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Oxidative stress response in the skin mucus layer of *Goodea gracilis* (Hubbs and Turner, 1939) exposed to crude oil: A non-invasive approach☆,☆☆

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ABSTRACT

The skin of the fish is the foremost target of oxidative stress due to the generation of Reactive Oxygen Species (ROS) originated in the environment and in the skin itself. In this study, a non-destructive assay was developed to evaluate the effects of crude oil (0.0001–0.1 mg/L, 96 h) on oxidative stress response in the Skin Mucus Layer (SML) of the dusky splitfin goodeid (*Goodea gracilis*). The response in the SML was compared with recognized target organs through the Integrated Biomarker Response (IBR*v*2) and a slight addition to the method was proposed. Crude oil was extremely toxic and elicited a clear induction of ROS in the SML, as in the brain, liver and muscle. By the exposure to crude, a significant change in the activities of Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx) as well as on lipid peroxidation (TBARS) and carbonyl protein (RC═O) levels was detected. Also, increases in the activity of EROD were found. The general IBR*v*2 proposed in this study (gIBR*v*2) showed that oil causes the higher oxidative response in the SML (60.049) under different concentrations of petroleum, which was greater in the brain (56.749), muscle (56.561) and liver (55.775). The results of the study revealed an organ-specific antioxidant defense response that was dependent on the load of petroleum. These results contributed to the understanding of the complexity of oxidative stress response in fish exposed to crude oil using the Skin Mucus Layer as a target for environmental monitoring studies.

1. Introduction

Crude oil (CO) is a complex mixture of olefin and paraffin hydrocarbons (alkanes and alkenes), mono-aromatic hydrocarbons (benzene, toluene, and xylene), phenols and heterocyclic compounds, containing nitrogen and sulfur (Saeed and Al-Mutairi, 1999). Also, heavy metals are present (Azeez et al., 2013). Crude oil is among the most important and abundant classes of pollutants found in aquatic environments. The accumulation of insoluble fractions of CO in fish species is extremely rapid by contact with the surfaces of animals, including the skin and the gills (Pacheco and Santos, 2001; Gravato and Santos, 2002; Barath et al., 2010). The oxidative stress response biomarkers are considered as suitable because they have shown both a dose- and time-dependent responses after an oil contamination, especially one related to Polycyclic Aromatic Hydrocarbons (PAHs) contained in the CO (Hannam et al., 2010; Nogueira et al., 2011; Milinkovitch et al., 2011). Between multiple toxic effects elicited by the CO, oxidative stress by an imbalance between pro-antioxidants and antioxidants prevails (Di Toro et al., 2001; Gravato and Santos, 2002; Simonato et al., 2008; Olsvik et al., 2010; Azeez et al., 2013). Some studies have shown toxic effects exerted by exposure to petroleum compounds on the liver, muscle, gills, brain and blood (Simonato et al., 2008; Oliveira et al., 2008; Duarte et al., 2010; Holth et al., 2011; Bui et al., 2012; Kumari et al., 2014). However, the skin is probably a major biological target of oxidative stress, due to its extension, by the generation of Reactive Oxygen Species (ROS), both in the environment, through chemical reactions and by the normal function of the skin (Trouba et al., 2002; Dzul-Caamal et al., 2013). In fish, the Skin Mucus Layer (SML) is a complex secretion composed of diverse substances such as cytokines (Lindenstrom et al., 2003), peptides, lysozyme, lipoprotein, molecules of the complement (Fernandes et al., 2004), lectins, proteases and antibodies (LaFrentz et al., 2002). Some components have a

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defensive purpose, whereas, others may act to modulate the organization and properties of the SML (Thornton and Sheehan, 2004). Thus, the SML in fish species may serve as a biological target for monitoring their physiological health status and for detecting alterations elicited by toxicants. Particularly, it is useful in monitoring endemic and endangered fish species without sacrificing them (Maltais and Roy, 2014; Dzul-Caamal et al., 2016).

The dusky splitfin (*Goodea gracilis* Hubbs and Turner, 1939) is a Mexican fish species included in the (International Union for Conservation of Nature) IUCN Red List as vulnerable (IUCN Red List Status Ref. 84930). This species, belonging to the family Goodeidae, is a filter feeder, live-bearing, demersal, freshwater fish species that inhabits temperate climates and usually lives in neutral or alkaline water in the center of Mexico. However, the natural habitats of this fish species have been impacted by different types of toxicants. In particular, the crude oil spills are estimated at, at least, 37 million barrels between 2000 and 2013 in the natural distribution areas of this fish species (http://cnh.gob.mx/_docs/derrames_fugas/Reporte_de_volumen_de_petroleo_crudo_derram ado_y_fugas_de_gas_natural.pdf.4). In addition, it is important to bear in mind that in the environment there are a lot of interactions among the water physiochemical properties and the xenobiotics able to modify the toxic effects exerted by a particular type of chemical compounds (Sweetman et al., 2005; Noyes et al., 2009). By these reasons, it is advisable to perform experiments under controlled conditions with the aim of reaching the first approximation about the toxic effects in aquatic organism, particularly in endemic fish species exposed to certain pollutants. In this regard, the dusky splitfin is useful as a study model in ecotoxicology (Dzul-Caamal et al., 2013; Olivares-Rubio et al., 2013). To the best of our knowledge, there are no previous studies about the toxic effects of acute exposure to crude oil in the SML in *G. gracilis*. Therefore, the aim of this study is to evaluate the pro-oxidant forces (O_2• and H_2O_2), oxidative stress biomarkers (TBARS and RC═O), activity of antioxidant defenses (SOD, CAT and GPx) and CYP 1A1 activity in the SML, liver, brain and muscle of *G. gracilis* exposed to crude oil for 96 h under controlled conditions. This approach was performed to probe the viability of SML as a sensitive tissue of fish for environmental monitoring.

2. Materials and methods

2.1. Experimental design

2.1.1. Experimental animals

For the test, specimens of the dusky splitfin (*G. gracilis*) born in the laboratory were used. The specimens were maintained in semi-synthetic water (0.22 g of $MgSO_4$, 0.18 g of $NaHCO_3$, 0.08 g of KCl and 0.13 g $CaSO_{4.2}H_2O$ per liter) with constant aeration, under natural photoperiod and temperature of the laboratory (18–24 °C). Every two weeks (fortnightly), 25% to 50% of the total volume of the water is renewed. The fish were fed ad libitum with pellets or with *Daphnia pulex* daily until the beginning of the experiments.

Sub-adult fish of about 48.23 ± 0.47 mm and 0.91 ± 0.03 g in non-reproductive state were used and were handled in accordance with the protocols for the Mexican Production, Protection and Welfare of Animals for Experimentation (SAGARPA, 2001).

2.1.2. Chemicals

All chemicals were purchased from Sigma-Aldrich®. The crude oil (CO) (Mayan crude petroleum) was obtained from the Mexican Petroleum Institute (IMP), a public center dedicated to basic and applied scientific research, the development of technologies applicable to the petroleum industry and the formation of specialized human resources. They also helped procure the support of the Secretary of Energy (SENER), Mexico.

2.1.3. Experimental treatments

2.1.3.1. Animal exposure. The fishes were not fed one day before and during the exposure in order to reduce fecal and food contamination. After acclimation, the dusky splitfin were subjected to toxicity tests. Even though we utilized fish born in the laboratory, it is recommended to use a reduced number of specimens due to the conservation status of this fish species and because the wild population of this species has been reduced (Dzul-Caamal et al., 2012). Six fishes per treatment were exposed and randomly distributed in 20 L glass container with 15 L dechlorinated (at a temperature of 24 ± 1 °C) and aerated semi-hard synthetic water were used to conduct the exposure tests. The nominal concentrations of CO dissolved in absolute ethanol were 0.0001, 0.001, 0.01 and 0.1 mg/L. The fishes were acutely exposed for 96 h (hours), while the absolute control group was exposed to clean semi-hard synthetic water in addition to the solvent control group (semi-hard water with ethanol at 0.001% of total volume). To guarantee the consistence of the experimental data, all the bio-assays and determination of oxidative stress response biomarkers, including both enzymatic and non-enzymatic parameters of crude oil, were conducted in triplicate.

