Japanese Journal of Veterinary Research 64(Supplement 2): S123-129, 2016

Lethrinas nebulosus fish as a biomarker for petroleum hydrocarbons pollution in Red Sea: Alterations in antioxidants mRNA expression

Mohamed Afifi[1,2,*], Haytham A. Ali[2], Taghred M. Saber[3] and Abd elhakeem El-Murr[4]

[1]Department of Biological Sciences, Faculty of Science, University of Jeddah, Saudi Arabia.
[2]Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.
[3]Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.
[4]Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.
* Corresponding author Email: abomariem_1983@hotmail.com

Abstract

Total Petroleum Hydrocarbons (TPHs) are environmental contaminants that are released into the marine water via oil spills and industrial activities. The mRNA expression profile of some antioxidant genes in livers, gills, skin and muscles of *Lethrinas nebulosus* was used as biomarker of TPHs pollution in six areas at Jeddah and Yanbu coasts in Kingdom of Saudi Arabia (KSA). TPHs were determined in Red Sea water and sediments collected from the studied areas. Ten fish of similar sizes were collected from each area for the mRNA expression of super oxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) and malondialdehyde (MDA) levels. The highest concentration of TPHs was observed in front of petromine refinery in Jeddah. The expression levels of antioxidant enzyme genes were highly significantly increased in the polluted areas. MDA levels were high in TPHs polluted areas. This study concluded that the expression profile of antioxidant enzyme genes in examined tissues of *L. nebulosus* could be utilized as a strong bioindicator of TPHs pollution in Jeddah and Yanbu coastal areas in KSA.

Keywords: Antioxidants, *Lethrinas nebulosus,* Total petroleum hydrocarbons.

Introduction

Petroleum hydrocarbons are chemical compounds which induce persistent environmental contaminants, especially in aquatic environments[14], including fish[3]. Lethrinidae family was selected for the present study, because they are distributed in tropical and subtropical Indo-Pacific area[23]. The biochemical changes can be used as an indicator for estimation of the environmental contamination degree by organic chemicals. It has been suggested that petroleum hydrocarbon exposure induces the reactive oxygen species (ROS) synthesis in aquatic organisms[5]. ROS are responsible for DNA damage, apoptosis, protein degradation and lipid peroxidation in

vertebrates[22].Transcription level alterations are the earliest sensitive bioindicators for biological responses to stress. Thus, genes with expression levels, that are altered in response to environmental stresses can be used for diagnosis and quantify the effects of these stresses[6]. These bioindicators can act as signs to pollutant exposure indicating the exposure levels and the magnitude of the organism's response to the toxic substances[7].

Antioxidant enzymes as SOD and CAT are often employed as pollutant bio-indicators by providing a measure of the physiological stress in animals when exposed to pollutants[15]. Furthermore; GR and GPx are usually used to assess the oxidative stress in biological system[13]. The aim of the present study was to evaluate the hazards effect of TPHs pollution on the antioxidant gene expression levels as a bioindicator for the pollution at sea coastal area of Jeddah and Yanbu provinces, Saudi Arabia.

Materials and Methods

This study was approved by the Ethical Committee of the King Fahed center for medical science, King Abdulaziz University, KSA. Six sampling areas were selected along the Red Sea coast at Jeddah and Yanbu provinces, four contaminated sites and two reference areas, as previously described by Afifi *et al.*[1]. Sediment, water and fish samples were collected from the studded sites during mid of March 2014 for extraction and determination of TPHs in accordance with the established procedures[12]. Fish sampling, ten fish of a similar size of *Lethrinas nebulosus* were collected from each studded site from overnight pre-held pots. Length and weight of each fish were recorded. Liver, gills and muscle samples were taken, kept in liquid nitrogen for molecular analysis and TPH determination. The MDA was analyzed using a TBARS assay kit (Catalog No. 10009055, Cayman, USA). Liver, gills, skin and muscle SOD, CAT, GR, GPx and GST genes expression were quantified using qPCR. Total RNA was isolated from tissue samples using the RNeasy Mini Kit Qiagen (Cat. No.74104). 0.5μg of total RNA, was used for production of cDNA using Qiagen Long Range 2 Step RT-PCR Kit, (Cat. No.205920). Five μL of total cDNA was mixed with 12.5 μL of 2x SYBR® Green PCR mix with ROX from BioRad and 10 pmol/μL of each forward and reverse primers for the examined genes. The house keeping gene β-actin was used as a constitutive control for normalization. Primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3/) as per the published GR, GST, GPx and β-actin genes sequences (XM_003445184, EU234530, EF206801and EU887951) of NCBI database all primers were provided by Sigma Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and are shown in table (1). PCR reactions were carried out in an AbiPrism 7300 (Applied Biosystems, USA). The

