Turkish Journal of Biology

Volume 36 | Number 5 Article 2

1-1-2012

Biomarkers of petroleum contamination in the digestive gland of Perna viridis (L.)

XIVANAND NARAYAN VERLECAR

KARMABEER JENA

SOMSHEKHER RAGHOBA DESAI

PRIYA BRATA DAS

GAGAN BIHARI NITYANANDA CHAINY

TÜBİTAK

Turk J Biol
36 (2012) 493-505

doi:10.3906/biy-1112-56

Biomarkers of petroleum contamination in the digestive gland of *Perna viridis* (L.)

Xivanand Narayan VERLECAR[1], Karmabeer JENA[2], Somshekher Raghoba DESAI[1], Priya Brata DAS[1], Gagan Bihari Nityananda CHAINY[3]

[1]Biological Oceanography Division, National Institute of Oceanography, Dona-Paula, Goa-403004 - INDIA

[2]Central Tasar Research & Training Institute, Central Silk Board, Ranchi, Jharkhand - INDIA

[3]Department of Zoology & Biotechnology, Utkal University, Bhubaneswar, Orissa - INDIA

Received: 30.12.2011 ● Accepted: 07.03.2012

Abstract: Oxidative stress responses were evaluated in the digestive gland tissue of *Perna viridis* exposed to water-accommodated fractions (WAFs) of petrol and diesel at 0.5% and 5% concentrations over 5-, 10-, and 15-day periods. Increased lipid peroxidation, protein carbonyl, DNA integrity, and hydrogen peroxide in WAF-exposed specimens are indicative of oxidative damage due to dissolved petroleum extracts. A consistent increase in superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, reduced glutathione, and ascorbic acid in WAF-exposed specimens signify the importance of antioxidants in protecting the cell against the oxidative damage arising from petroleum contamination. The responses of field specimens collected from petroleum-contaminated sites support the observed laboratory experimental results. This research recommends the use of these oxidative stress indices in the digestive gland tissue of *Perna viridis* as biomarkers of petroleum contamination.

Key words: *Perna viridis*, water-accommodated fraction, petroleum contamination, oxidative stress, digestive gland, biomarkers

Introduction

Tropical coastal waters that sustain rich benthic population diversity have been threatened by repeated incidents of oil pollution over the past few decades. Besides the Macondo well blowout in April 2010 (1), which greatly challenged the US Protection Agencies, other such incidents are not uncommon along European, Gulf, and South Asian countries' shores (2,3). As Fingas stated, an estimated 48% of the marine oil pollution is derived from fuels, 29% from crude oil, and some 5% from tanker accidents (4). In India, a significant amount of oil spillage into the sea originates from bilge discharges and leakages of petrol and diesel used in the operation of fish trawlers, tourist speed boats, land vehicles, industrial processes, and harbor activities. This results in the presence of high amounts of petroleum hydrocarbons in water ranging from 10 to 2440 μg L^{-1}, in inshore sediments from 0.4 to 570 μg g^{-1}, and in beach sand up to 11,059 μg g^{-1}, all of which affect the benthic and pelagic populations (5-7).

Among some of the recent incidents of massive oil spills along the Goa coast in India was that reported by Vethamony et al. regarding the collision of an iron ore barge with an oil tanker on 23 March 2005 (8). Other examples of oil pollution include regular depositions of a tar-like material on Goan beaches due to coastal perturbation during the monsoon. These examples of

oil pollution pose a continuous threat to the coastal beach fauna, which is reflected in the high polycyclic aromatic hydrocarbon (PAH) content in some of the gastropod tissues along the Goa coast, with values ranging from 22.32 to 53.78 μg g^{-1} (9). Similarly high PAH values of up to 186 mg g^{-1} in bivalves are also observed near Vishakhapatnam Harbor, where the average water PAH content is reported to be 108 μg L^{-1} (10). The Goa coast is ecologically sensitive as it sustains a large number of benthic crustaceans (prawns and crabs), bivalves, flat fish, and pelagic fisheries (11). The bivalve mollusk, a sedentary organism living close to the intertidal region, comes in direct contact with various contaminants that induce oxidative stress in their tissues (12-15). Adverse effects, including genotoxicity, of water-soluble petroleum products on marine life are well documented (16,17). However, most of this information pertains to bioaccumulation and other toxicological aspects. Petroleum hydrocarbons are known to produce different metabolites as well as reactive oxygen species (ROS) causing oxidative damage to cells (18,19). This induces antioxidative responses to protect cells against the unfavorable effects of ROS. To this end, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-S-transferase (GST) constitute some of the enzymatic responses, while the nonenzymatic antioxidants include reduced glutathione (GSH) and ascorbic acid (ASA) as main scavengers of oxyradicals (20). Lipid peroxidation (LPX), protein carbonyl (PC), and DNA damage are the results of oxidative stress when antioxidants fail to eliminate the ROS (15,19). Bivalve mollusks have a well-developed system to sustain oxidative stress presented by contaminants such as organic pollutants, heavy metals, and abiotic changes (14,15,18). These properties permit them to be the ideal organisms to study biomarker responses to environmental changes (21-24).

The digestive gland is a major tissue in the metabolic function of this bivalve, involved in xenobiotic uptake and oxyradical generation (12). Our earlier experiments on the measurement of antioxidants in natural populations of *Perna viridis* L. in relation to seasonal changes and laboratory exposures to contaminants such as mercury and temperature stress also showed well-defined responses in the digestive gland of this species (14,15,24). However, in this tropical region, little information is available on the changes in antioxidants of the digestive gland of these bivalves with reference to petroleum hydrocarbons in field and laboratory specimens. Therefore, the aim of this research was to investigate the antioxidant sensitivity of the digestive gland tissue of *Perna viridis* to water-accommodated fractions (WAFs) of petrol and diesel under laboratory conditions, and then to compare these results with natural specimens to evaluate the use of these antioxidant as pollution biomarkers.

Laboratory experiments were designed to measure oxidative stress responses in the digestive gland of *P. viridis* exposed to the WAF of petrol (WAF-P) and the WAF of diesel (WAF-D) for a continuous period of 15 days. The oil-exposed animals were dissected periodically to measure the antioxidant responses in the digestive gland. The parameters measured included LPX, PC, hydrogen peroxide (H_2O_2), GSH, ASA, and antioxidant enzymes such as SOD, CAT, GPX, GR, and GST as indices of oxidative stress, and DNA strand breaks as an index of genotoxic stress. These results were examined with reference to our field observations on the same species collected from polluted and unpolluted locations.