2.1.4. Obtaining the Skin Mucus Layer (SML) and tissue samples

After exposure, the weight (g) and standard length (mm) were evaluated. The SML was collected from 36 fishes as described by Dzul-Caamal et al. (2013). The mucus was removed carefully from the dorsal surface of the body with a plastic spatula and subsequently placed in Phosphate Buffer Solution (PBS1×) to a volume of 750 μL. The mucus of the ventral skin was not collected to avoid intestinal contamination or damage. After that, the fish were euthanized by fast freezing using ice, followed by dry ice. Necropsy was done immediately to obtain the liver, brain and muscle (weighed to within 0.1 mg) and it was homogenized 1:5 (w/v) with Phosphate Buffer Solution (PBS1×) using Teflon micropestles. Both the SML and tissue sample homogenates were divided into two portions; one was centrifuged at 9000 *g* and 4 °C for 15 min in a Hermle Labnet Z216MK centrifuge to obtain the S9 fraction. The uncentrifuged portion and the S9 fraction were stored at −70 °C until the biomarker assay (less than two weeks). The S9 fraction was used for ROS quantification as well as for the enzymatic assays and the uncentrifuged fraction for the evaluation of oxidative damage biomarkers.

2.2. Evaluation of biomarkers

2.2.1. Pro-oxidant (O_2• and H_2O_2) forces

Quantification of ROS (O_2• and H_2O_2): This measurement was performed as in previous reports with 20 μL of S9 fraction of both the SML and tissue samples, using a final concentration of 7.0 μmol of DHE and DHF-DA according to previous reports (Dzul-Caamal et al., 2013). The concentration of H_2O_2 was calculated using a calibration curve from 0 to 7.0 mol. For O_2•, a molar extinction coefficient of 4669 mol^{-1} cm^{-1} was used for its quantification considering a light path of 0.67 cm in 96-well plates with 200 μL of final volume. Results were expressed as mol/g fish.

2.2.2. Oxidative stress biomarkers (RC═O, TBARS)

The lipid peroxidation evaluated as TBARS was assessed in the uncentrifuged fractions of the SML and tissues using the method of Buege and Aust (1978). The results of lipid peroxidation were expressed as Thiobarbituric Acid Reactive Substances (TBARS), with a molar extinction coefficient of 156,000 mol^{-1} cm^{-1}, and presented as μmol TBARS/g fish. The oxidation of proteins was evaluated by the method of Levine et al. (1994) which is based on the reaction of carbonyl groups of oxidized proteins with 2,4-dinitrophenyl hydrazine (DNPH) in an acid medium to generate dinitrophenyl hydrazones with maximum absorbance of 366 nm. The concentration of protein carbonyls (RC═O) was

estimated with the molar extinction coefficient of 22 $mmol^{-1}\ cm^{-1}$ and is reported as μmol RC═O/mg protein/g fish.

2.2.3. Antioxidant defenses: SOD, CAT and GPx

The activity of three antioxidant defense enzymes was evaluated in S9 fraction of the SML and tissues. SOD (EC 1.15.1.1) activity was calculated using a standard curve, which was obtained from the activity of SOD 3.73–18.65 U of pure bovine erythrocytes (Sigma). The results were expressed as mmol/min/mg protein/g fish according to the method of Misra and Fridovich (1972). Catalase (CAT; EC 1.11.1.6) was assessed by the method of Radi et al. (1991) and was estimated using the molar extinction coefficient of H_2O_2 (0.043 $mmol^{-1}\ cm^{-1}$). The results obtained were expressed as mmol/min/mg protein/ g fish. The activity of glutathione peroxidase (GPx; EC 1.11.1.9) was evaluated with the method of Lei et al. (1995), using a test coupled with 1 U of glutathione reductase, 0.03 mol NADH, 3.5 mmol GSH and 40 mmol H_2O_2 followed by a reading at 340 nm. Enzyme activity was calculated using the molar extinction coefficient of NADH (6.22 $mmol^{-1}\ cm^{-1}$). The results were expressed as mmol/min/mg protein/g fish. All tests were performed in triplicate.

The total protein content was determined using the Thermo Scientific Pierce® 660 nm Protein Assay.

2.2.4. Activity of CYP 1A1

The activity of cytochrome P450 isoform 1A1 (CYP 1A1; EC 1.14.14.1) was evaluated through the specific metabolism of 7-ethoxy resorufin O-deethylase activity (EROD) according to the method of Parrot et al. (1999) with modifications for microplates. Fluorescence of resorufine was evaluated at 520 nm excitation and 585 nm emission, using a Biotek Synergy Mx spectrofluorometer. Resorufine was calculated by a calibration curve (0.05–0.25 μmol) and results were presented as mmol/min/mg protein/g fish.