Table 1. Oligonucleotides sequences of primers

Gene	Forward 5'->3'	Reverse 5'->3'	Ampliconn
CAT	TCCTGAATGAGGAGGAGCGA	ATCTTAGATGAGGCGGTGATG	232
SOD	GGTGCCCTGGAGCCCTA	ATGCGAAGTCTTCCACTGTC	377
GPx	CCAAGAGAACTGCAAGAACGA	CAGGACACGTCATTCCTACAC	180
GR	CATTACCGAGACGCGGAGTT	CAGTTGGCTCAGGATCATTTGT	420
GST	TAATGGGAGAGGGAAGATGG	CTCTGCGATGTAATTCAGGA	640
ß actin	CAATGAGAGGTTCCGTTGC	AGGATTCCATACCAAGGAAGG	280

The SPSS version 20, Statistical packages (IBM, New York, NY, USA) was used for statistica l analysis of the obtained data, that were presented as a mean ± SD, n=10. One-way analysis of variance (ANOVA) was used for determination of statistical differences between the groups. Duncan's test was used for testing the inter-grouping homogeneity. Statistical significance was set at $p<0.05$.

Table 2. Total petroleum hydrocarbons in sea water ($\mu g.L^{-1}$) and sediment ($mg.K^{-1}$ dry weight) in the different areas.

	I	II	III	IV	V	VI
Sea water	1.3±0.3	6.2 ± 1^{a}	13.4 ± 1.4^{ab}	2.08±0.3	9.8 ± 1^{ac}	20.2 ± 1.7^{abc}
Sediment	14.8±2.6	130 ± 12.5^{a}	170 ± 15.2^{ab}	20.2±3	167 ± 10^{ac}	212 ± 8^{abc}

I; reference area at Yanbu coast, II; collected close to Yanbu industrial harbor, III; close to oil refineries and petrochemical factories at Yanbu coast, IV; reference area at Jeddah coast,V; north of Jeddah Islamic seaport and VI; in front of petromine refinery in Jeddah. Values are presented as mean ±SE (n=10).The levels of significance ($p<0.05$) found in the same column are: a = in versus to the reference group, b = comparison of group III and VI versus group II and V are compared respectively, c = when compare Jeddah groups versus the corresponding Yanbu groups.

RNA concentration in each sample was determined from the threshold cycle (Ct) values. The mRNA expression levels were calculated relative to ß-actin gene mRNA levels using the $2^{-DD\,CT}$ method.

Results and Discussion

In the present study, we determined the expression levels of some antioxidant enzymes in *L. nebulosus* that was grown in the selected areas of the study and their relation to TPHs pollutants. The selected areas of the study were characterized by high PHs pollution due to the crude or refined petroleum or combustion sources[4]. Our result showed that the TPHs concentrations were higher in the polluted areas if compared with their references areas, with the highest concentrations in the area in front of petromine refinery at Jeddah (10.49 folds higher than the reference area) (Table 2 and 3).

Table 3. Total petroleum hydrocarbons ($\mu g.g^{-1}$) in liver, gills, skin and muscle of *Lethrinas nebulosus*.