Material and methods

Chemicals

Thiobarbituric acid (TBA), bovine serum albumin (BSA), cumene-hydroperoxide, 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), horseradish peroxidase (HRP), and streptomycin sulfate were procured from Sigma Chemical Co. (USA). Reduced nicotinamide adenine dinucleotide phosphate (NADPH), oxidized glutathione (GSSG), GSH, 1-chloro-2,4-dinitrobenzene (CDNB), phenylmethylsulfonyl fluoride (PMSF), and 2,4-dinitrophenyl hydrazine (DNPH) were obtained from SISCO Research Laboratory (India). All other chemicals used were of analytical grade.

Experimental animals and maintenance

Green-lipped mussels (*Perna viridis* L.), with a length of 8-10 cm, were collected from the Bambolim beach in Goa (15°27′N, 73°51′E), along the western coast of India. Animals were transported to the laboratory in

polythene bags filled with water from the seacoast. Fouling organisms were removed from the external part of the shells. In the laboratory, the mussels were acclimatized for 2-3 weeks in seawater with salinity of 31‰, pH 8.0, and temperatures ranging from 23 to 25 °C. The light and dark regime was kept constant at 12 h and 12 h. The mussels were fed with algae species *Chlorella* sp. twice daily during the acclimation and exposure period. To avoid fecal contamination, the water was exchanged every 24 hours and oxygen was provided by a continuous air-bubbling system.

Preparation of WAFs and experimental setup

The WAFs of oils (petrol and diesel) were prepared according to the method of Phatarpekar and Ansari (25). In a volumetric flask, 1 part oil to 9 parts filtered, autoclaved seawater (1:9, v v^{-1}) was mixed. The flask was tightly capped with a stopper and covered with aluminum foil to reduce the evaporation of volatile petroleum hydrocarbons. The solution was mixed at a slow speed with a magnetic stirrer for 20 h at room temperature. After mixing, the oil and water phases were allowed to separate for 8 h in a separating funnel and then the aqueous phase was drained out. This preparation was used as the 100% stock solution. The WAF range was selected based on earlier observations on the effect of WAFs of oil on *P. viridis* (26).

PAH analysis in WAF

For the measurement of total PAH, the WAF-P and WAF-D samples were extracted in double distilled hexane and quantified by spectrofluorometry using a Shimadzu 1501 spectrofluorometer. Bombay high crude oil was used as the reference material for the quantification of the hydrocarbons. The total petroleum hydrocarbon was measured in triplicates with the standard error of analysis as less than 1.05%.

Experimental designs

Experiment A

Group 1: Control mussels, untreated.

Group 2: Mussels treated with 0.5% WAF-P.

Group 3: Mussels treated with 5% WAF-P.

Experiment B

Group 1: Control mussels, untreated.

Group 2: Mussels treated with 0.5% WAF-D.

Group 3: Mussels treated with 5% WAF-D.

For each of these treatments, 3 replicates were kept consisting of 45 mussels in a 90-L plastic tank (2 L of seawater per mussel). The test solutions were exchanged and the accumulation of fecal matter was removed at regular intervals to avoid any bacterial contamination. The mussels were sacrificed for biochemical tests after a period of 5, 10, and 15 days.

Sample preparations

The digestive gland was carefully excised, surface-dried with tissue paper, thoroughly washed with phosphate buffer (50 mM; pH 7.4), and homogenized with 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.15 M KCl, and 0.01% (w v^{-1}) PMSF. Homogenization was carried out at 4 °C using 12-15 strokes of a motor-driven Teflon Potter homogenizer, after which the samples were centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was used for biochemical analysis (LPX, H_2O_2, SOD, CAT, GPX, GR, and GST).

Lipid peroxidation measurement

LPX level was assayed by measuring the amount of malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acid, and determined by the thiobarbituric acid (TBA) reaction as described by Ohkawa et al. (27). The absorbance was read at 532 nm after the removal of any flocculated material by centrifugation. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (28) and expressed as nmol TBARS formed mg $protein^{-1}$.

Estimation of protein carbonyl

Samples were homogenized in 50 mM phosphate buffer (pH 7.4) containing 2 mM EDTA, 0.1% (w v^{-1}) digitonin, and 0.46 mM PMSF. The homogenate was centrifuged at 6000 × *g* for 15 min at 4 °C and the supernatant was used for determination. For the carbonyl quantification, the reaction with DNPH was performed as described by Mecocci et al. (29). The PC content was calculated from the absorbance measurement at 366 nm with the use of a molar absorption coefficient of 22,000 mol cm^{-1} and expressed as nmol mg $protein^{-1}$.

Alkaline unwinding assay

The alkaline DNA unwinding assay was performed according to the method of Shugart with slight

modification (30,31). Approximately 100-150 mg of the tissue was homogenized using 1 mL of 1 N NH_4OH in 0.2% Triton X-100. The homogenate was transferred to a centrifuge tube containing 2 mL of water and 6 mL of phenol/chloroform/isoamyl alcohol (PCI) (25:24:1, v v^{-1} v^{-1}), and the tube was allowed to stand for 10-15 min. The homogenate was then centrifuged at 10,000 × *g* for 20 min at 4 °C. The aqueous layer was then digested by 5 µL of ribonuclease A (10 mg mL^{-1}) for 30 min at 37 °C and extracted successively by an equal volume of PCI recovered by a Sephadex G-50 column (1 cm i.d., 4-mL settled bed volume) and equilibrated in a G-50 buffer (0.15 M NaCl, 0.01 M Tris (pH 7.4), 1 mM $MgCl_2$, and 5 mM EDTA). The DNA sample was diluted and separated into 3 equal proportions. Each tube contained 100 µL of the DNA sample and 50 µL of 0.05 N NaOH. The contents were mixed and maintained at 4, 80, and 38 °C for 25 min for the fluorescence determination of double-stranded DNA (ds DNA), single-stranded DNA (ss DNA), and partially unwound DNA (au DNA) using a spectrofluorometer (Shimadzu RF-501) (excitation: 360 nm, emission: 450 nm).

The assay of each sample was performed in duplicate and the averaged fluorescent values were used for the determination of the F-value with the following equation: (au DNA - ss DNA) / (ds DNA - ss DNA).