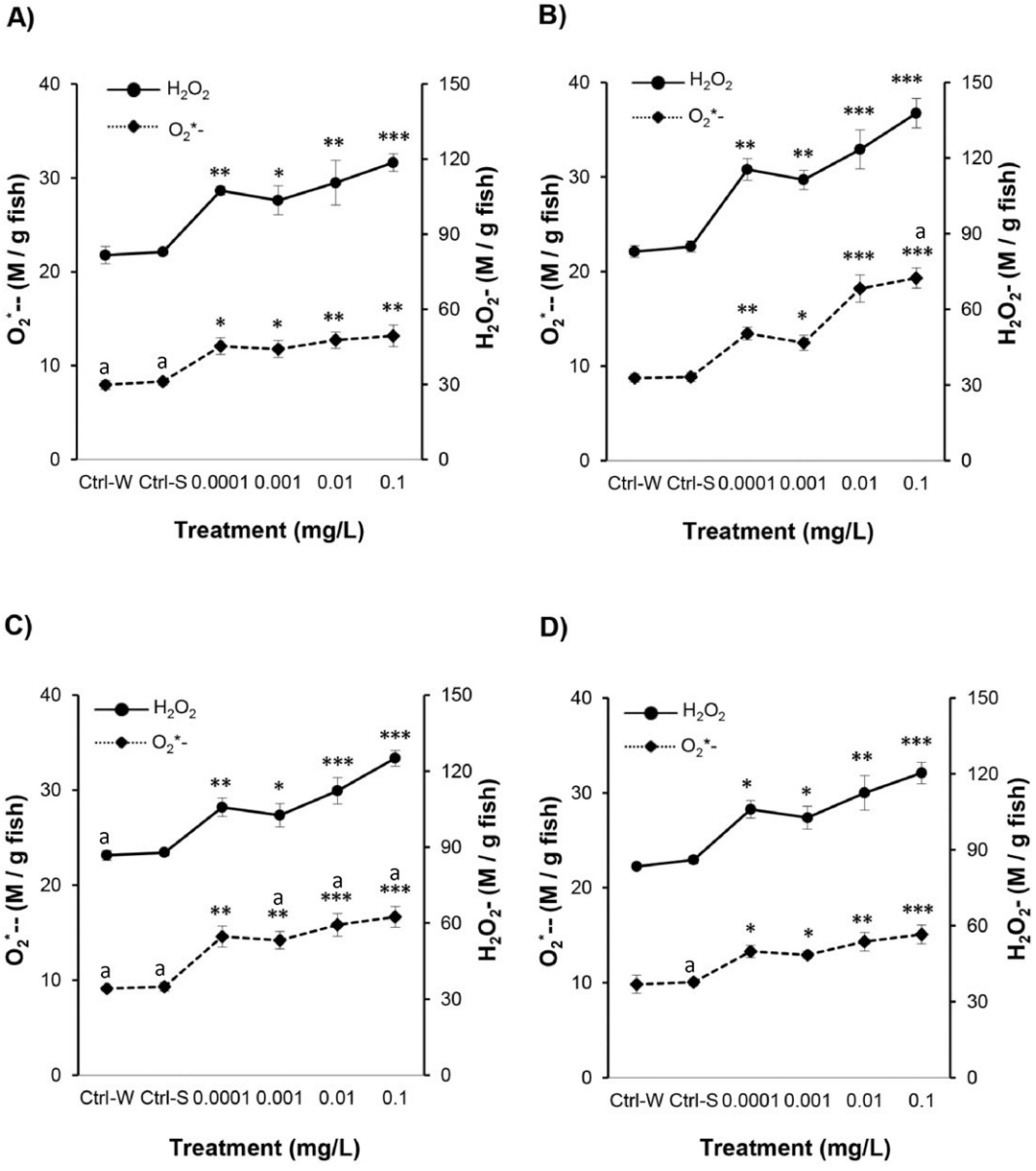

Fig. 1. Induction of Reactive Oxygen Species (ROS) in (A) the Skin Mucus Layer, (B) liver, (C) brain, and (D) muscle of *G. gracilis*, treated with crude oil. The black dotted lines indicate the contents of H_2O_2 and the solid black lines indicate the content of $O_2^{\bullet}$. Data are expressed as mean ± standard deviation. CTRL-W = absolute control (semi-hard water). CTRL-S = solvent control (semi-hard water with ethanol 0.001%). The asterisks denote significant differences compared with the control fish, both water and solvent, * $p \le 0.05$, ** $p \le 0.01$ and *** $p \le 0.001$. The lowercase letters denote statistical differences ($p \le 0.05$) between the SML with respect to other tissues of the fish exposed to the same concentration of crude oil.

All the biomarker responses were normalized per gram of fish because it is not possible to relate some biomarkers such as ROS concentration and TBARS with the amount of total proteins (Dzul-Caamal et al., 2013).

2.3. Statistical

2.3.1. Calculation of the Integrated Biological Response version 2 (IBRv2)

In this study, we used the index named "Integrated Biological Response version 2" (IBR*v*2) developed by Sanchez et al. (2013), which is an algorithmic approach, to compare the specific biomarkers and their relations with environmental variables or under gradients of pollutants. It is helpful in the interpretation of biomarkers to evaluate the integrated biological response, both in the field and under controlled conditions (Olivares-Rubio et al., 2013). In the current study, this index allowed us to compare the response of fish exposed to crude oil with regard to solvent control group. The table was constructed using the values of *A* obtained for the SML and tissue biomarkers, which was then compared with the area of crude oil contents following the criteria of the IBR*v*2 method. We proposed a slight addition to IBR*v*2, named general IBR*v*2, which is calculated as the summation of all IBR*v*2 involved in a particular situation (gIBR$v2 = \Sigma$ IBR$v2\ i = 1$ to $i = n$) to integrate the total effects as a single value. This approach could be useful for environmental assessment, considering the sum of the effects into different scenarios, as well as for estimating the differential responsiveness of the biological targets under controlled conditions. In both cases, Excel software was used.

2.3.2. Statistical analysis

All data were expressed as means ± s.e.m. (standard error of the mean) corresponding to groups and analyzed by the SPSS software (version. 16.0, SPSS Company, Chicago, USA). Tests for normality (Kolmogorov–Smirnov) and homogeneity of variances (Levene) were applied. One way ANOVA following Tukey's test was performed to

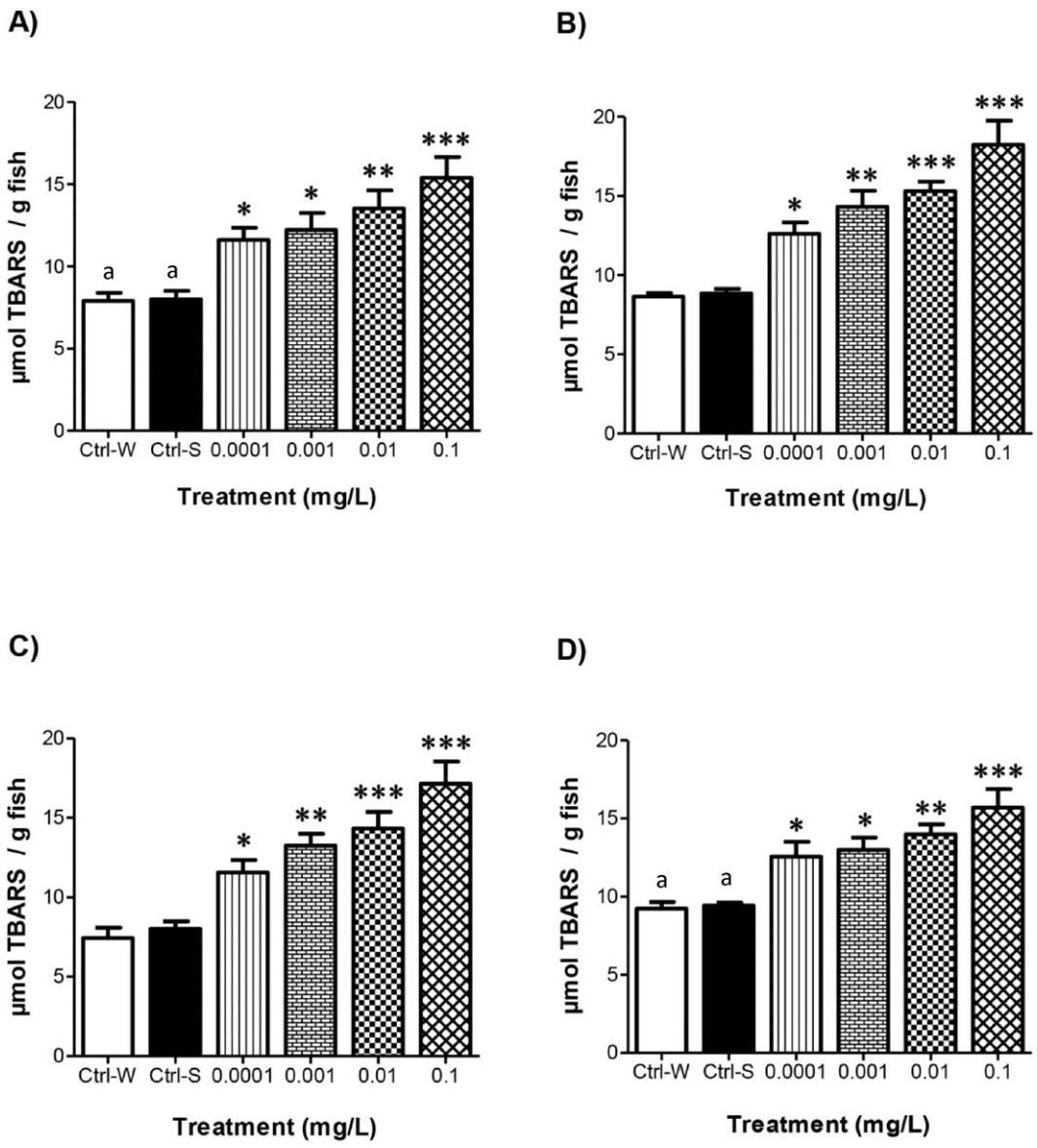

Fig. 2. Levels of lipid peroxidation evaluated as TBARS in (A) the Skin Mucus Layer, (B) liver, (C) brain, and (D) muscle of *G. gracilis*, treated with crude oil. Data are expressed as mean ± standard deviation. CTRL-W = absolute control (semi-hard water). CTRL-S = solvent control (semi-hard water with ethanol 0.001%). The asterisks denote significant differences compared with the control fish, both water and solvent * $p \le 0.05$, ** $p \le 0.01$ and *** $p \le 0.001$. The lowercase letters denote statistical differences ($p \le 0.05$) between the SML with respect to other tissues of the fish exposed to the same concentration of crude oil.

signal the significant differences between the groups and among tissues. The significant difference was set at $p \leq 0.05$ and $p \leq 0.01$, respectively. Correlations between biomarkers evaluated in the SML were estimated using the Pearson's correlation coefficient by the SPSS v.12.0 software. These results were considered significant at $p \leq 0.05$.

