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

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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 *Lethrinus 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, *Lethrinus nebulosus*, Total petroleum hydrocarbons.

Introduction

Petroleum hydrocarbons are chemical compounds which induce persistent environmental contaminants, especially in aquatic environments¹⁴, including fish³. Lethrinidae family was selected for the present study, because they are distributed in tropical and subtropical

Indo-Pacific area²³. 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 organisms⁵. ROS are responsible for DNA damage, apoptosis, protein degradation and lipid peroxidation in

vertebrates²²⁾. 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 stresses⁶⁾. These bioindicators can act as signs to pollutant exposure indicating the exposure levels and the magnitude of the organism's response to the toxic substances⁷⁾.

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 pollutants¹⁵⁾. Furthermore; GR and GPx are usually used to assess the oxidative stress in biological system¹³⁾. 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 studied sites during mid of March 2014 for extraction and determination of TPHs in accordance with the established procedures¹²⁾. Fish sampling, ten fish of a similar size of *Lethrinus nebulosus* were collected from each studied 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, EF206801 and 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'	Amplicon
CAT	TCCTGAATGAGGAGGAGCGA	ATCTTAGATGAGGCGGTGATG	232
SOD	GGTGCCCTGGAGCCCTA	ATGCGAAGTCTTCCACTGTC	377
GPx	CCAAGAGAACTGCAAGAACGA	CAGGACACGTCATTCCTACAC	180
GR	CATTACCGAGACGCGGAGTT	CAGTTGGCTCAGGATCATTTGT	420
GST	TAATGGGAGAGGGAAGATGG	CTCTGCGATGTAATTCAGGA	640
β actin	CAATGAGAGGTTCCGTTGC	AGGATTCCATACCAAGGAAGG	280

The SPSS version 20, Statistical packages (IBM, New York, NY, USA) was used for statistical 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 ($\mu\text{g.L}^{-1}$) 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 $\pm$ 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 (C_t) values. The mRNA expression levels were calculated relative to β -actin gene mRNA levels using the $2^{-\Delta\Delta C_t}$ 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 sources⁴). 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

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

petromine refinery at Jeddah (10.49 folds higher than the reference area) (Table 2 and 3).

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 livings¹). 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 respectively¹⁶). 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 *Lethrinus 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 only⁹). 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 peroxidation²⁰) (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 peroxidation¹⁷). The high levels of lipid peroxidation products induced GPx activity²¹). This increase in GSH utilization, producing oxidized glutathione (GSSG) and induced GR activity for reduced

equivalents and normal redox homeostasis in the living cells¹⁰). 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 studies¹⁹), but others reported no significant alterations¹¹), or decreased activity¹⁹). The effect of chemicals on GSTs activities in living fish was yielded conflicting results⁸). 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.

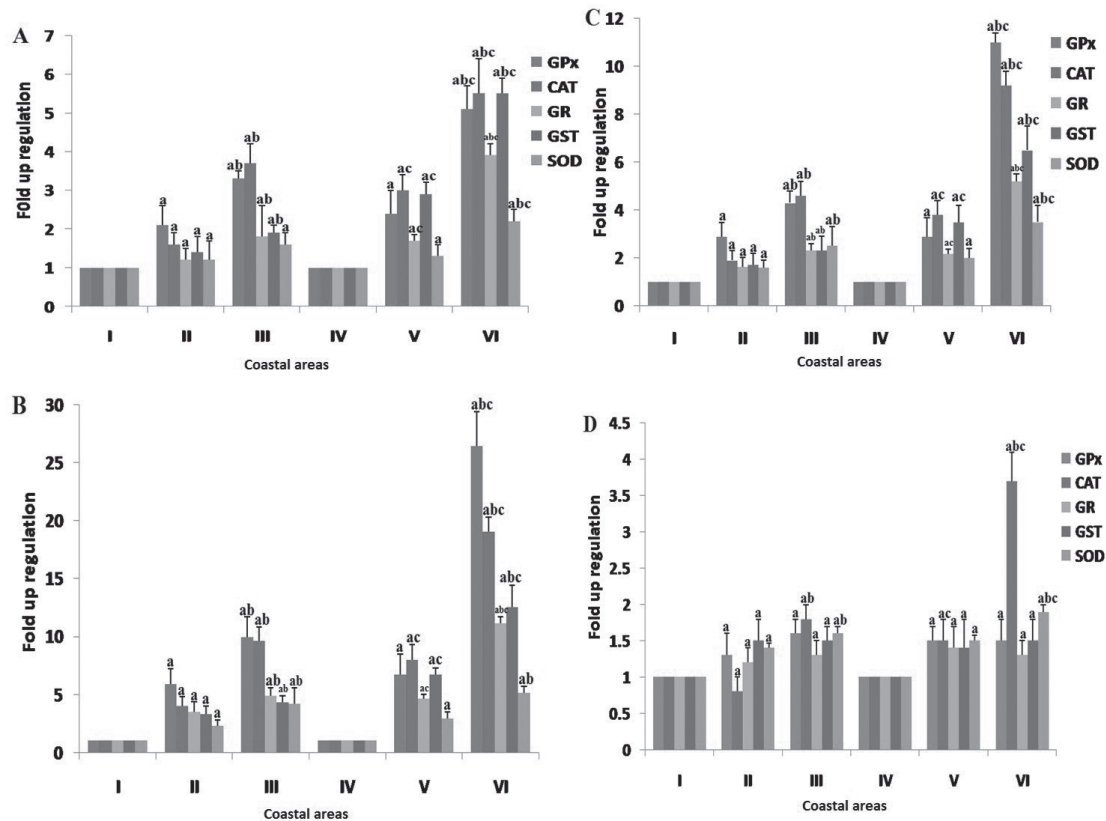


Fig. 1 Antioxidant genes expression (fold of up regulation versus to the reference areas) in skin (A), Gills (B), Liver (C) and Muscle (D).

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 $\pm$ 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.

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