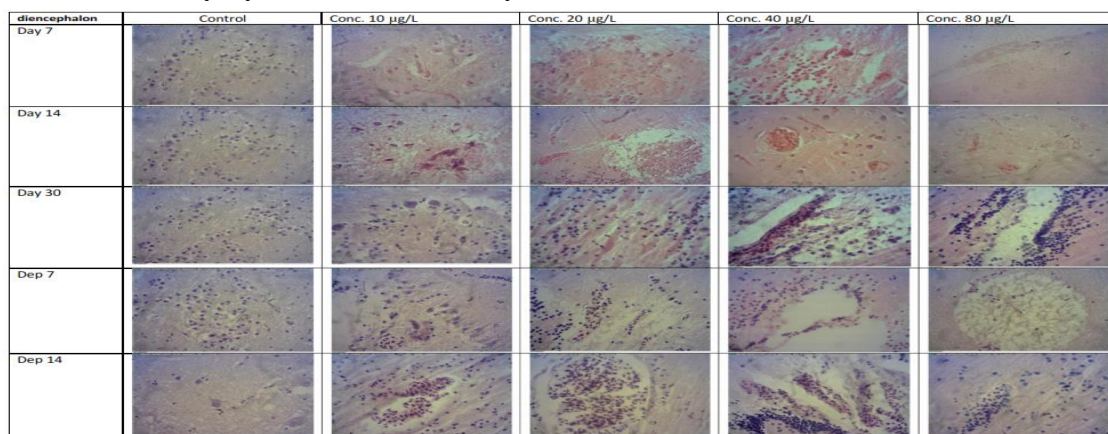


Figure 10: Diencephalon from orange spotted grouper exposed to 0,10,20,40 and 80 µg L⁻¹ of methylmercury chloride after 7, 14 and 30 days and depuration of 7 and 14 days (H&E, X3000). (Dep: depuration, Conc: concentration).

Fig. 11 shows the medulla of *E. coioides*. As seen in the exposures, the Nissl bodies show karyolysis of nucleus and the neuroglial cells are defected by necrosis, however the neuropil depicts

vacuolation and swelling of gray matter besides hypertrophy of neuronal processes. Depuration experiments show swelling and degeneration of the neuropil and Nissl bodies.

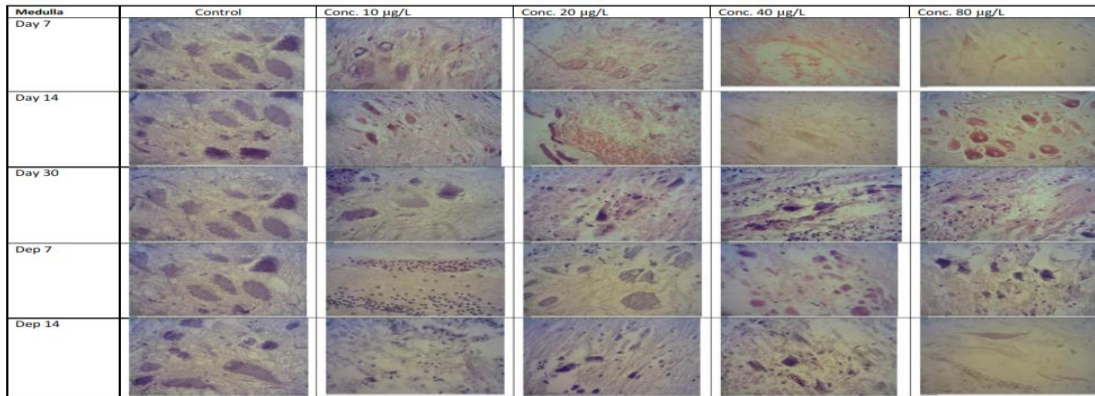


Figure 11: Medulla of *Epinephelus Coioides* in response to 0, 10, 20, 40 and 80 µg L⁻¹ of methylmercury chloride exposure after 7, 14 and 30 days of exposure and depuration of days 7 and 14 (H&E, X3000). (Dep: depuration, Conc: concentration).

Fig. 12 shows the high and low histopathological defects brought by the pollution of Zangi Creek on the brain of *E.coioides*. A comparison is done between the high and low in order to see the severity of the defects in Zangi Creek. In order to see where this amount of pollution fits with the methylmercury exposure contamination

a graph of histopathological alteration index has been drawn in Fig. 13 and it shows that Zangi creek falls below the lowest concentration and is closer to the control fish. This index indicates that the histopathological defects of methylmercury on fish brain are severe and irreversible (no rescue of phenotype is shown).

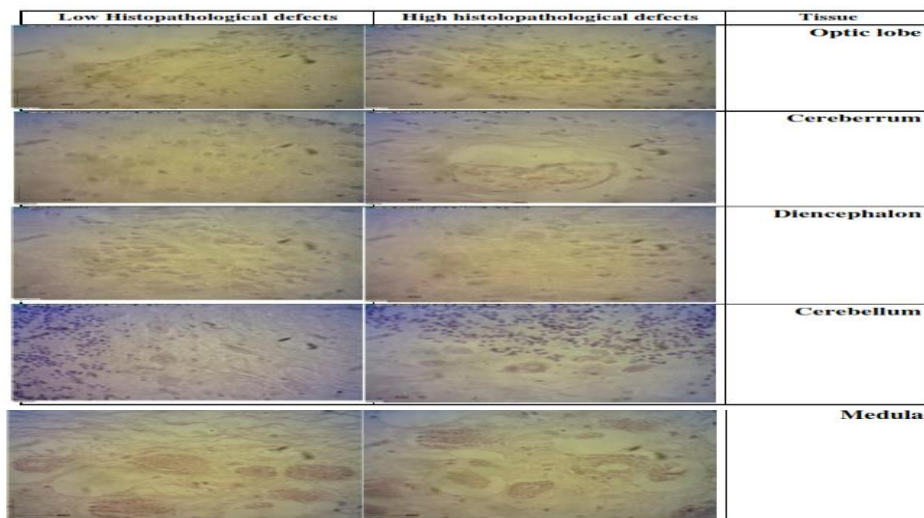


Figure 12: The histopathological defects of Zangi Creek *Epinephelus coioides* brain. The defects are shown as low and high compared to each other.