3. Results

3.1. Induction of pro-oxidant forces (O_2• and H_2O_2)

The basal levels of O_2• and H_2O_2 of the control fishes were higher in the liver (Fig. 1B), brain (Fig. 1B) and SML (Fig. 1C) than in the muscle (Fig. 1D). In the fishes treated with crude oil above 0.0001 mg/L, a statistical induction of O_2• and H_2O_2 in all targets were observed ($p \leq 0.05$).

3.2. Oxidative stress biomarkers of damage (TBARS and RC═O)

Basal levels of the lipid peroxidation and oxidation of proteins were higher in the liver (Fig. 2B), brain (Fig. 2C) and SML (Fig. 2A) than in the muscle (Fig. 2D). By exposure to CO, a significant increase ($p \leq 0.05$) of lipid peroxidation as TBARS (Fig. 2) and protein oxidation as R═O (Fig. 3) was observed. Both, lipid peroxidation and oxidized proteins presented a concentration-dependent response in the SML as well as in the tissues under study. The higher oxidative damage was observed at 0.1 mg/L.

3.3. Activities of antioxidant enzymes (SOD, CAT and GPx)

The activities of enzymes involved in the antioxidant defense presented differences in their metabolic rate in the different tissues.

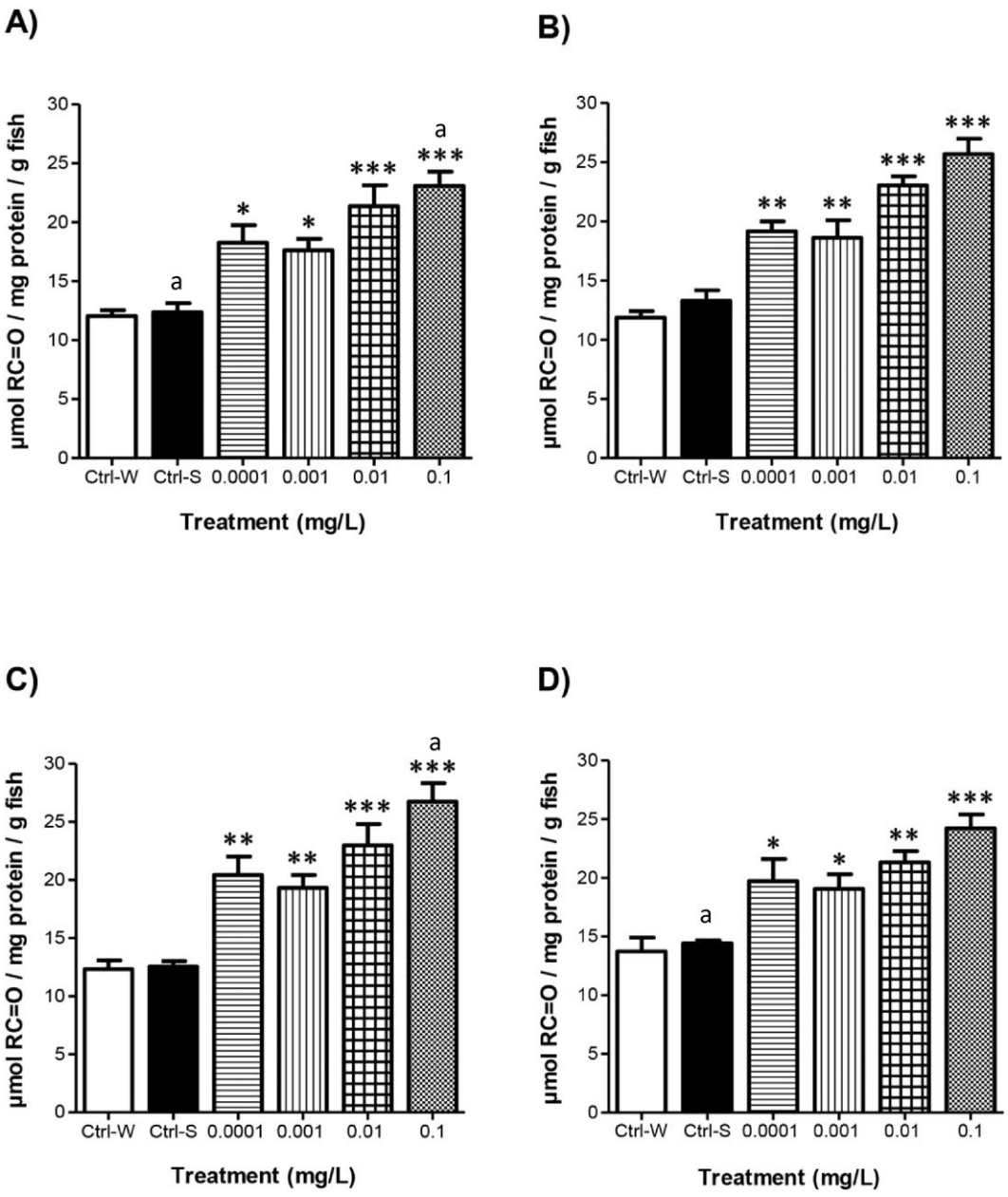

Fig. 3. Levels of oxidized proteins evaluated as RC═O in (A) the Skin Mucus Layer, (B) liver, (C) brain, and (D) muscle of *G. gracilis*, treated with crude oil. Data are expressed as mean ± standard deviation. CTRL-W = absolute control (semi-hard water). CTRL-S = solvent control (semi-hard water with ethanol 0.001%). The asterisks denote significant differences compared with the control fish, both water and solvent * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. The lowercase letters denote statistical differences ($p \leq 0.05$) between the SML with respect to other tissues of the fish exposed to the same concentration of crude oil.

SOD activity was greater in the SML, followed by the brain (Fig. 4). In SML and in all tissues under study, CAT activity was increased compared to control; however, a peak was observed at 0.01 mg/L (Fig. 5). By the effect of CO, the great increase in CAT activity was detected in the brain, followed by the liver and the SML. CAT activity in the muscle was the least responsive to the treatment (Fig. 5D). The hepatic GPx activity was significantly higher in the liver and brain, followed by the SML at 0.001 mg/L, compared to control values (Fig. 6A to C). In muscle, GPx activity was lower than that of the other tissues (Fig. 6D).

3.4. Activity of CYP 1A1

The activity of EROD in the SML, liver, brain and muscle of *G. gracilis* exposed to Maya crude oil was greater than in control groups (absolute and solvent control). The maximum activity of this enzyme was found at 0.001 mg/L in the SML and in the other tissues under study at 0.01 mg/L with high significance. Above these treatments, the metabolism of EROD was slightly reduced (Fig. 7). The activity of EROD on the SML was statistically correlated ($p \leq 0.001$) with O_2• and H_2O_2, with oxidative stress biomarkers (TBARS and RC═O) and with antioxidant defenses (SOD, CAT and GPx) in the SML.

3.5. Integrated biological response version 2 (IBRv2) and regression analyses

Results of IBR*v*2 are presented in Table 1 showing a clear discrimination of the biomarkers according to treatments and tissues. Interestingly, the integrated biological response was greater in the SML than in the brain, muscle and liver of *G. gracilis* exposed to different treatments (Table 1). Positive values of *A* were observed in the levels of O_2• and H_2O_2, which allows an increase in the oxidative damage (TBARS, RC═O). The general IBR*v*2 proposed in this study (gIBR*v*2) showed that crude oil causes the higher oxidative response in the SML (60.049) under different concentrations of petroleum, which was greater in the brain (56.749), muscle (56.561) and liver (55.775).