Areas	Length	Weight	Total petroleum hydrocarbon			
			Liver	Gills	Skin	Muscle
I	48±5	393±64	4.2±0.03	5.4±0.8	6.65±0.9	3.6±0.01
II	43±57	386±35	60.5 ± 10^{a}	78.6 ± 8.5^{a}	108.8 ± 20^{a}	29.6 ± 5^{a}
III	46.3±6	380±40	185 ± 20^{ab}	211.6 ± 11^{ab}	229.7 ± 22^{ab}	50.8 ± 8^{ab}
IV	45±2.3	390±20	4.8±0.03	6.65±0.05	7.3±1	6±0.04
V	48±3	383±8	84.6 ± 11^{ac}	110.6 ± 10^{ac}	151 ± 13^{ac}	47 ± 6^{ac}
VI	47±4.5	393±28	219.5 ± 22^{abc}	273.9 ± 13^{abc}	302.3 ± 25^{abc}	94 ± 10^{abc}

I; reference area on Yanbu coast, II; collected close to Yanbu industrial harbor, III; close to oil refineries and petrochemical factories at Yanbu coast, IV; reference area at Jeddah coast, V; north of Jeddah Islamic seaport and VI; in front of petromine refinery in Jeddah. Values are presented as mean ±SE (n=10).The levels of significance ($p<0.05$) found in the same column are: a = in versus to the reference group, b = comparison of group III and VI versus group II and V are compared respectively, c = when compare Jeddah groups versus the corresponding Yanbu groups.

This has been reflected on the biochemical levels of MDA and expression levels of antioxidant genes. The highest levels of MDA in liver, gills, skin and muscle were observed in fish collected from the area in front of the petromine refinery in Jeddah (Table 4). TPHs are known to increase the oxidative stress in aquatic livings[1]. The CAT, SOD, GPx, GR and GST activities/expression were increased as a protective response to oxygen free radicals. They act through reduction of superoxide radicals by SOD and of hydrogen peroxide and organic hydroperoxides by CAT and GPx respectively[16]. In the present study, the mRNA expression levels of these antioxidant genes were studied using qPCR in livers, gills, skin and muscle. The result showed high expression levels in *L. nebulosus* tissues collected from the polluted areas in both Jeddah and Yanbu coastal areas if compared with fish collected from reference areas (Fig.1).

Table 4. MDA level (nmol.g^{-1} wt.w. tissue) in studied tissues of *Lethrinas nebulosus*

Areas	Skin	Gills	Livers	Muscles
I	2.7±0.4	2.2±0.5	1.3±0.3	0.4±0.09
II	5.5±0.8^{a}	4.1±0.3^{a}	2.5±0.2^{a}	0.8±0.06^{a}
III	7.6±0.9^{ab}	5.8±0.4^{ab}	3.5±0.6^{ab}	1.1±0.08^{ab}
IV	3±0.5	2.3±0.4	1.38±0.2	0.4±0.07
V	6.6±1.3^{a}	5±1^{a}	3±0.6^{a}	0.96±0.02^{a}
VI	10.5±1.6^{abc}	7.9±1.2^{abc}	4.7±0.9^{abc}	1.5±0.08^{abc}

I; reference area on Yanbu coast, II; collected close to the Yanbu industrial harbor, III; close to oil refineries and petrochemical factories at Yanbu coast, IV; reference area at Jeddah coast, V; north of Jeddah Islamic seaport and VI; in front of petromine refinery in Jeddah. Values are presented as mean ±SE (n=10). The levels of significance ($p<0.05$) found in the same column are: a = in versus to the reference group, b = comparison of group III and VI versus group II and V are compared respectively, c = when compare Jeddah groups versus the corresponding Yanbu groups.

