Evaluation of acetylcholinesterase transcript level as a biomarker of methylmercury in orange spotted grouper (*Epinephelus coioides*) brain

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Abstract

The bioavailability of methylmercury (MeHg) in the brains of orange spotted groupers, captured from four creeks of Mahshahr embayment was measured. Then the effects of this pollutant on the regulation of gene expression, acetylcholinesterase transcript levels was chosen in order to monitor the amounts of methylmercury concentrations in the creeks, and the fluctuations of mRNA expressions in the brain and their effect on fish health. Fishes were collected from Zangi, Ghanam, Marymous and Petrochemical Creeks, and their brains were removed by dissection. In parallel with these experiments some fishes were exposed to methylmerucry chloride in the Fisheries center and the amount of their gene expression was assessed via Real-Time PCR method. The lethal concentration of methylmerucry causing the mortality of half of the fish population after 96 hr ($LC_{50\text{-}96}$) was assessed and gene expression of sub-lethal concentration (more and less than 10% of $LC_{50\text{-}96}$) were analyzed. Gene expression studies revealed that the most polluted creek was the Petrochemical Creek, and the least polluted one was Marymous Creek. This regulation was assessed by the effect of MeHg on the gene expression, meaning the more gene expression, the less polluted and vice versa. From this study we concluded that acetylcholinesterase gene expression can serve as a biomarker of the effect of methylmercury, which can provide a good estimation of the amount of methylmercuric availability in the brain of *Epinephelus coioides* and its effect on the brain neurotransmission pathway.

Keywords: Acetylcholinesterase (AChE), Methylmercury (MeHg), *Epinephelus coioides,* Orange spotted grouper, Transcript level, Brain

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Introduction

Acetylcholinesterase is an enzyme that through its hydrolytic activity degrades the neurotransmitter acetylcholine into its components choline and acetate. This enzyme is found in the neuromascular junctions and cholinergic nervous system. It is mainly involved in the termination of synaptic transmission and has a high catalytic activity. It hydrolyses 25000 acetylcholine molecules per second (Purves *et al*., 2008). The activity of this enzyme has been shown to reduce upon methylmercury exposure in Medeka fish (Liao *et al*., 2006).

Methylmercury is the organic form of the heavy metal mercury which is much more toxic compared to mercury itself. This is due to the lipophilicity of methylmercury that penetrates through the plasma membrane of cells and especially the blood-brain barrier and accumulates into the tissues (Erni and Geier, 1979; Govindaswamy *et al*., 1992). This toxicant affects many tissues of the fishes (Liao *et al*., 2006), yet the brain is the organ which is more susceptible and sensitive to this pollutant (Liao *et al*., 2006). It has been shown that methylmercury accumulates more in the brain, and affects the activity of acetylcholinesterase more than other tissues of the body (Liao *et al.,* 2006).

This study showed that by exposure of orange spotted grouper brain to methylmercury and acetylcholinesterase the gene expression reduced by this toxicant (Alves Costa *et al*., 2007). It also demonstrated the correlation between the amount of reduction of AChE gene expression and the concentration of dissolved MeHgCl in water. Moreover this study proved that acetychloinesterase is a good indicator of MeHg exposure, as well as the bioavailability of MeHg in the brain of orange spotted grouper in response to reduction of AChE mRNA expression. Therefore one can assess the amount of MeHg exposed in the water through its relationship with the AChE transcript levels.

Meterials and methods

Accumulation

Organic interferences were removed from homogenized seafood by acetone wash followed by toluene wash. Protein-bound methyl Hg was released by addition of HCl and extracted into toluene. Toluene extract was analyzed for CH_3HgCl by electron capture GC.

Chromatography

The following procedure should be followed in order to check and verify the proper operation of the GC system. First inject5µL of the standard solution containing 0.005µg Hg/mL into the GC system. The difference between the CH_3HgCl peak heights for 2 injections should be ≤%. Then check the detector linearity by chromatographing all working standard solutions. Inject 5µL of standard solution with concentration approximately equal to or slightly greater than concentration of the extract. Immediately after the CH3HgCl

peak appears, inject another 5µL extract. Immediately after the CH_3HgCl and background peaks for the extract appear, inject another 5µL of standard solution. Because the column performance and the peak height slowly decrease with time, calculate each Hg concentration in each test sample by comparing the peak height for each of the test extracts to average peak height for standard solutions injected immediately after the test extract. Correct the height of CH_3HgCl peak for the test extract by subtracting the height of the peak for the blank method obtained at the same attenuation and recorder sensitivity. Calculate the methyl-bound Hg content of the test sample expressed as µg Hg/g (ppm Hg) by comparing the height of the peak from injection of test extract to the average height of the peak from duplicate injections of standard solution by the following formula (AOAC International, 1995):

µg Hg/g fish = (R/R¢)×(C¢/C)×50

where R= corrected height of CH_3HgCl peak from injection of test extract,

R¢=average height of CH_3HgCl peak from duplicate injections of standard solution,

C= weight (g) of test portion,

C¢= concentration (µg/mL) of Hg in standard solution, and

50 = final volume (mL).