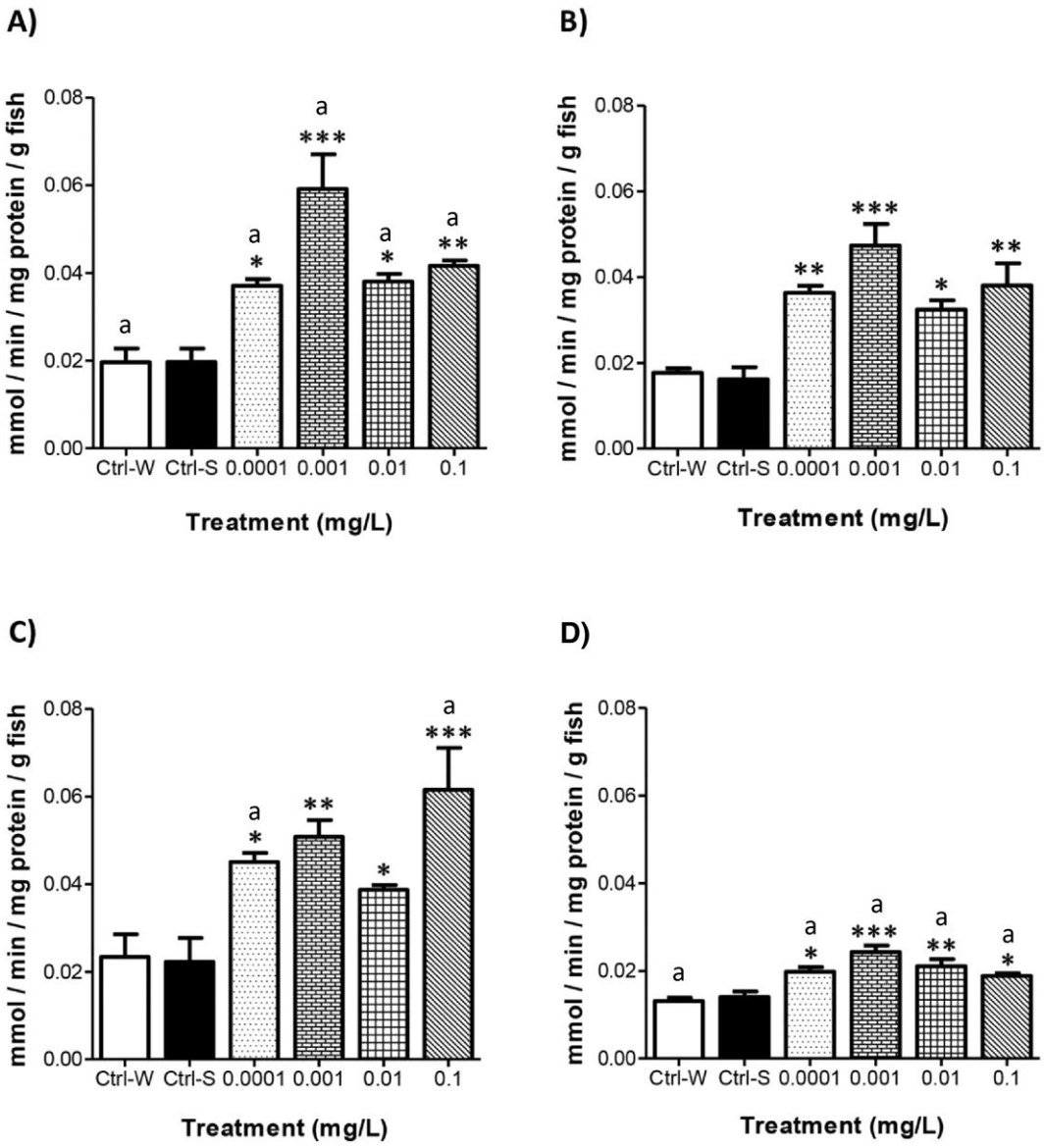

Fig. 4. SOD activity in different organs: (A) the Skin Mucus Layer, (B) liver, (C) brain, and (D) muscle of *G. gracilis*, treated with crude oil. Data are expressed as mean ± standard deviation. CTRL-W = absolute control (semi-hard water). CTRL-S = solvent control (semi-hard water with ethanol 0.001%). The asterisks denote significant differences compared with the control fish, both water and solvent * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. The lowercase letters denote statistical differences ($p \leq 0.05$) between the SML with respect to other tissues of the fish exposed to the same concentration of crude oil.

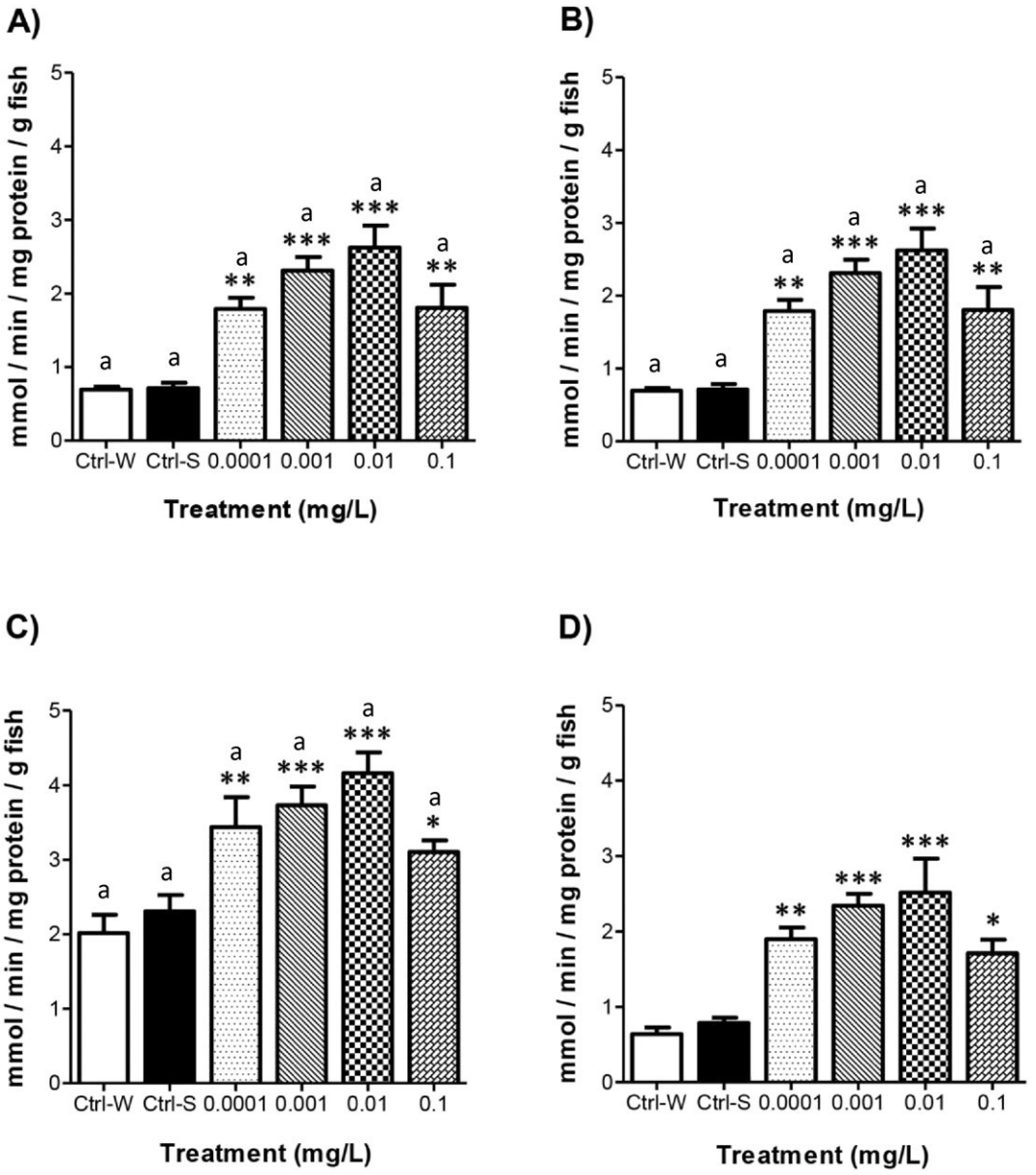

Fig. 5. CAT activity in different organs: (A) Skin Mucus Layer, (B) liver, (C) brain, and (D) muscle of *G. gracilis*, treated with crude oil. Data are expressed as mean ± standard deviation. CTRL-W = absolute control (semi-hard water). CTRL-S = solvent control (semi-hard water with ethanol 0.001%). The asterisks denote significant differences compared with the control fish, both water and solvent * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. The lowercase letters denote statistical differences ($p \leq 0.05$) between the SML with respect to other tissues of the fish exposed to the same concentration of crude oil.