The coastal area in front petromine refinery in Jeddah showed the highest expression levels of antioxidant enzymes at all. To the best of our knowledge; we are the first who performed the full study about the gene expression of the bioindicators for TPHs pollution in Saudi Arabia; as other authors evaluated these indicators in relation to the cellular activities only[9]. The oxidative stress bioindicators responses, in our work, were mostly accused in Jeddah coastal (The highest hydrocarbons polluted area) areas than Yanbu coastal areas, an explanation for the induced expression of bio-indicators as CAT and GR in the skins, gills and liver this was paralleled to the data obtained by Vicente-Martorell *et al.*[18]. The possible explanation for GPx and GR gene expression induction is the increase of lipid peroxidation[20] (manifested in this study by increase of MDA) that induced by the increase of mitochondrial membranes polyunsaturation in *L. nebulosus*. The membranes polyunsaturation raises the mitochondrial respiratory rates that enhance the ROS synthesis, inducing membrane lipid peroxidation[17]. The high levels of lipid peroxidation products induced GPx activity[21]. This increase in GSH utilization, producing oxidized glutathione (GSSG) and induced GR activity for reduced equivalents and normal redox homeostasis in the living cells[10]. So there is a relationship between the increase in the TPHs concentrations and the high concentrations of MDA and high expression profile of antioxidant genes in *L. nebulosus* tissues. The increase of GST activity in fishes tissues after exposure to PHs has been reported in several studies[19], but others reported no significant alterations[11], or decreased activity[19]. The effect of chemicals on GSTs activities in living fish was yielded conflicting results[8]. We demonstrated a high increase in the GST expression level in the tissues of *L. nebulosus* at the TPHs polluted areas.

In conclusion, this study confirmed the presence of a significant correlation between the intensity of the TPHs pollution and high MDA levels with the high expression levels of SOD, CAT, GR, GPx and GST genes in *L. nebulosus* tissues. Also, these findings recommended the bioavailability of *L. nebulosus* as a useful biomarker for TPHs pollution in coastal areas. Further studies are crucial to investigate the role of *L. nebulosus* as a bioindicator of other environmental pollutants.