Antioxidant enzymes

The activity of total SOD was determined by the method of Paoletti et al. by monitoring the rate of oxidation of reduced nicotinamide adenine dinucleotide (NADH) (32). One unit of SOD activity was defined as a 50% inhibition of NADH oxidation in the reaction. The absorbance was recorded at 340 nm and expressed as unit mg $protein^{-1}$. CAT activity was determined according to the method of Aebi by monitoring the decrease in the absorbance of H_2O_2 at 240 nm, and enzyme activity was expressed as nkat mg $protein^{-1}$ (33). The activity of GPX was determined by the method described by Paglia and Valentine, which uses cumene-hydroperoxide as the substrate, and enzyme activity was monitored by recording the oxidation of NADPH at 340 nm (34). Enzyme activity was expressed as nmol NADPH oxidized min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}. The activity of GR was assayed according to the rate of NADPH oxidation at 340 nm in the presence of GSSG (35). Enzyme activity was expressed as nmol NADPH oxidized min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}. GST activity was measured according to the method of Habig et al. using CDNB as a substrate (36). The change in the absorbance was recorded at 340 nm and enzyme activity was expressed as nmol CDNB conjugate formed min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Measurement of small antioxidant molecules

A 10% tissue homogenate was prepared in 5% trichloroacetic acid (w v^{-1}) and centrifuged at 1000 × *g* for 30 min. The deproteinized supernatant was used for the assay of GSH (37). Ascorbic acid was measured according to the method of Mitusi and Ohata (38).

Hydrogen peroxide

The H_2O_2 content in postmitochondrial fractions was determined spectrophotometrically according to the method of Pick and Keisari using HRP and phenol red (39). The protein content was estimated by the Folin-phenol reaction as described by Lowry et al. using BSA as the standard (40).

Field observations

The present laboratory results were compared with earlier similar studies on the antioxidant status of field specimens of *Perna viridis* collected from the following 3 coastal locations in Goa (13): Bambolim beach (BB; 15°270′N, 73°510′E), located away from commercial and industrial activities with low pollution levels; Malim fish jetty (ML; 15°300′N, 73°500′E), a major fish-landing jetty; and Mormugao Harbor (MH) (15°240′N, 73°480′E), a major port along the central western coast of India (Figure 1).

Statistical analysis

The experiments were conducted with 3 replicate groups per treatment and the results were expressed as mean ± standard deviation (SD). The difference among the means of the control and treated means were analyzed by 2-way analysis of variance (ANOVA), followed by Duncan's new multiple range test. Differences were considered statistically significant when $P < 0.05$.

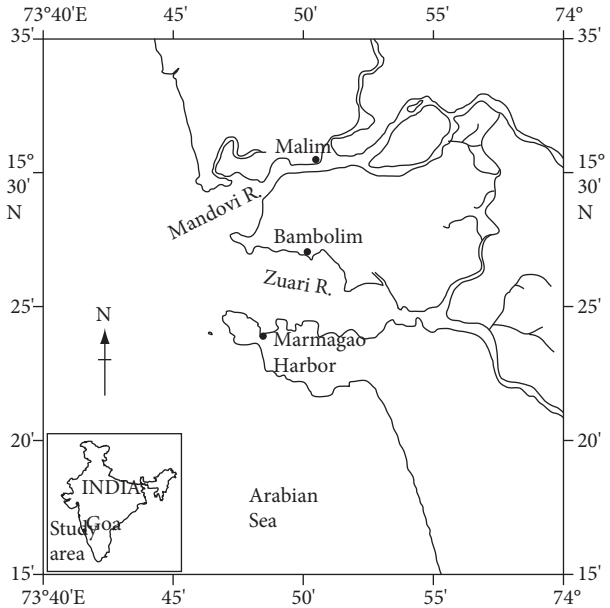

Figure 1. Location of the sampling sites for the field collection of *P. viridis* in the waters near the shore of the Goa coast.

Results

Petroleum hydrocarbon content in the WAF of oils

Analysis of the WAFs used in our exposure experiments showed a total petroleum hydrocarbon (TPH) content of 30 μg L^{-1} in the 0.5% solution and 300 μg L^{-1} in the 5% solution of WAF-P, and 22.5 μg L^{-1} in the 0.5% solution and 227 μg L^{-1} in the 5% solution of WAF-D. Gas chromatography-mass spectrometry (GCMS) results, as described under Desai et al., indicated a diverse fraction of TPH from C9 to C36 in petrol and WAF-P (41). Petrol constituted 20.1% of C09 to C15, 77.5% of C16 to C25, and 2.1% of C26 to C36 fractions. The major fraction of TPH in WAF-P was C09 to C15, constituting 97.8%, while the solubilities of the other 2 fractions were 1.8% and 0.4%, respectively. Detailed analysis of the hydrocarbon compounds in aqueous extracts of gasoline, diesel, and crude oil, using GCMS combined with high-performance liquid chromatography and UV fluorescence, indicated a comparative absence of aliphatic compounds with greater water solubility of the more polarizable aromatics (16). Therefore, water contaminated with gasoline (petrol) contains highly conjugated aromatics such as naphthalene and alkenyl benzenes (i.e., toluene, xylene, methyl styrene, ethyl xylene, naphthalene, and methyl naphthalene) with component ratios varying slightly as per the brand mark. Of these, methyl naphthalenes dominated by up to almost 95% in water extracts of standard diesel, while crude oil indicated lower volatility similar to diesel, as compared to gasoline (16). Pacheco and Santos demonstrated that the majority of PAHs measured displayed higher levels in water extracts of petrol than diesel (42). While naphthalene was the most abundant component in both of the fractions, the difference displayed was particularly evident for PAHs such as benzo(ghi)perylene, pyrene (P), and benzoic(a)preen, as they were several-fold higher in the water extracts of petrol than diesel. This is in agreement with our GCMS analytical results, which showed a high content of fused-ring hydrocarbons such as naphthalene, anthracene, phenanthrene, and their derivatives in the WAF-P (41).

Lipid peroxidation

A significant increase in LPX content was seen on day 5 in *P. viridis* exposed to WAF-P concentrations of 0.5% and 5% in the digestive gland tissue in relation to the control (Figure 2A). LPX continued to increase up to day 10 in the 5% solution while the rest of the values did not differ significantly in relation to the control. As for WAF-D exposure, a significant increase in LPX was seen at both of these concentrations with reference to the control during all sampling occasions (Figure 2B).

Protein carbonyl content

A significant increase in PC content was recorded in the mussels exposed to 0.5% WAF-P on day 5, which remained almost similar to the control conditions for the rest of the period. The changes in PC were more consistent at 5% WAF-P, showing a significant increase during the entire exposure period up to day 15 (Figure 2C).

Although the WAF-D-exposed mussels showed increasing PC values during all sampling occasions, significantly high values could only be recorded on day 10 for both concentrations and on day 15 for the 5% WAF-D (Figure 2D).

DNA strand break

The decrease in F-values, although evident in both WAF-P concentrations throughout the exposure period, attained significant difference only on day 10 at 5% and day 15 at 0.5% WAF-P in relation to the control (Figure 2E).

As seen with WAF-P, the decreasing trend in the F-value was apparent with increasing concentrations in WAF-D-exposed mussels throughout the experimental period. However, a statistically significant decrease could only be observed on day 15 with the 5% WAF-D in relation to the control (Figure 2F).