4. Discussion

Most crude oil-treated groups of *G. gracilis* in this study showed significantly altered biochemical responses after a short-term exposure to crude oil (CO), indicating the oxidative stress-inducing potential of the Mayan petroleum. However, the most outstanding finding of this study was the concentration-dependent oxidative stress response observed in the Skin Mucus Layer (SML) of this fish species. Similar results were documented in the SML of the dusky splitfin goodeid exposed under laboratory condition to three halomethanes (Dzul-Caamal et al., 2013). Nevertheless, before a wide recognition about the usefulness of the SML on environmental monitoring studies, the validation of these responses with regard to a reference target organ is needed.

The production of ROS and other free radicals induces oxidative stress, cell death and tissue injury (Vlahogianni et al., 2007; Yin et al., 2007; Eriyamremu et al., 2008). In the case of oxidative stress response elicited by CO, it is important to bear in mind that petroleum contains PAHs, among a lot of components, as well as other products generated by its auto-oxidation (National Renewable Energy Laboratory, 2009). These substances are responsible for triggering ROS generation in fish species that are exposed to this super mix of substances (Achuba and Osakwe, 2003; Lushchak, 2011). Vega-López et al. (2009a) reported that in the presence of molecular oxygen, O_2• can be generated by decoupling the flow of electrons during the redox processes of the cytochrome P450 isoenzymes. In addition to the biotransformation process of CO, the fish skin is probably a major target of the oxidative stress due to Reactive Oxygen Species (ROS), originating both in the environment, such as in the skin itself (Trouba et al., 2002; Dzul-Caamal et al., 2016). The levels of ROS and oxidative damage (lipid peroxidation and oxidation of proteins) detected in the SML of the dusky splitfin goodeid suggest that the CO could potentially induce structural and functional impairments of the mucus composition and functions. Although, many studies are necessary to evaluate the total impact related to mucus dysfunction and to the skin, a recent study by our team shows that

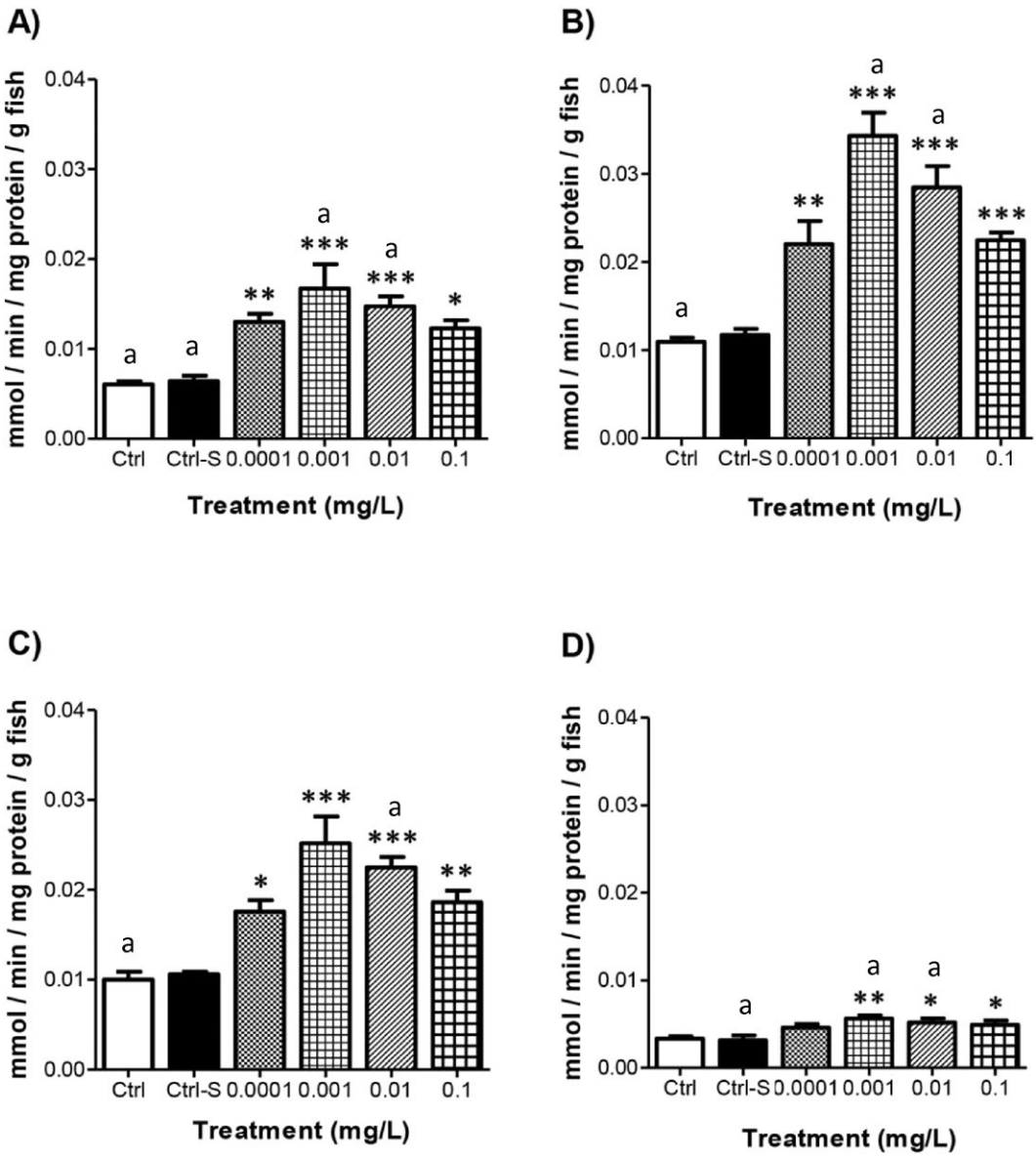

Fig. 6. GPx activity in different organs: (A) the Skin Mucus Layer, (B) liver, (C) brain, and (D) muscle of *G. gracilis*, treated with crude oil. Data are expressed as mean ± standard deviation. CTRL-W = absolute control (semi-hard water). CTRL-S = solvent control (semi-hard water with ethanol 0.001%). The asterisks denote significant differences compared with the control fish, both water and solvent, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. The lowercase letters denote statistical differences ($p \leq 0.05$) between the SML with respect to other tissues of the fish exposed to the same concentration of crude oil.

the fish skin of the wild *Girardinichthys viviparus* is an active site involved in bio activation of compounds such as PAHs and total phenols (Dzul-Caamal et al., 2016). In addition to the oxidative stress, it is the widely recognized impairment of the immune function of fish species. In this regard, the protective role of the mucosa and its components in the immune system of some fish species was documented (Palaksha et al., 2008). However, other studies are required to demonstrate the probable dysfunction of the skin immune response that CO entails as the alteration of the skin mucus production elicited by CO.