Gene expression

Fish brains were dissected from orange spotted groupers from both field and laboratory. The brains were kept in liquid nitrogen tanks until stored in -80ºC freezer. RNA was extracted from Total RNA Extraction Kit PP-210S). Three brains were pulled in one sample in order to obtain more RNA due to the small size of cerebellum and brain. cDNA was synthesized using AccuPower RocketScript PreMix lyophilized tubes (catalogue no. K-2101). Oligo dT primers were used to synthesize the cDNA strand. The primers were designed by Primer 3 software and they were as follow:

AChE primer (target gene):

Forward Primer: 5'-TATCGTGTTGGAGCTTTTGG-3'

Tm: 58.77

Reverse Primer: 5'-GCTCCTGCTTGCTGTAATGG-3'

Tm: 60.93

16S rRNA primer (reference gene):

Forward Primer: 5'-CCTGCCCTGTGACTATATGTTT-3'

Tm: 58.15

Reverse Primer: 5'-CATTCATACAGGTCCCCATTT-3'

Tm: 58.65

The PCR reaction was carried out using 0.375 mM forward and reverse primers, 1.5 mM $MgCl_2$ cDNA 1.5 µg. This reaction was done by AccuPower GreenStar qPCR Master Mix (SybrGreen based methods) from Bioneer company (catalogue no. K-6251) with the correction of 0.7 µL Rox dye. Step one software V.2.0 was used to analyze the data. The analysis of the data was performed by $2^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001).

Lethal concentration

Lethal concentration of 50% of fish after 96 hrs (LC_{50-96}) was assessed by using MeHgCl from Sigma diluted in double distilled water. A stock solution of 0.1 gr/ml and a working solution of 1 mg/ml was used. Concentrations 280, 300, 320, 340 and 380 were used and the amount of fish death was recorded after 96 hrs. The LC_{50-96} was measured using Finney's probit analysis in SPSS v. 16 (Finney, 1947; Boudou and Ribeyre, 1997).

Results

The areas studied were Mahshahr Creeks, part of Musa Creek, with another 4 different creeks namely, Zangi, Ghanam, Marymous and Petrochemical Creeks. Their position is shown in Fig. 1.

In order to estimate the contamination of the area, the amount of MeHg accumulation in the brain of orange spotted grouper was calculated using GCMS-ECD technique (Fig. 2).

To measure the amount of gene expression alterations Real-Time PCR technique was used. In this technique the melt curve plots show the expression of the transcripts and the specificity of the experiment (showing the amount of transcripts).

Alteration in AChE transcript levels in the brain *of E. coioides* in different creeks was obtained by Real Time PCR method. Petrochemical and Ghanam Creeks had the least expression while Zangi and Marymous Creeks had higher transcript levels. It seems that MeHg concentration had a negative effect on transcript level.

The transcript level alterations of AChE in cerebellum was assessed (Fig. 5). All the values on this plot have a significant difference compared to each other except for the Zangi and Marymous Creeks. These values are less than those of the brain and it seems that cerebellum reacts more severely towards MeHg.

Correlation between the methylmercury concentration in the brain and gene expression alterations was assessed (Fig. 6). These factors had a negative correlation with the amount of 92%.

Probit analysis used in SPSS software revealed that lethal concentration of 50% mortality after 96 hours is 300.438 µg/L.

Transcript levels of AChE reduced in response to the MeHg dose and time exposures in the brain of *E.coioides.* This reduction was more upon higher doses and later days of exposure meaning the least amount of AChE transcript levels was at 80µg/L at day 30. There was an irregular pattern at day 14 which showed higher amounts of AChE transcript compared to the control and other exposures.

Transcript levels increased during depuration experiments showing the highest amount at 7 days after depuration (day 37) and lowering at 14 days of depuration (day 44) but still higher than the control levels (thousand times more). Exposure of day 30 is also shown in order to compare the depuration studies with exposure experiments (Fig. 9).

Figure 1: Different creeks studied from Mahshahr Creek: Zangi, Ghanam, Marymous and Petrochemical Creeks (picture obtained from Google Earth).