Hydrogen peroxide

H_2O_2, which is an intermediate product of the dismutation of the superoxide radical by superoxide dismutase and catalase, showed increasing values during the first 10 days, but on day 15 the changes remained insignificant in relation to the control (Figure 3A). Although the H_2O_2 values in WAF-D-exposed specimens remained higher than in the control during the entire experimental period, the significant difference in values was only noticed on day 15 for both concentrations and on days 5 and 10 for the 5% and 0.5% WAF-Ds, respectively (Figure 3B).

Superoxide dismutase

A significant increase in SOD values in relation to the control was seen all throughout the exposure period for 5% WAF-P, while at 0.5% exposure such a difference was observed only on day 5 (Figure 3C). For the WAF-D-exposed mussels, a somewhat

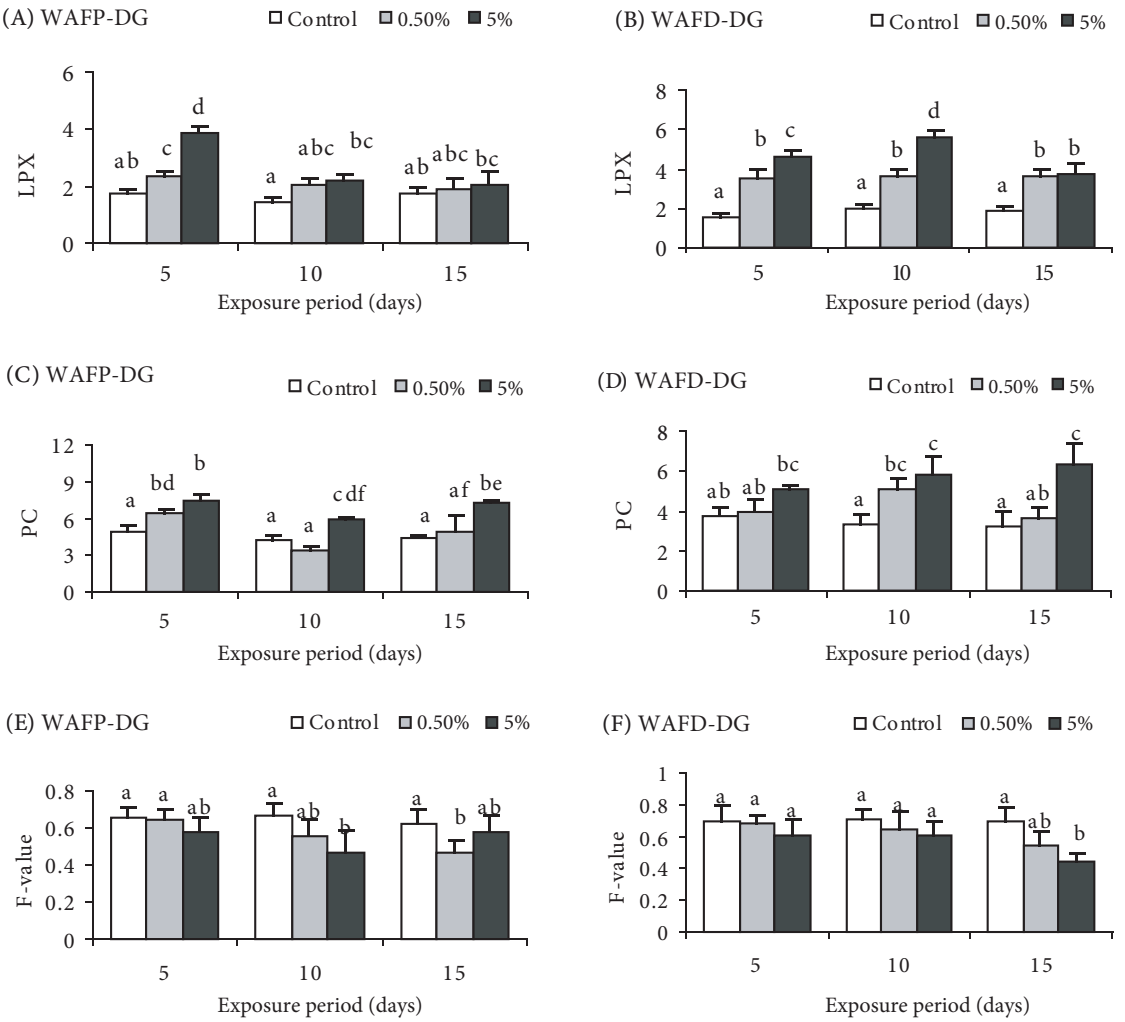

Figure 2. Effect of WAF-P and WAF-D on lipid peroxidation (A and B; nmol TBARS mg protein^{-1}), protein carbonyl (C and D; nmol mg protein^{-1}), and DNA damage (E and F; F-value) in the digestive gland of *P. viridis* (data expressed as mean ± SD; n = 3). Superscripts of different letters are significantly different from each other at $P < 0.05$.

delayed SOD response could be observed at 0.5% exposure showing an increase only on day 15, but persistent elevated enzyme activity was observed at 5% for the entire period of 5 to 15 days (Figure 3D).

Catalase

Catalase is involved in catalyzing H_2O_2 to water and molecular oxygen. Although elevated values were evident in WAF-P-exposed specimens, the differences were minor except for a significant change on day 10 at the 5% level (Figure 3E). A somewhat delayed catalase action was observed for WAF-D-exposed mussels with values attaining a significant difference on day 10 onwards at both treatment levels (Figure 3F).

Glutathione peroxidase

GPX is also a scavenger of oxyradicals like CAT. The activity of GPX was more defined during the entire exposure period with increased values from days 5 to 15 at both WAF-P concentrations (Figure 4A). As seen with CAT, delayed GPX action was observed under the WAF-D exposures, with values recording significant increases on day 10 in both concentrations and day 15 only at the 5% level (Figure 4B).