The oxidation of polyunsaturated fatty acids on the cellular membrane is a process related with cellular dysfuntions and with generation of highly active metabolites that are able to react with some biomolecules, such as the case of the DNA (Cossu et al., 1997; van der Oost et al., 2003; Simonato et al., 2008; Circu and Aw, 2010; Nogueira et al., 2011; Otitoloju and Olagoke, 2011). However, its damage depends on the nature and concentration of the oxidant, fluctuating from the local reductions in membrane fluidity to full disruption of bi-layer integrity (Hermes-Lima, 2004). The results of this study showed a concentration-dependent response attended by a high lipid peroxidation on the SML, which was similar to those observed in the liver of the dusky splitfin goodeid. This finding demonstrates the usefulness of the SML for evaluation of toxic effect provoked by CO with regard to the liver. Notwithstanding, a preceding report about the effects of CO in the SML lipid peroxidation does not exist; current results suggest that the alterations in this target could be associated with both the integrity and the function of the skin. Lipid peroxidation on the endoplasmic reticulum, as in the mitochondrial membranes, may cause an uncontrolled Ca^{2+} efflux, alternating some cellular functions. The released calcium could be involved in the activation or de-activation of some enzymes and biomolecules (Hermes-Lima, 2004). In this regard, it is possible to have an impairment of the cytokines, lysozyme, lipoprotein, molecules of the complement, lectins, proteases and antibodies involved in the skin immune system of the fish (LaFrentz et al., 2002; Lindenstrom et al., 2003; Fernandes et al., 2004). In addition to uncontrolled activation of Ca^{2+}-dependent proteases, phospholipases, and endonucleases are able to degrade enzymes, membranes, and DNA as

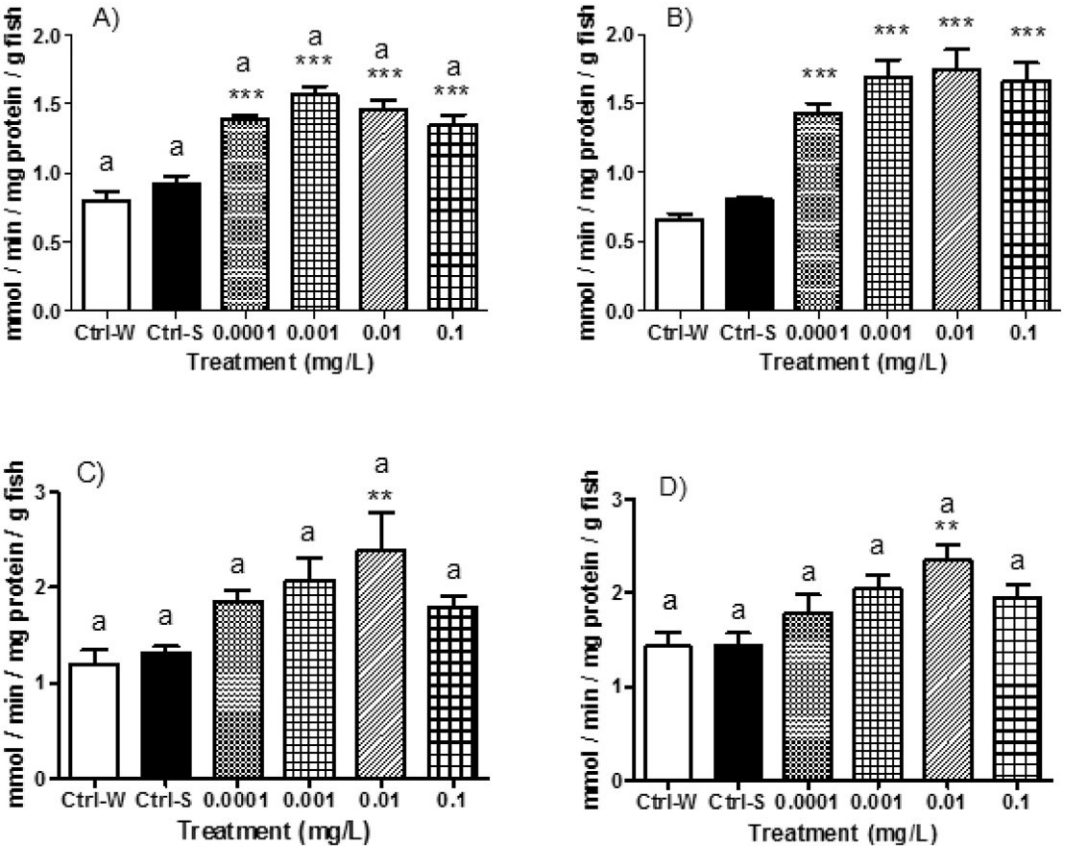

Fig. 7. EROD activity in different organs: (A) the Skin Mucus Layer, (B) liver, (C) brain, and (D) muscle of *G. gracilis*, treated with crude oil. Data are expressed as mean ± standard deviation. CTRL-W = absolute control (semi-hard water). CTRL-S = solvent control (semi-hard water with ethanol 0.001%). The asterisks denote significant differences compared with the control fish, both water and solvent, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. The lowercase letters denote statistical differences ($p \leq 0.05$) between the SML with respect to other tissues of the fish exposed to the same concentration of crude oil.

well as activate the nitric oxide synthase (Hermes-Lima, 2004), which in the present study may probably impact the fish skin. Moreover, the calcium signaling may cause the cellular death by apoptosis (Lang et al., 2007). Many studies are required to clarify the toxic effects of CO on the fish skin.

A similar trend that was observed on lipid peroxidation was found on oxidized proteins evaluated as RC═O, which was greater in the SML as in the liver and brain of the dusky splitfin goodeid treated with CO. Related response in the SML of *G. gracilis* exposed to disinfection by-products (Cl_2CH_2, $BrCl_2CH$ and Cl_3CH) was documented (Dzul-Caamal et al., 2013). In this study, the oxidative damage in proteins could be a result of free-radical mediated mechanisms. Terminal products of lipid peroxidation as malondialdehyde, hexanal, 4-hydroxy-2, 3-trans-nonenal, 4-hydroxy-2, 3-trans-hexenal, and acrolein; in addition to sugar oxidation products, metabolites nitric-oxide derived and metal-catalyzed oxidation systems are responsible for the oxidation of proteins (Hermes-Lima, 2004).

However, it is important to bear in mind that the oxidation of proteins as a natural process occurred under aerobic conditions. About 10% of cellular proteins have a carbonyl group in their structures, in addition to cellular mechanisms involved in both repairing the oxidized protein and providing protection against its damage (Stadtman and Levine, 2000). It has been considered that a rate of about two- to three-fold higher in a carbonyl protein level could be associated with dysfunctional proteins, contributing to cell damage and death (Hermes-Lima, 2004). Taking into account this information, the CO could induce an irreversible damage in the structure and functions of the proteins of the SML, as in the liver, brain and muscle of the dusky splitfin goodeid.

As a consequence of ROS induction and oxidative damage on PUFAs of the dusky splitfin goodeid, specific antioxidant enzymes were induced. In this study, it was found that the activity of enzymes involved in antioxidant defense in the SML was greater regarding to other tissues under study. In addition, different patterns of responses in these enzymes are due to the high dynamism of ROS and oxidative stress (Lushchak, 2011). In the dusky splitfin goodeid exposed to the greater CO concentration, the brain activity of SOD reached a peak at 0.1 mg/L. This finding was in agreement with diminution of CAT and GPx activities as was observed in the brain of the freshwater fish *Heteropneustes fossilis* exposed to cadmium (Radhakrishnan, 2008). Although SOD and CAT metabolize different ROS, the diminution of CAT and GPx activities in the brain of *G. gracilis* seems to indicate an imbalance in the antioxidant defenses in this fish species and the influence of other factors still not identified as observed in the liver of *Ameca splendens* exposed to PCBs (Vega-López et al., 2009b). In this regard, Kitlar et al. (1994) reported that mammalian CAT has a specific binding site for NADPH that protects this enzyme from attack by H_2O_2. Likely, the abolishment of the activity of CAT in the brain of dusky splitfin goodeid exposed to 0.1 mg CO/L is due to oxidative damage on the binding site of this enzyme preventing NADPH protection. However, contradictory reports have been documented on the sturgeon (*Acipenser naccarii*) and on the trout (*Oncorhynchus mykiss*), where basal activities of SOD, CAT and GPx on the skin were lower than in the gills, heart, digestive tract, liver, white muscle, red blood cells and swim bladder (Trenzado et al., 2006). Despite toxic effects elicited by CO, the differences about the basal values between the activities of the antioxidant enzymes in the SML of the dusky splitfin goodeid and these fish species probably are explained by water temperature in their habitats. *G. gracilis* typically

Table 1
"A" values of integrated biological response version 2 (IBR*v*2) on the skin mucus layer (SML), liver, brand and muscle of *G. gracilis* exposed to crude oil.