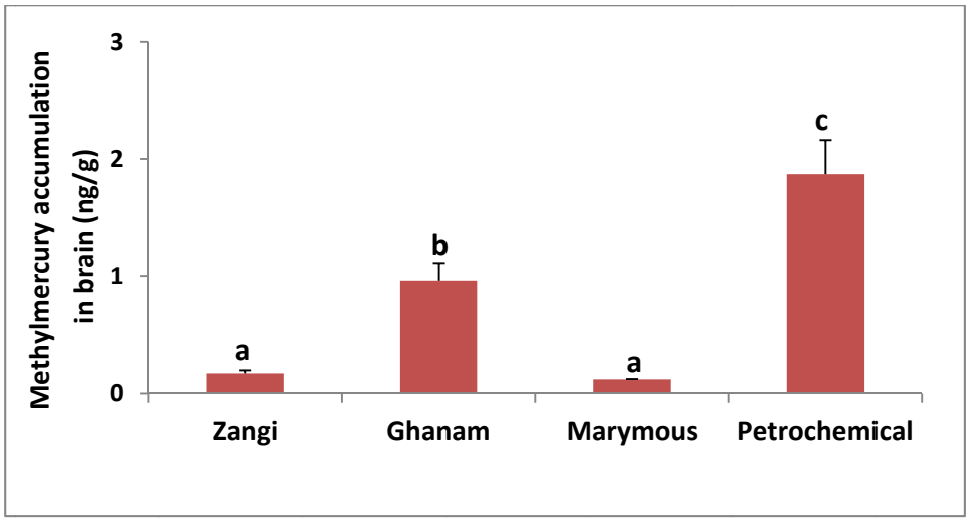

Figure 2: Accumulation of methylmercury in brain of *Epinephelus coioides* in Mahshahr Creeks. Different letters indicate significant difference (p<0.05).

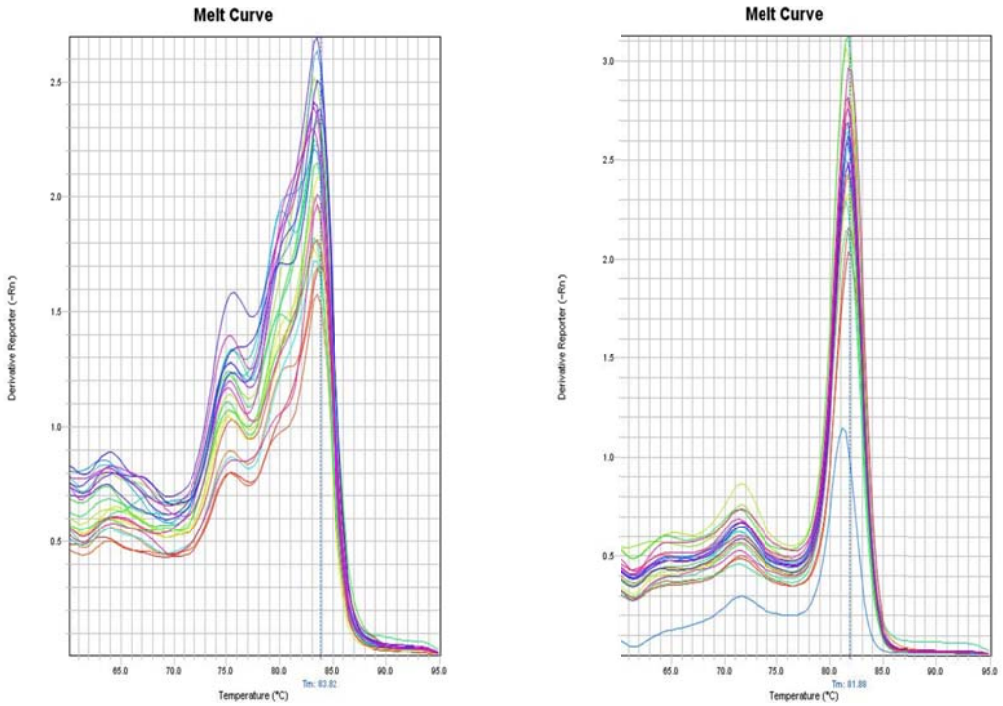

Figure 3: Melt curve plots of target gene, AChE (left) and reference gene, 16S rRNA (right). The peaks on each plot indicate the specific bands.

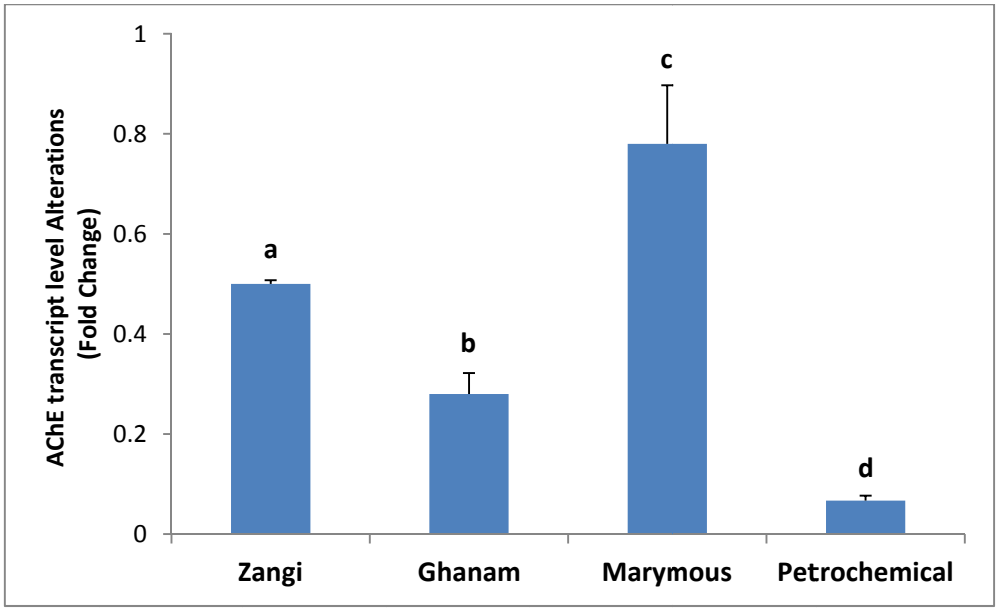

Figure 4: AChE transcript level alterations in the brain of *Epinephelus coioides* in different creeks. Different letters indicate significant differences (p<0.05).