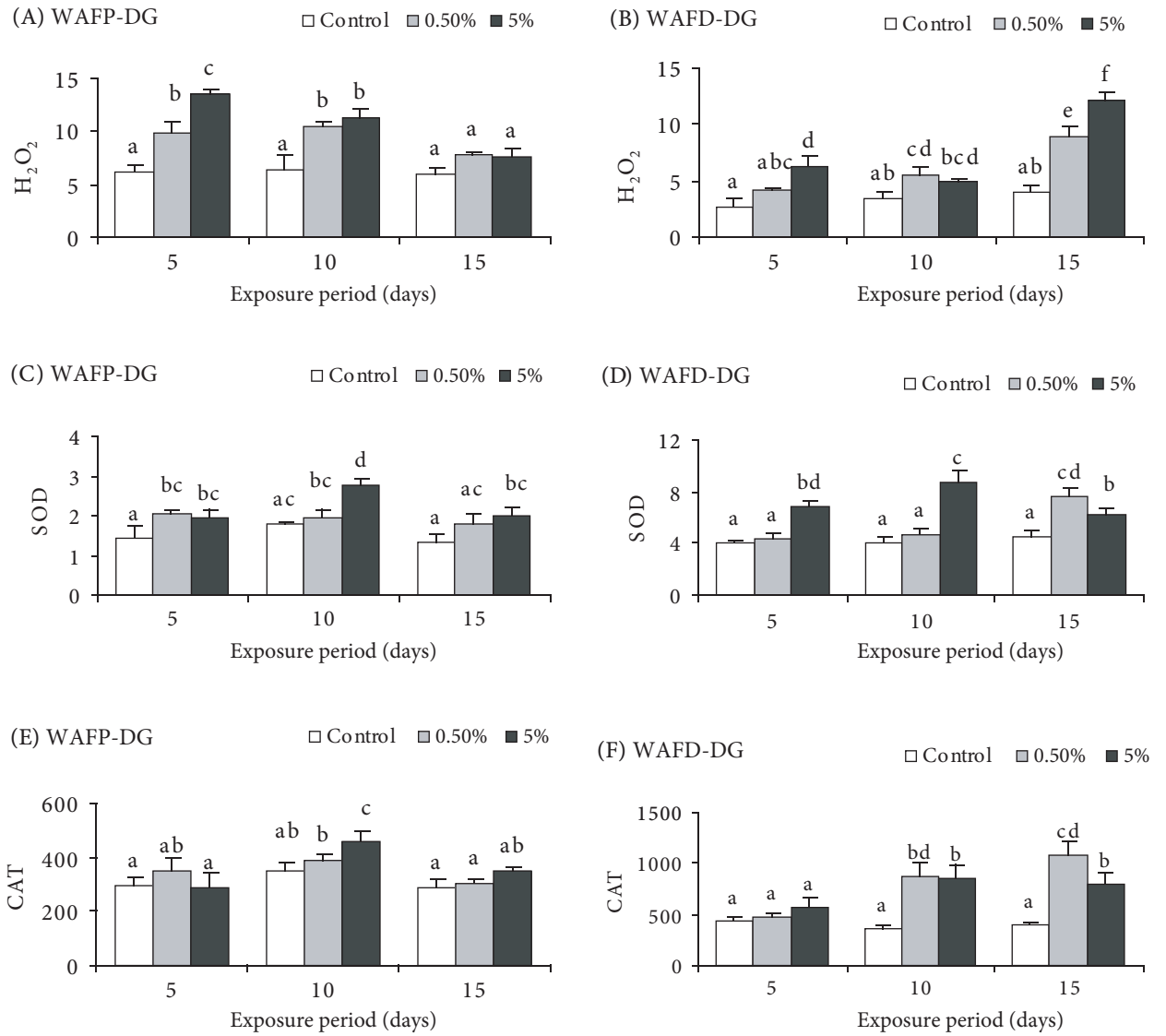

Figure 3. Effect of WAF-P and WAF-D on hydrogen peroxide (A and B; nmol H_2O_2 mg protein^{-1}), superoxide dismutase (C and D; unit mg protein^{-1}), and catalase (E and F; nkat mg protein^{-1}) in the digestive gland of *P. viridis* (data expressed as mean ± SD; n = 3). Superscripts of different letters are significantly different from each other at $P < 0.05$.

Glutathione S-transferase

GST, which takes part in the detoxification process of oxyradicals, showed elevated activity immediately on day 5 at both WAF-P concentrations. Thereafter, the increase could only be recorded on day 10 at 5% with reference to the control (Figure 4C). The GST increase showed a significant difference in relation to the control at both WAF-D concentrations, except at the 0.5% level on day 5 (Figure 4D).

Glutathione reductase

GR, which is involved in the regeneration of GSH from GSSG, also showed promising activity at both concentrations of WAF-P and WAF-D, registering a significant increase in relation to the control during the entire period, except on day 5 at 0.5% WAF-D (Figures 4E and 4F).

Reduced glutathione

GSH, which is a nonenzymatic antioxidant, showed a significant increase in activity only on day 5 at both WAF-P concentrations and remained comparable to the control during the rest of the period (Figure 5A). Under WAF-D exposure, elevated GSH responses could be observed during the entire period except on day 15 at 0.5%, indicating a prominent role of this antioxidant in the ROS scavenging process (Figure 5B).