Treatments (mg/L)	Biomarkers	SML	Liver	Brain	Muscle
0.0001	O_2^*	1.509	1.506	0.847	1.396
	H_2O_2	3.035	2.898	3.584	2.649
	TBARS	1.508	1.738	1.333	1.327
	RC═O	1.621	1.874	0.720	1.392
	SOD	2.274	2.685	3.483	1.780
	CAT	2.008	1.058	0.576	2.487
	GPX	3.063	2.163	2.485	2.578
	CYP 1A1	0.428	0.115	0.678	0.847
	IBR*v*2	**15.446**	**14.035**	**13.703**	**14.456**
0.001	O_2^*	1.684	1.789	1.027	1.525
	H_2O_2	3.022	3.032	3.787	2.797
	TBARS	1.472	1.270	0.426	1.170
	RC═O	1.845	1.983	1.090	1.548
	SOD	2.928	3.664	4.287	2.713
	CAT	1.570	1.631	0.032	3.451
	GPX	3.028	3.805	4.885	3.499
	CYP 1A1	0.260	0.739	0.071	0.234
	IBR*v*2	**15.810**	**17.912**	**15.606**	**16.937**
0.01	O_2^*	1.556	0.385	0.305	1.055
	H_2O_2	2.998	2.651	3.184	2.378
	TBARS	1.231	1.021	0.105	0.832
	RC═O	1.352	1.190	0.064	1.037
	SOD	1.802	2.259	2.463	2.046
	CAT	2.278	1.873	0.696	3.778
	GPX	2.821	3.114	4.129	3.115
	CYP 1A1	0.520	0.841	1.001	0.398
	IBR*v*2	**14.558**	**13.334**	**11.946**	**14.639**
0.1	O_2^*	1.629	0.162	0.042	0.818
	H_2O_2	3.171	2.242	2.460	2.068
	TBARS	0.623	0.371	1.302	0.305
	RC═O	0.971	0.788	1.081	0.451
	SOD	2.477	2.852	5.566	1.541
	CAT	1.850	1.169	1.261	2.014
	GPX	2.413	2.236	2.863	2.876
	CYP 1A1	1.102	0.673	0.919	0.458
	IBR*v*2	**14.235**	**10.494**	**15.494**	**10.529**
gIBR*v*2		**60.049**	**55.775**	**56.749**	**56.561**

Abbreviations: O_2^*, superoxide anion; H_2O_2, hydrogen peroxide; LPOX, lipid peroxidation; RC═O, protein carbonyls; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; CYP 1A1, activity of cytochrome P450 isoform 1A1. Positive values of the concentrations denote induction of the biomarker, and negative represents inhibition of the biomarker regarding the control fish.

inhabits temperate freshwater ecosystem (Jelks et al., 2008); in contrast, the trout and sturgeon inhabit cold water (Page and Burr, 1991; Kottelat and Freyhof, 2007). Notwithstanding, there are no comparative studies about the activities of the enzymes involved in the antioxidant defense in the SML between fish species from temperate or cold water, in some studies, alterations by the effects of temperature in the entire organisms as in the liver have been documented. The activities of CAT, SOD and GPx in the early developmental stages of marble trout (*Salmo marmoratus*) were more dependent on temperature increase than in juveniles (Simčič et al., 2015), leading to the need for more comparative studies about basal values of the antioxidant enzymes on the SML of fish species, as well as exposure to toxicants such as the case of CO.

It is well documented that the isoform 1A1 of cytochrome P450 assessed as EROD activity is a sensitive biomarker in fish for exposure to PAHs and similar chemical compounds (van der Oost et al., 2003; Vega-López et al., 2009a, 2009b). In the current study, we found that the activity of CYP 1A1 was greater in the SML, liver, brain and muscle of treated fish than crude oil with regard to both controls. Recent studies have recorded similar findings in other marine and freshwater fish species exposed to crude oil or its fractions on different tissues than the SML (Deér et al., 2010; Danion et al., 2014; Holth et al., 2014; Frantzen et al., 2015). Interestingly, the activity of the SML of *G. gracilis* was significantly different in brain and muscle but not in the liver. This finding contributes to support the hypothesis that SML is a suitable tissue for health fish monitoring. Moreover, the activity of the CYP1A1 on the SML could be a source of ROS and of the consequent oxidative stress. The gene expression of CYP 1A1 in *Girardinichthys viviparus* and other fish species belonging to the family Goodeidae from the center of Mexico was demonstrated (Dzul-Caamal et al., 2016). In this way, it is feasible that crude oil metabolism carried out in the skin of the dusky splitfin goodeid might be linked with the oxidative stress response observed in the SML as was demonstrated by the Pearson's correlation coefficient.

The findings discussed above support the usefulness of the SML in the evaluation of oxidative stress response in monitoring programs in fish species exposed to CO. Nevertheless, the main advantage of gIBR*v*2 is to reduce the number of individual data obtained from the calculation of IBR*v*2 as a sole data by tissue or environmental condition (when applicable). In this study this strategy was employed to provide integrated and quantitative information about the toxic effects elicited by CO in the SML, with regard to other widely recognized target organs. Marigómez et al. (2013) calculated the IBR and IBI to assess the effect of the Prestige Oil Spill (POS) that occurred in Galicia and the Bay of Biscay with basis in the Mussell Watch monitoring program (2003–2006). The obtained results of the IBR and IBI indicate that mussel health was strongly affected in 2003; however, in the year after, basal values showed an improvement. In this regard, the former authors proposed that IBR and IBI provide complementary information concerning the mechanisms of biological response to environmental insults. Milinkovitch et al. (2013) used the biomarker assessment to compare responses between different treatments of dispersed oil in the juvenile golden grey mullet (*Liza aurata*). The study revealed that exposure to dispersed oil (whether mechanically or chemically dispersed) within 48 h (hours) elicited toxic effects detectable even after 14 days in the depuration period. The comparative biomarker responses assessed in this study suggested that the chemical dispersion induced similar toxic effects with regard to mechanical dispersion. Because of this, it is possible to remark that the natural dispersion under turbulent conditions (for example, waves) could exert the same effect as using a dispersant. Using an experimental design on caged mussels, it has been suggested that an IBR value greater than 10 with regard to the reference site would be linked to toxic effects (Damiens et al., 2007).

Considering our results, it is possible to remark a better responsiveness of the SML, comparing it with traditional target organs due to the greater gIBR*v*2 value which was found on this target (60.049) under different concentrations of petroleum, which was higher in the brain (56.749), muscle (56.561) and liver (55.775) of *G. gracilis* treated with Mayan crude oil. Current results are in agreement with previous reports, where it has been stated that the IBR possesses enough sensitivity to discern the effects of complex mixtures of chemicals present, even at concentrations below or near their detection limits (Brooks et al., 2011).

5. Conclusions

The integration of the biological effects data using the gIBR*v*2 revealed that the Skin Mucus Layer (SML) of *G. gracilis* treated with Mayan crude oil suffered the greater oxidative damage mediated by ROS generation than the liver, brain, and muscle. However, what stands out is the need for a lot of studies aimed at validating the usefulness of non-invasive approach as is the case of the SML for contributing to the protection and avoiding unnecessary sacrifice of fish, particularly, those endemic or endangered species.

Conflict of interest

None.

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