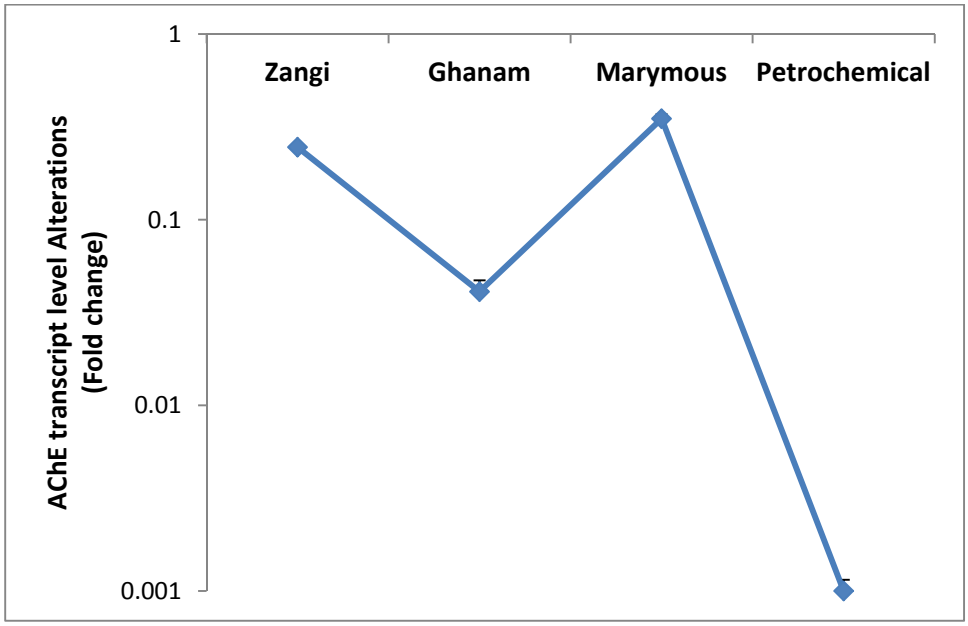

Figure 5: AChE transcript level alterations in the cerebellum of *Epinephelus coioides* in different creeks. Different letters indicate significant differences (p<0.05). The plot has been drawn on a logarithmic scale.

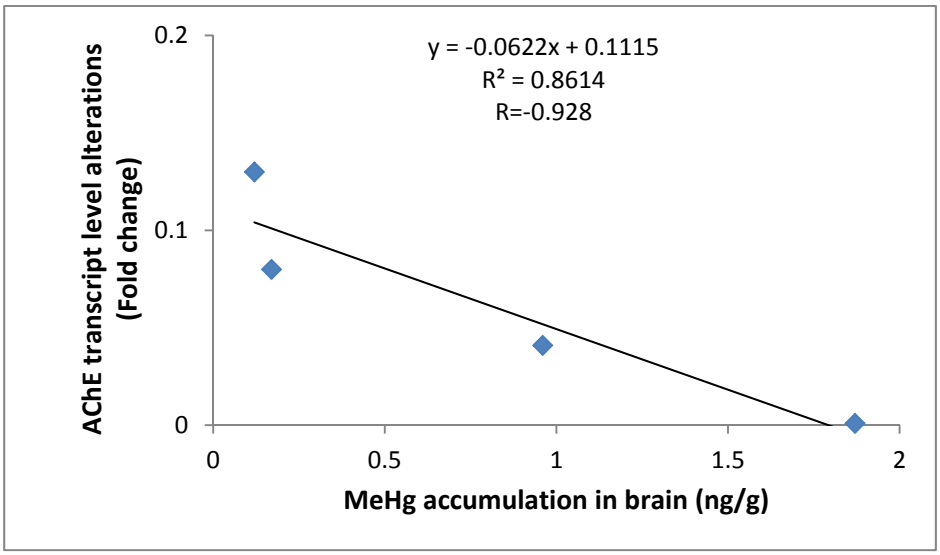

Figure 6: Correlation between methylmercury concentration in the brain and gene expression alterations.