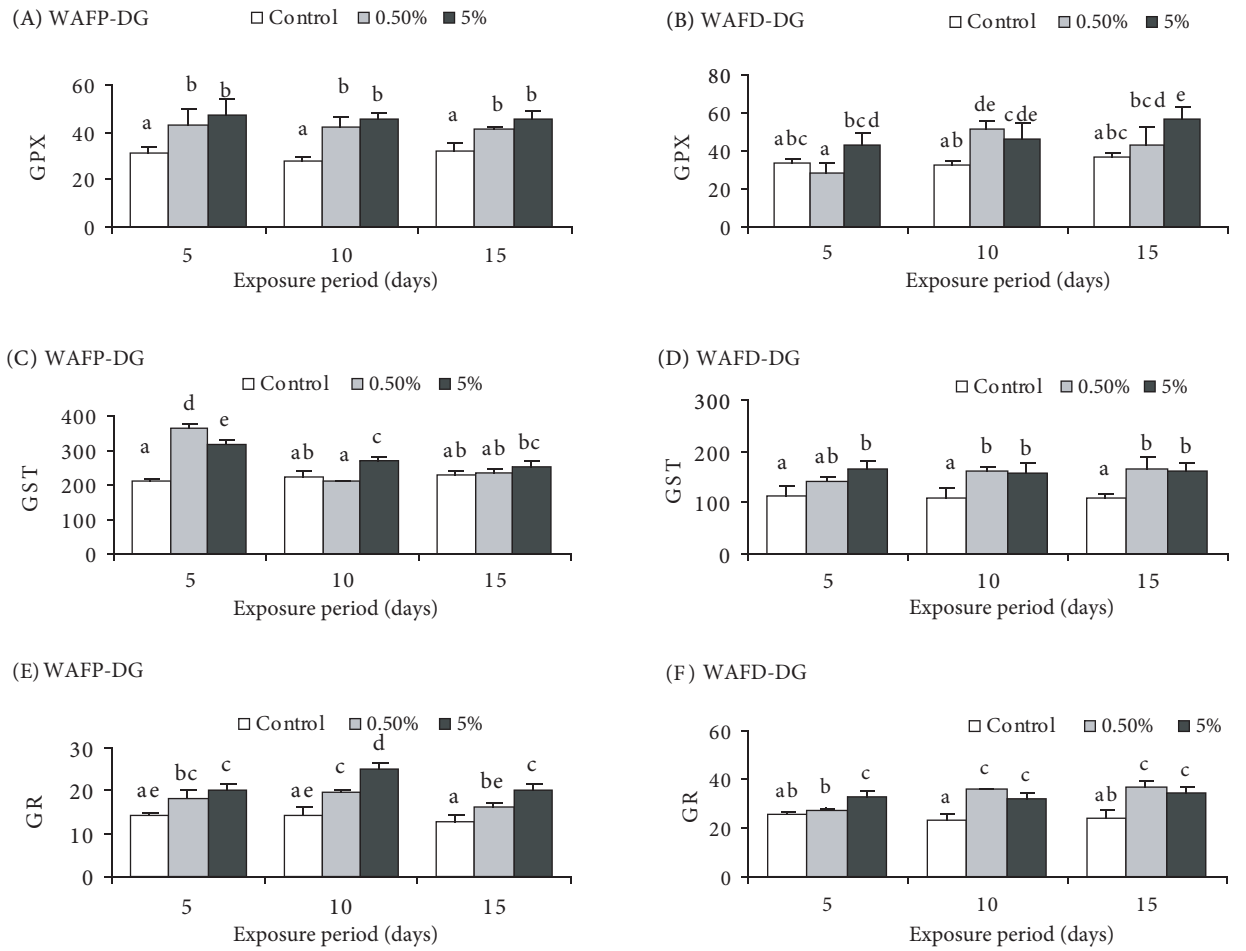

Figure 4. Effect of WAF-P and WAF-D on glutathione peroxide (A and B; nmol NADPH oxidized min^{-1} mg $protein^{-1}$), glutathione S-transferase (C and D; nmol CDNB conjugate formed min^{-1} mg $protein^{-1}$), and glutathione reductase (E and F; nmol NADPH oxidized min^{-1} mg $protein^{-1}$) in the digestive gland of *P. viridis* (data expressed as mean ± SD; n = 3). Superscripts of different letters are significantly different from each other at $P < 0.05$.

Ascorbic acid

ASA, which is also a scavenger of superoxide and the hydrogen peroxide radical, showed a small change in activity under WAF-P exposure, but in the case of WAF-D, significant increase in values could be recorded on day 10 at 0.5% and on days 5 and 15 at 5% (Figures 5C and 5D).

Antioxidant responses in field specimens

Our observations of field specimens indicated that TBARS and other enzymatic parameters such as SOD, CAT, GPX, GST, and GR maintained higher values at the 2 contaminant-prone sites of MH and ML as compared to BB. This difference in the values of antioxidants is caused either by trace metals or PAH content (43,44). The monthly changes in water-soluble PAH values from September to April, covering 3 seasons of monsoon and the post- and premonsoon times, ranged from 3.26 to 25.44 μg L^{-1} (17.37 ± 6.15) at BB (44). The time series data collected during a 24-h period once a month at MH during this period indicated wide fluctuation in values between 2.76 and 299.9 μg L^{-1}, although the average monthly concentrations remained low, varying from 4.99 to 19.39 μg L^{-1} (Figure 6) (44). Seasonal changes in PAH values recorded at ML ranged from 55 to 78.42 μg L^{-1} (65.56 ± 8.6) and the highest value recorded during the observations was 170.4 μg L^{-1}, with an average of 78.31 ± 28.49 μg L^{-1} (43). Thus, the average PAH concentration at ML remains much higher than even that at MH. This probably reflects the water

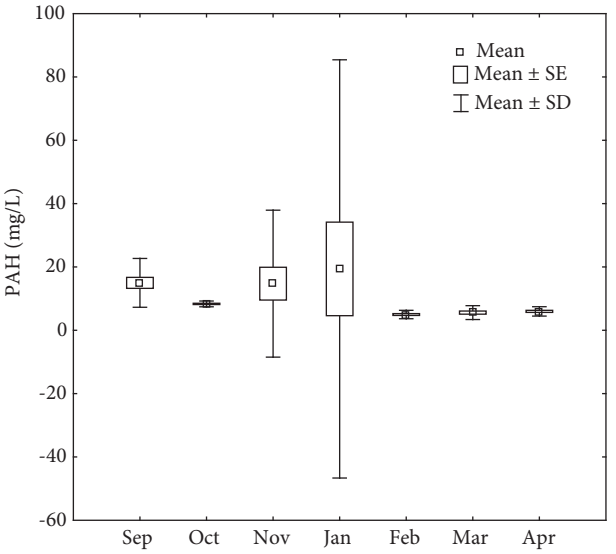

Figure 6. Time series observations of water soluble PAH (μg L^{-1}) at Mormugao Harbor.

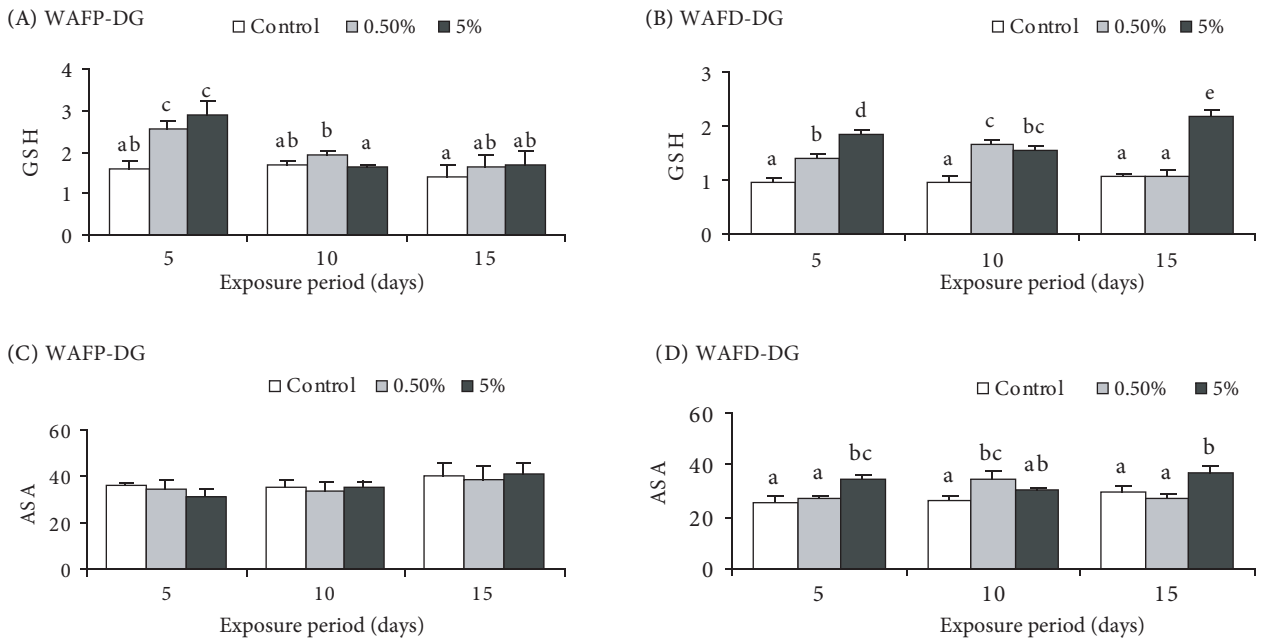

Figure 5. Effect of WAF-P and WAF-D on reduced glutathione (A and B; μmol g wet tissues^{-1}) and ascorbic acid (C and D; μg g wet tissues^{-1}) in the digestive gland of *P. viridis* (data expressed as mean ± SD; n = 3). Superscripts of different letters are significantly different from each other at $P < 0.05$.