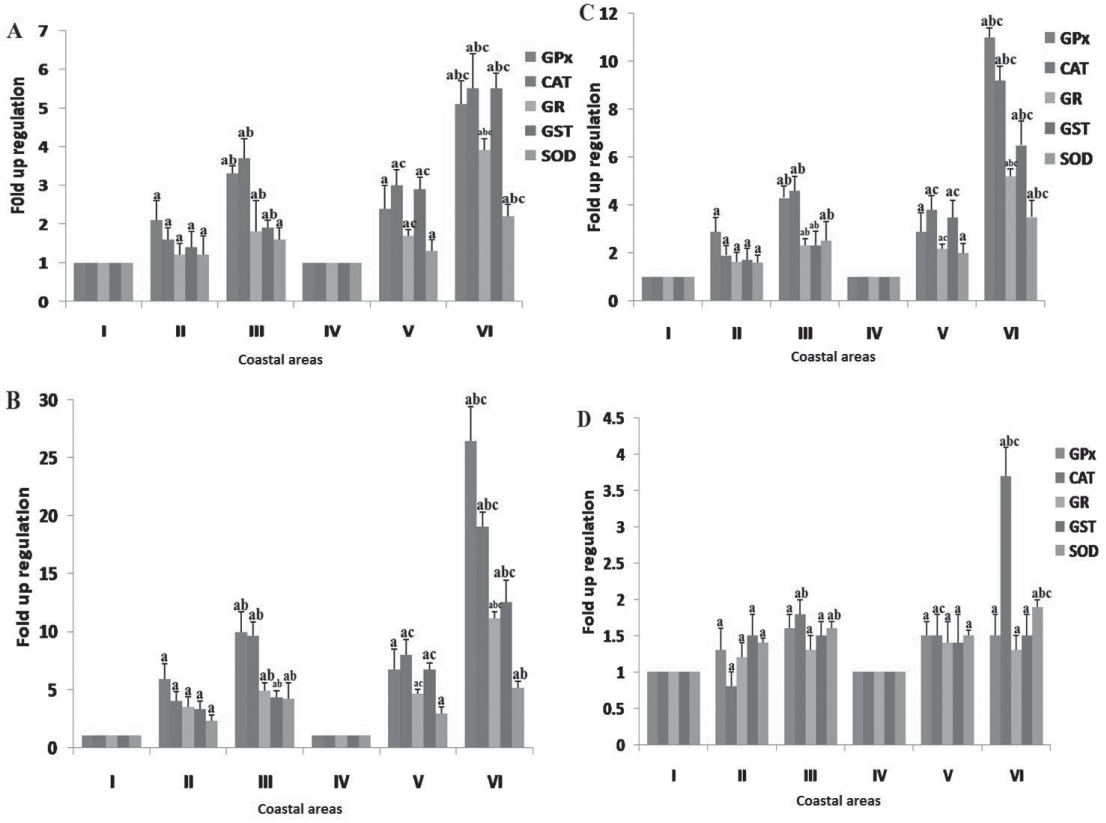

Fig. 1 Antioxidant genes expression (fold of up regulation versus to the reference areas) in skin (A), Gills (B), Liver (C) and Muscle (D).

I; reference area on Yanbu coast, II; collected close to Yanbu industrial harbor, III; close to oil refineries and petrochemical factories at Yanbu coast, IV; reference area at Jeddah coast, V; north of Jeddah Islamic seaport and VI; in front of petromine refinery in Jeddah. Values are presented as mean ±SE (n=10).The levels of significance ($p<0.05$) found in the same column are: a = in versus to the reference group, b = comparison of group III and VI versus group II and V are compared respectively, c = when compare Jeddah groups versus the corresponding Yanbu groups.

References

1) Afifi, M., Alkaladi, A., Abu Zinada, O. and Couderchet, M. 2015. Alteration in antioxidant genes expression in some fish caught from Jeddah and Yanbu coast as a bio-indicator of oil hydrocarbons pollution. *Saudi J. Biol. Sci.* doi: 10.1016/j.sjbs.2015.06.014 (In Press).

2) Afifi, M., Alkaladi, A., Abu-Zinada, O. and Zahed, N. 2014. Histopathological and ultrastractural alterations in skin, gills, liver and muscle of Siganus canaliculatus and Epinephelus morio caught from Jeddah and Yanbu coast as bioindicators of oil hydrocarbons pollution. *Life Sci. J.,* **11**: 880-888.

3) Balk, L., Hylland, K., Hansson, T., Berntssen, M. H., Beyer, J., Jonsson, G., Melbye, A., Grung, M., Torstensen, M. E., Borseth, J. F., Skarphedinsdottir, H. and Klungsoyr, J. 2011. Biomarkers in natural fish populations indicate adverse biological effects of offshore oil production. *PLoS One,* **6**: e19735.

4) Cheung, C., Zheng, G. J., Li, A. M., Richardson, B. J. and Lam, P. K. 2001. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of

marine mussels, Pernairidis. *Aquat. Toxicol.,* **52**: 189-203.

5) Crowe, K. M., Newton, J. C, Kaltenboeck, B. and Johnson, C. 2014. Oxidative stress responses of gulf killifish exposed to hydrocarbons from the Deepwater Horizon oil spill: Potential implications for aquatic food resources. *Environ. Toxicol. Chem.,* **33**: 370-374.

6) Todorcević, M., Skugor, S. and Ruyter, B. 2010. Alterations in oxidative stress status modulate terminal differentiation in Atlantic salmon adipocytes cultivated in media rich in n-3 fatty acids. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.,* **156**: 309-318.

7) Dondero, F., Dagnino, A., Jonsson, H., Capri, F., Gastaldi, L. and Viarengo, A. 2006. Assessing the occurrence of a stress syndrome in mussels (Mytilus edulis) using a combined biomarker/ gene expression approach. *Aquat. Toxicol.,* **78**: S13-S24.

8) Machala, M., Drabeck, P., Neca, J., Kolarova, J. and Svobodova, Z. 1998. Biochemical markers for differentiation of exposures to non planar polychlorinated biphenyls, organochlorine pesticides, or 2,3,7,8-tetrachlorodibenzo-p-dioxin in trout liver. *Ecotoxicol. Environ. Saf.,* **41**: 107-111.

9) Nishimoto, M., Eberhart, B. T., Sanborn, H. R., Krone, C., Varanasi, U. and Stein, J. E. 1995. Effects of a complex mixture of chemical contaminants on hepatic glutathione, L-cysteine and g-glutamylcysteine synthetase in English sole (Pleuronectes etulus). *Environ. Toxicol. Chem.,* **14**: 461-469.