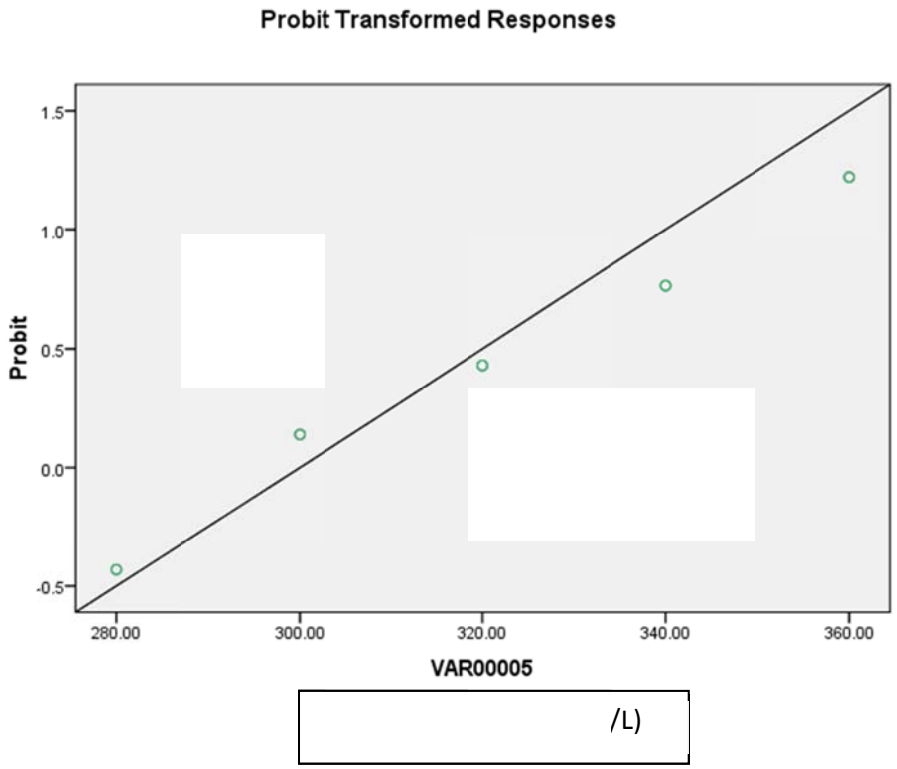

Figure 7: Probit analysis of lethal concentration of 50% mortality after 96 hours.

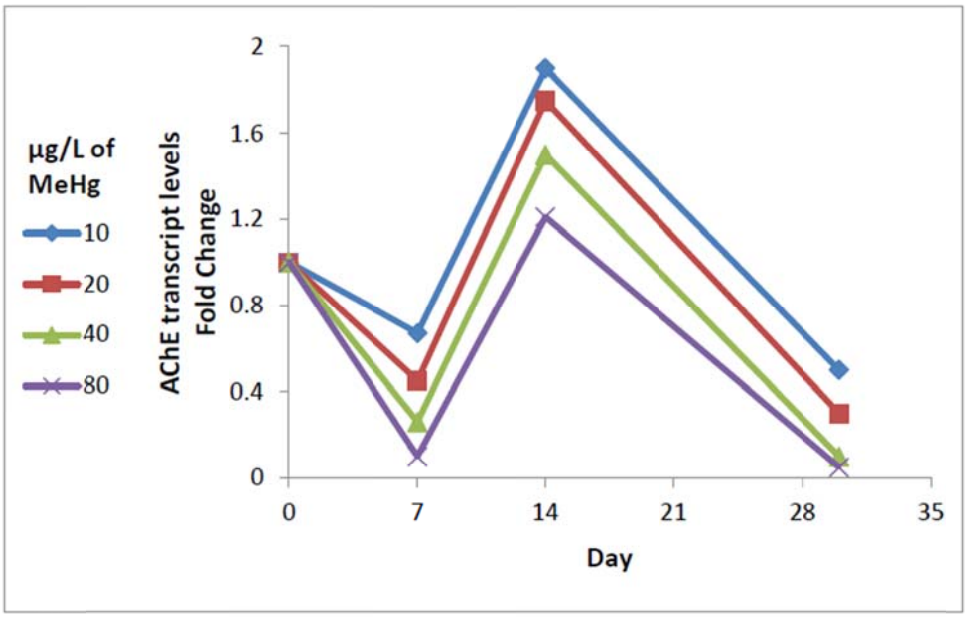

Figure 8: AChE transcript levels compared to control at days 7, 14 and 30 of different MeHg exposures in brain of *Epinephelus coioides*.