exchange systems prevailing at these 2 locations. MH is located near the mouth of the Zuary estuary, which offers a better dispersion of contaminants as compared to ML, which is located more inside the Mandovi estuary. Thus, the water exchange patterns at ML are more sluggish than at MH and offer limited scope for the pollutants to dissipate faster. As already discussed, the sediment trace metals remained slightly higher at the 2 pollution-affected sites than at BB (13). However, the fluctuation in PAH values appeared to be more distinct at MH and ML, which could also contribute substantially to the observed higher antioxidant content in mussels at these 2 locations compared to BB.

Discussion

PAH compounds in the WAF of oil interfere with enzyme processes in aquatic animals, resulting in typical responses in their gills or liver tissues (18,21). The observed enhanced levels of TBARS, PC, and DNA strand breaks suggest increased ROS production as a result of the mussels' exposure to the WAF of petroleum oils. Similar biomolecular damaging effects of petroleum products in mollusks have been reported in earlier studies (19,22,42,45,46).

The LPX responses were sharper in diesel extracts and evidently prevailed during the entire experimental period. This suggests that the dissolved naphthalene components of WAF could induce oxidative stress, resulting in damage to cell lipids. These LPX products can react with amino acid groups of proteins and other molecules to form a variety of adducts (47,48). It has been established that direct damage to proteins or the chemical modification of amino acids in proteins during oxidative stress can give rise to protein carbonyls (49,50). Protein carbonyl responses are positive at both concentrations of WAF-P and WAF-D. This suggests that protein oxidation occurs during petroleum pollution. Our observations on increased PC during oil exposure are in agreement with the results of Labieniec and Gabryelak with reference to their work on mussels exposed to tannins (46).

Increased DNA strand breaks were seen during the entire exposure period with a significant difference on day 5 with 0.5% WAF-P, and on days 10 and 15 with 0.5% and 5% WAF-D, with reference to the control. This suggests that the mussels were highly sensitive to even low concentrations of petrol or diesel inducing mutagenic activity in their tissues. The presence of pyrene and other carcinogenic derivatives in these extracts could be responsible for these mutagenic changes (22,42). DNA strand breaks in mussels exposed to petroleum-related toxicants were also reported (22,45). Field experiments on marine snail *Cronia contracta* also showed similar impairment of DNA integrity (up to 39% in the coastal waters of Goa), which was mainly related to petroleum contamination (9).

The increased activity of SOD, CAT, GPX, GST, and GR seen in the present experiments is indicative of the adaptive response of the mussel cells to avoid oxidative stress induced by oil exposure. The significantly high values of H_2O_2 that were recorded during most of the exposure period for both concentrations of WAF-P and WAF-D show the extent of oxidative damage that could be produced in mussel cells. Alternatively, the SOD activity is equally consistent with the generation of H_2O_2 and other cellular damage parameters such as LPX, PC, and F-values, which indicates the well-balanced role of this enzyme in scavenging ROS. The increased SOD activity seen here corroborates with that of Cheung et al. for mussels exposed to PAH (51).

H_2O_2 can further alter the cell physiology through the formation of $OH^{\bullet}$ by the Fenton reaction (52). Interestingly, cells sustain a mechanism to enhance the activity of antioxidant synthesis in response to oxidative stress conditions (53,54). The significant levels of CAT and GPX activities seen in this study under the exposure of WAFs of both petrol and diesel ensured quick removal of H_2O_2. In this, GPX appeared to be more active in eliminating the H_2O_2 than CAT. These results are in agreement with those of Reid and MacFarlane and Pan et al., who also observed increased GPX activity in mollusks exposed to petroleum products (19,55).

Our observations on increased GST activity in test specimens treated with the WAF of oils are similar to those reported by Lima et al. in mussels collected from an area contaminated with petrochemicals (56). The increased GST activity observed in our study could be directly involved in detoxifying the petroleum compounds, thereby relieving the tissue from oxidative stress (57). Cheung et al. also

documented significant relationships between GST and PAH body burdens in mussels (51).

GR converts GSSG to its reduced form, GSH, at the expense of the oxidation of NADPH to $NADP^+$ (20). Therefore, increased GR activity is vital during stress conditions, and this was prominently seen during the longest exposure period in the present study (Figures 3E and 3F). Our results are in agreement with those of Zhang et al., who also recorded a significant increase in GR activity on days 7 and 10 in goldfish (*Carassius auratus*) exposed to WAF-D (58). The elevated GR activity during the exposure to the pollutant is a part of the protective response of the tissue to maintain its intracellular thiol status. GSH and GR maintained elevated activity during most of the WAF exposure period in our study. This suggests that GSH is active either as a substrate to accelerate the function of other antioxidant enzymes or as a scavenger of ROS by binding to them directly. Interestingly, the significant induction of glutathione-related enzymes (GPX, GST, and GR) in the digestive gland of *P. viridis* exposed to the WAF of oils observed in our study indicates that the glutathione pathway is more competently used by the organism to protect its cells from ROS toxicity in an oil-contaminated environment.

Sufficiently higher ASA activity was seen under WAF-D exposure as compared to WAF-P in the present study. This contaminant-specific increase in ASA content may be due to the increased availability of GSH, which reduces dehydroascorbate to ascorbate (59). Further studies along these lines may be required to understand the relationship between ASA content and stress generation in mussels due to oil exposure.

Our laboratory experimental results, which showed elevated LPX and antioxidant responses to the WAFs of oils, are well supported by the field observations displaying similarly higher values in *Perna viridis* at the 2 PAH-contaminated locations, ML and MH. However, nonenzymatic antioxidants such as GSH and ASA showed a reverse trend, with values remaining higher at BB than at MH or ML. This is probably because of the direct involvement of both of these antioxidants in scavenging for ROS or metal ions at the contaminated sites, since GSH is also utilized in the synthesis of enzymatic antioxidants (13). Under similar laboratory studies, Luchmann et al. concluded that bioaccumulation of aliphatic and aromatic hydrocarbons and antioxidant responses in oyster *Crassostrea brasiliana* exposed to WAF-D could serve as biomarkers of petroleum toxicity (60). Thus, the increasing trend in cell damage parameters such as LPX, PC, DNA unwinding, and antioxidant enzymes under petroleum stress in field and laboratory conditions observed here could be utilized as biomarkers for the detection of petroleum by-product pollution.