10) Oliva, M., Gonza´lez, M. L., Canales, A., Gravato, C., Guilhermino, L. and Perales, J. A. 2010. Biochemical effects and polycyclic aromatic hydrocarbons (PAHs) in senegal sole (Soleasenegalensis) from a Huelva estuary (SWSpain). *Ecotoxicol. Environ. Saf.,* **73**: 1842-1851.

11) Oliveira, M., Pacheco, M. and Santos, M. A. 2008. Organ specific antioxidant responses in golden grey mullet (Liza aurata) following a short-term exposure to phenanthrene. *Sci. Total Environ.,* **396**: 70-78.

12) Parsons, T. R., Maita, Y. and Lalli, C. M. 1984. *A manual of chemical and biological methods for seawater analysis.* Pergamon Press, Oxford.

13) Regoli, F., Principato, G. B., Bertoli, E., Nigro, M. and Orlando, E. 1997. Biochemical characterization of the antioxidant system in the scallop Adamussium colbecki, a sentinel organism for monitoring the Antarctic environment. *Polar Biol.,* **17**: 251-258

14) Reynaud, S. and Deschaux, P. 2006. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: a review. *Aquat. Toxicol.,* **77**: 229-238.

15) Ricciardi, F., Binelli, A. and Provini, A. 2006. Use of two biomarkers (CYP450 and acetyl choline esterase) in zebra mussel for the biomonitoring of Lake Maggiore (northern Italy). *Ecotoxicol. Environ. Saf.,* **63**: 406-412.

16) Sarkar, A., Ray, D., Shrivastava, A. N. and Sarker, S. 2006. Molecular biomarkers: their significance and application in marine pollution monitoring. *Ecotoxicol.,* **15**: 333-340.

17) Song, B. J., Akbar, M., Abdelmegeed, M. A., Byun, K., Lee, B., Yoon, S. K. and Hardwick, J. P. 2014. Mitochondrial dysfunction and tissue injury by alcohol, high fat, nonalcoholic substances and pathological conditions through post-translational protein modifications. *Redox Biol.,* **29**: 109-123.

18) Tanabe, S., Tatsukawa, R. and Phillips, D. J. 1987. Mussels as bioindicators of PCB pollution: a case study on uptake and release of PCB isomers and congeners in green-lippedmussels (Perna iridis) in Hong Kong waters. *Environ. Pollut.,* **47**: 41- 62.

19) Vicente-Martorell, J. J., Galindo-Riano, M. D., Garcıa-Vargas, M. and Castro-Granado, D. 2009. Bioavailability of heavy metals monitoring water, sediments and fish species from a polluted indestuary. *J. Hazard. Mater.,* **162**: 823-836.

20) Vieira, L. R., Sousa, A., Frasco, M. F., Lima, I.,

Morgado, F. and Guilhermino, L. 2008. Acute effects of Benzo (a) pyrene, anthracene and a fuel oil on biomarkers of the common goby Pomatoschistus microps (*Teleostei, Gobiidae*). *Sci. Total Environ.,* **395**: 87-100.

21) Wang, B., Feng, L., Jiang, W. D., Wu, P., Kuang, S. Y., Jiang, J., Tang, L., Tang, W. N., Zhang, Y. A., Liu, Y. and Zhou, X. Q. 2014. Copper-induced tight junction mRNA expression changes, apoptosis and antioxidant responses via NF- κ B, TOR and Nrf2 signaling molecules in the gills of fish: Preventive role of arginine. *Aquat. Toxicol.,* **6**: 125-137.

22) Woo, S., Lee, A., Denis, V., Chen, C. A. and Yum, S. 2014. Transcript response of soft coral (Scleronephthya gracillimum) on exposure to polycyclic aromatic hydrocarbons. *Environ. Sci. Pollut. Res.,* **21**: 901-910.

23) Young, P. C. and Martin, R. B. 1982. Evidence for protogynous hermaphroditism in some lethrinid fishes. *J. Fish Biol.,* **21**: 475-484