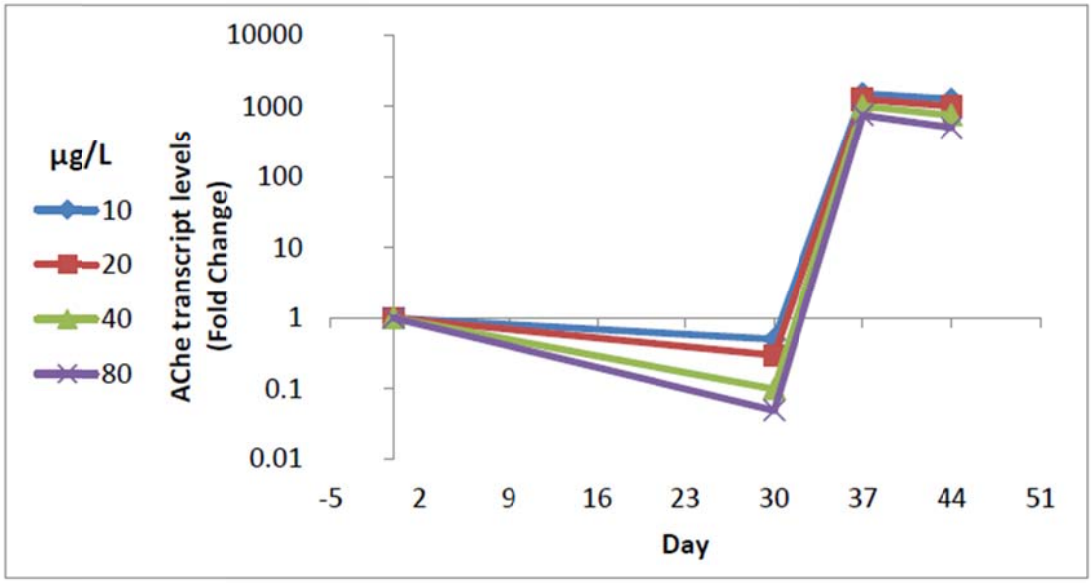

Figure 9: AChE transcript levels at depuration studies (37 and 44) compared to the control and day 30 of exposure period in brain of *Epinephelus coioides*.

Fig. 10 shows the amount of transcript levels compared to control at days 7, 14 and 30 of different concentrations of exposure in the cerebellum of *E. coioides*. The amount of reduction here was more than the brain at days 7 and 30.

Depuration studies in the cerebellum showed more increase in transcript levels compared to the brain. Cerebellum was more sensitive towards exposure and depuration experiments.

Fig. 12 shows Correlation between MeHg concentrations in water and AChE transcript level alterations. This correlation was negative and was about 91%.

Comparison between the AChE transcript levels of different creeks and exposure concentrations (µg/L) are depicted in Fig. 13. Ghanam and Petrochemical Creeks had a level equivalent to 40 and 80 µg/L of MeHg which was higher than 10% of $LC_{50\text{-}96}$ (20-40 µg/L) and they were assessed as being at high risk. Zangi and Marymous were above 10 µg/L and they were considered as safe creeks.

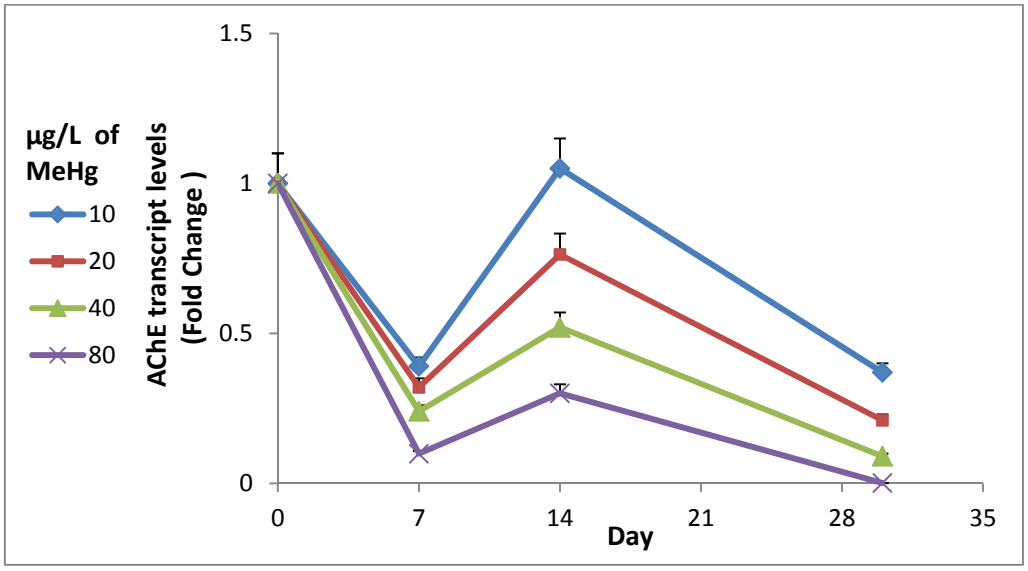

Figure 10: AChE transcript levels compared to control at days 7, 14 and 30 of MeHg exposure studies in cerebellum.