Discussion

In a study the effect of dietary methylmercury on zebra fish brain was assessed and based on a transmission electron microscopic observation defects such as an impairment of the optical tectum integrity, with a decrease of the nuclear area in contaminated granular cells compared to control cells, and a lower density of cells in the contaminated tissue were seen (Cambier *et al.*, 2012). A potential functional significance of these defects might be impaired vision and therefore lower adaptability of fish to their environment. However in our experiments more severe results were yielded in the optic tectum showing hyperanemia, hemorrhage, karyolysis and necrosis.

Another study confirmed the histopathological evidence of mercury and methylmercury on arctic charr (*Salvelinus alpinus*) by scanning electron microscopy of the olfactory epithelial surface compared to the control fish and visualized the lack of cilia on the surface of olfactory epithelial (Oliveira Ribeiro *et al.*, 2002). In our studies electron microscopy was not used but endothelium hypertrophy where the endothelial was disrupted and fallen apart was obvious and more severe defects such as necrosis in the stage of karyolysis, hyperanemia and hemorrhage were detected.

In all the exposures karyolysis and necrosis was obvious and this is due to the effect of methylmercury. In other experiments performed in rats necrosis with gliosis have been apparent in

cerebrum and cerebellum (Ranjan *et al.*, 2015). Necrosis is a form of cell injury that results in the premature death of cells in living tissue. Necrosis is caused by external factors towards the cell or tissue, such as infection, toxins, or trauma that result in the unregulated digestion of cell components. Cells that die due to necrosis do not follow the apoptotic signal transduction pathway but rather various receptors are activated that result in the loss of cell membrane integrity and an uncontrolled release of products of cell death into the intracellular space (Proskuryakov *et al.*, 2003). This initiates an inflammatory response in the surrounding tissue: Nearby phagocytes are prevented from locating and engulfing the dead cells. (Kasper and Zaleznik, 2001). The result is a build-up of dead tissue and cell debris at, or near, the site of the cell death. In necrosis, small blebs form and the structure of the nucleus changes, the blebs fuse and become larger, no organelles are located in the blebs, the cell membrane ruptures and releases the cells' content and the organelles are no longer functional (Craft *et al.*, 2013). In addition Karyolysis is the complete dissolution of the chromatin matter of a dying cell due to the activity of DNase. The whole cell will eventually stain uniformly with eosin after karyolysis. It is usually preceded by karyorrhexis and occurs mainly as a result of necrosis (Cotran *et al.*, 1999). These two phenomena occurred due to methylmercury exposure in the brain of *E. coioides* and showed the effect of

this organic pollutant on brain tissue implying the deteriorate effect of methylmercury and the severe defects brought by such a pollutant. As seen in the Figs the more the concentration of the pollutant rises, the more severe the defect appears. Moreover the longer the duration of exposure becomes the heavier the abolished effect brought by methylmercury appears. Due to necrosis and karyolysis, hyperchromatin and nuclear dust is seen among all tissues especially cerebellum and diencephalon.

Significantly almost all the exposures of different concentrations to MeHg showed hyper-anemia and hemorrhage in the tissues. The highest amount of hemorrhage was seen in the cerebellum. Cerebellum seems to show the most defects among other tissues and is seen to be more affected by this pollutant. In addition diencephalon and cerebrum showed high amounts of hemorrhage as shown in Figs. 10 and 7, respectively. The depuration experiments showed the hemorrhage more obvious than the exposures as seen in all the Figs. Perhaps these stages had more time to take up the effect of methylmercury and the effect became more settled in them by the time of depuration, than the actual exposure experiments.

Generously, certain amounts of vacuolation, cloudy swelling and hydropic degeneration were apparent in all tissues. Diencephalon, medulla and optic lobe showed more swelling in the form of edema, rather than cloudy swelling seen in cerebrum and olfactory lobe. The white space

surrounding around the nucleus and cells are due to shrinkage not vacuolation.

Endothelium hypertrophy is seen among the olfactory lobe, cerebrum and cerebellum where the endothelial has been disrupted and fallen apart as seen in the Figs.

Although depuration studies after 7 and 14 days did not show any rescue of the phenotype and release of the defects, they showed more swelling, water accumulation and hydropic degeneration of the tissues to the extent of edema.

In general methylmercury showed to have a deteriorating effect on all parts of the brain. However more severe in the cerebellum which could serve as the spatial part of the brain to the biomarker. It was apparent that with rise in concentration and the duration of the exposure, the defects brought about by the pollutant increases. Moreover the depuration experiments not only did not show any rescue and release of the defected phenotype yet as a conclusion showed more flaws due to more time to inhale the defect, such as more rates of hemorrhage in these experiments; meaning that the defect was not washed out but became more settled. Hemorrhage is more severe in depuration studies rather than the exposures; perhaps there was more time to take up the defect in these experiments. Zangi Creek falls between the lowest concentration and the control, showing still some hopes meaning that the area is not as polluted and can be considered a safe creek.

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