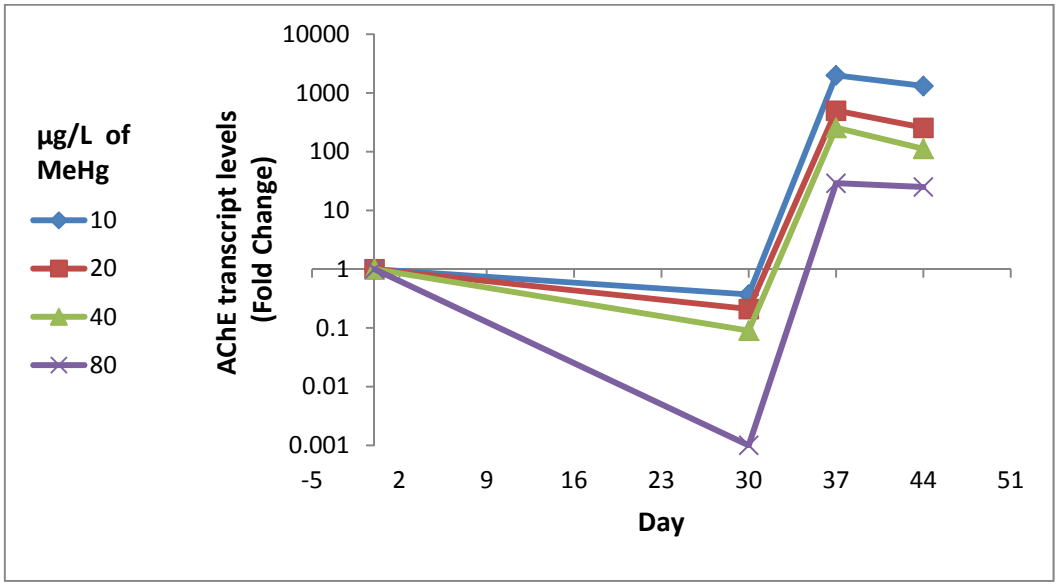

Figure 11: AChE transcript levels compared to control during depuration studies (days 37 and 44) and at day 30 of the exposure in the cerebellum of *E. coioieds*.

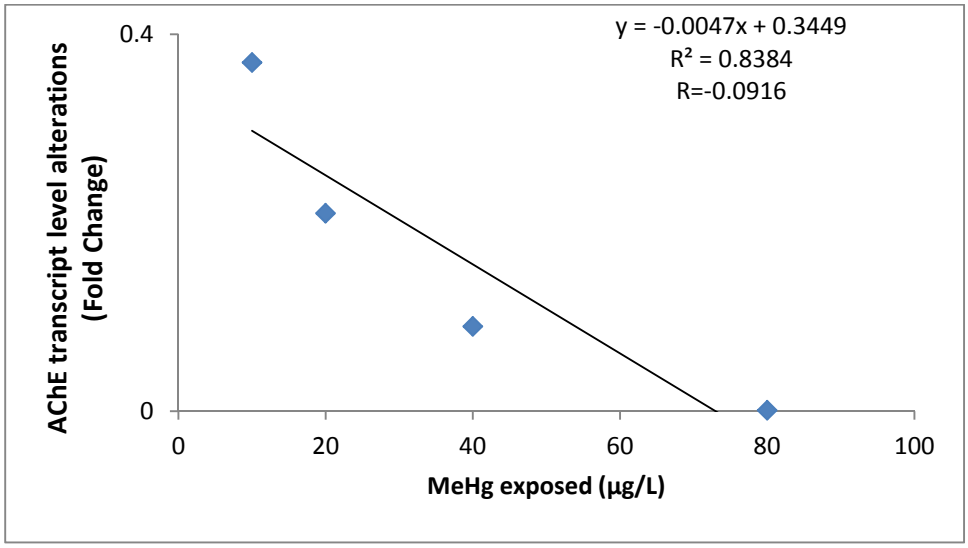

Figure 12: Correlation between MeHg exposed to water and the AChE transcript level alterations.

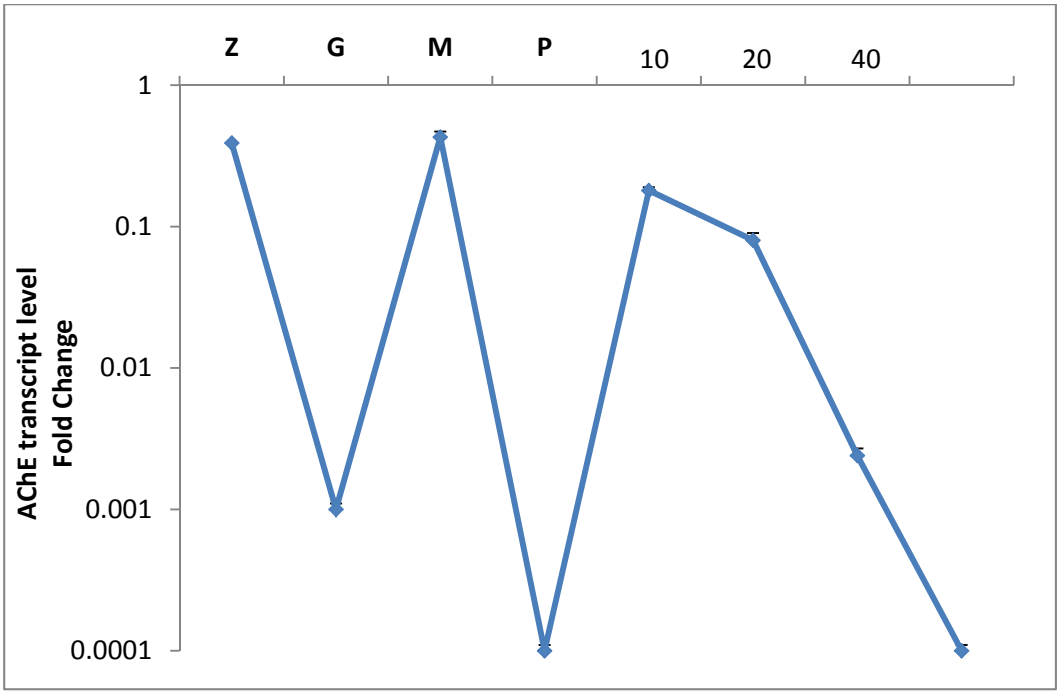

Figure 13: Comparison of the AChE transcript levels in Mahshahr Creeks and different MeHg exposures (µg/L). Z: Zangi, G: Ghanam, M: Marymous, P: Petrochemical.

Discussion

The amount of gene expression was analyzed in cerebellum and the brain (brain – cerebellum). Cerebellum has shown to accumulate more MeHg in itself than the rest of the brain (Coccini *et al.,* 2000). In addition AChE has shown to be tissue specific (Cooper and Huasman, 2013; Karp, 2013) meaning it expresses more in cerebellum rather than other tissues. Therefore in this study cerebellum was analyzed apart from the rest of the brain. This study revealed that the more AChE gene expression in brain tissue, the less the fish has been exposed to MeHg, whether less time exposure or concentration. The reason for the reduction of AChE transcript levels in response to MeHg is because MeHg has a high affinity for thiol groups (-SH) and most transcription factors contain cysteine amino acids that possesses sulfur. Once MeHg binds to the sulfur groups it diminishes its activity through a conformational change brought by this attachment (Guo, 2001; Rodgers *et al*., 2001; Dixit *et al*., 2004). Thus blockage of transcription factors leads to less mRNA expression and lower transcript levels. In this experiment 2 bands appeared in the Real Time PCR method showing different isoforms of AChE in response to MeHg exposure.

The biological reason that AChE drops more in the cerebellum compared to the rest of the brain could be due to the role of cerebellum in maintaining balance and contraction of the muscles (Genten *et al*., 2009).

In the creeks the fishes collected were about the same size and age (however the amounts were normalized). The accumulation of MeHg in their brain showed that the Petrochemical area was the most polluted and Marymous was the least polluted creek (the sediment and water pollution confirmed the same results; data not shown here). Gene expression analyses revealed the same results. Petrochemical Creek had the least amount of AChE transcript level in the brain and cerebellum whereas Marymous Creek had the most AChE transcript folds. The correlation between AChE transcript level and MeHg accumulation in the brain of Mahshahr Creek fishes was 92% and it was negative meaning that when the transcript levels are high there is less accumulation and vice versa. This high correlation indicates the significance of this experiment and how the factors examined are interconnected.

The amount of transcript level reduction in the cerebellum was more than the brain and this shows the specificity of this tissue (Lodish *et al*., 2012; Alberts *et al*., 2013) towards MeHg accumulation compared to other tissues. For example in the Petrochemical Creek there was 10 fold difference between cerebellum and the brain. Therefore AChE transcript levels are to be introduced as a biomarker of MeHg in the cerebellum rather than the whole brain, since they give more reasonable and sensitive values than the rest of the brain. This is because AChE expresses differently (gene specific) in

various parts of the brain and MeHg as well accumulates differently in parts of brain (tissue specific).

In the exposure experiments the more the concentration of MeHg, the less the amount of AChE transcripts. In these experiments the amount of MeHg doubled during each phase starting from 10 to 20, 40 and up to 80 µg/L but the levels of the transcript dropped from 10 up to 100 folds. The amounts of AChE transcripts in cerebellum for the longest exposure duration and the doses mentioned were 0.37, 0.21, 0.09 and 0.001, respectively. These amounts indicate that the transcript levels drop exponentially since there are 2 strands of DNA and in each cycle the amount of regression would be 2 to the power of alterations.

In the exposure studies the amount of AChE mRNA levels at day 14 increased with higher doses whereas they were expected to fall. This irregular pattern had also been seen in the activity of AChE by other scientists (Liao *et al*., 2006) and it could be due to the onset of AChE reduction or a compensation mechanism to retaliate for the loss. The most reductions have been observed at day 30 which indicate that time exposure is significantly correlated with reduction.

The reason for the exposure studies in parallel with the field studies is to gain a standard for health and risk assessment evaluations. In the exposure studies the $LC_{50\text{-}96}$ revealed 300.438 µg/L which 10% of that was the level that the animals can tolerate and knowing this information the sublethal doses were chosen above and beneath this dose (30 µg/L). The transcript levels of the creeks were less than 10% of $LC_{50\text{-}96}$ range (between 20 to 40 µg/L of MeHg exposure). This indicates that Ghanam and Petrochemical creeks are at risk and the aquatic animals in this ecosystem were not able to tolerate the MeHg pollution discharged into these creeks. Off course it is acceptable to have such high MeHg levels at these sites since the petrochemical industry and the chloralkali division discharge their waste, which are full of Hg, into these waters. Zangi and Marymous Creeks were less polluted and fell behind the warning range (threat range) and they are considered safe from MeHg contamination. In this study AChE transcript levels in cerebellum is introduced as a biomarker of MeHg exposure.

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