Our results showed that WAFs of petrol and diesel could retain aromatics and PAHs in significant quantities. Considerable increases in LPX, PC content, and DNA damage in mussels measured over 5, 10, and 15 days of exposure to WAF-P and WAF-D indicated the toxic nature of these petroleum products to *P. viridis*. This is reflected in the consistent increase in the activity of antioxidant enzymes such as SOD, CAT, GPX, GST, and GR. Similar responses of antioxidant enzymes obtained in field specimens collected from petroleum-contaminated sites as in laboratory-exposed samples support the hypothesis that dissolved petroleum products in WAFs produce oxidative stress in the digestive gland tissue of *Perna viridis*. The results in field and laboratory investigations support the fact that the cellular damage and oxidative stress indices in the digestive gland tissue of *Perna viridis* could suitably be used as biomarkers of petroleum pollution during coastal ecological investigations.

Acknowledgments

The authors are grateful to the Director of the National Institute of Oceanography (NIO) for help. This work has been funded through CSIR Network Project NWP-0018 and forms Contribution No. 5154 of NIO.

Corresponding author:

Xivanand Narayan VERLECAR

Environmental Laboratory,

Biological Oceanography Division,

National Institute of Oceanography,

Dona-Paula, Goa-403004 - INDIA

E-mail: verlecar@nio.org

References

1. The Deepwater Horizon Study Group. Final Report on the Investigation of the Macondo Well Blowout. DHSG. Berkeley, California; 2011. Available at http://ccrm.berkeley.edu/pdfs_papers/bea_pdfs/DHSGFinalReport-March2011-tag.pdf.
2. Laffon B, Rabade T, Pasaro E et al. Monitoring of the impact of *Prestige* oil spill on *Mytilus galloprovincialis* from Galician coast. Environ Int 32: 342-8, 2006.
3. Al-Thukair AA, Abed RMM, Mohamed L et al. Microbial community of cyanobacteria mats in the intertidal zone of oil-polluted coast of Saudi Arabia. Mar Pollut Bull 54: 173-9, 2007.
4. Fingas M. The Basics of Oil Spill Cleanup. Lewis Publishers. Boca Raton, Floria; 2001.
5. Verlecar XN, Desai SR, Sarkar A et al. Biological indicators in relation to coastal pollution along the Karnataka coast, India. Water Res 40: 3304-12, 2006.
6. Ingole B, Sivadas S, Goltekar R et al. Ecotoxicological effect of grounded *MV River Princess* on the intertidal benthic organisms off Goa. Environ Int 32: 284-91, 2006.
7. Shirodkar PV, Pradhan UK, Vethamony P et al. Impact of water quality changes on harbor environment due to port activities along the west coast of India. Proceedings of Second International Conference on Coastal Zone Engineering and Management, 2010. Available at http://drs.nio.org/drs/bitstream/2264/3759/1/Int_Conf_Arabian_Coast_2010_Shirodkar.pdf.
8. Vethamony P, Sudheesh K, Babu MT et al. Trajectory of an oil spill off Goa, eastern Arabian Sea: field observations and simulations. Environ Pollut 148: 438-44, 2007.
9. Sarkar A, Gaitonde DCS, Sarkar A et al. Evaluation of impairment if DNA integrity in marine gastropods (Cronia contracta) as a biomarker of genotoxic contaminants in coastal water around Goa, West coast of India. Ecotoxicol Environ Saf 71: 473-82, 2008.
10. Chandramohan P, Raghuprakash R. Concentration of petroleum hydrocarbons in bivalve *Mytilopsis sallei* and in the harbour waters of Visakhapatnam, east coast of India. Indian J Mar Sci 27: 496-8, 1998.
11. Sen Gupta R, Desa E. The Indian Ocean: A Perspective. A.A. Balkema Publishers. Rotterdam, the Netherlands; 2001.
12. Livingstone DR, Garcia Martinez P, Michel X et al. Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* L., and other molluscs. Functional Ecol 4: 415-24, 1990.
13. Jena KB, Verlecar XN, Chainy GBN et al. Application of oxidative stress indices in natural populations of *Perna viridis* as biomarker of environmental pollution. Mar Pollut Bull 58: 107-13, 2008.
14. Verlecar XN, Jena KB, Chainy GBN et al. Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature. Chem Biol Interact 167: 219-26, 2007.
15. Verlecar XN, Jena KB, Chainy GBN et al. Modulation of antioxidant defences in digestive gland of *Perna viridis* (L.), on mercury exposures. Chemosphere 71: 1977-85, 2008.
16. Groner M, Murokosy AR, Myrick ML et al. Identification of major water-soluble fluorescent components of some petrochemicals. Mar Pollut Bull 42: 935-41, 2001.
17. Francioni E, Wagener ALR, Scofield AL et al. Polycyclic aromatic hydrocarbon in inter-tidal mussel *Perna perna*: space-time observations, source investigation and genotoxicity. Sci Total Environ 372: 515-31, 2007.
18. Martinez PG, Livingstone DR. Benzo[a]pyrene-dione-stimulated oxyradical production by microsomes of digestive gland of the common mussel, *Mytilus edulis* L. Mar Env Res 39: 185-9, 1995.
19. Pan LQ, Ren J, Liu J et al. Response of antioxidant systems and LPO level to benzo(a)pyrene and benzo(k)fluoranthene in the haemolymph of the scallop *Chlamys ferrari*. Environ Pollut 141: 443-51, 2005.
20. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. Oxford University Press. New York; 2001.
21. Doyotte A, Cossu C, Jacquin MC et al. Antioxidant enzymes, glutathione and lipid peroxidation relevant biomarkers of experimental or field exposure in gills and the digestive gland of the fresh water bivalve *Unio tumidis*. Aquat Toxicol 39: 93-110, 1997.
22. Perez-Cadahia B, Laffon B, Pasaro E et al. Evaluation of PAH bioaccumulation and DNA damage in mussels (*Mytilus galloprovincialis*) exposed to spilled *Prestige* crude oil. Comp Biochem Physiol 138: 453-60, 2004.
23. Alves de Almeida E, Celso Dias Bainy A, Paula de Melo Loureiro A et al. Oxidative stress in *Perna perna* and other bivalves as indicators of environmental stress in the Brazilian marine environment: antioxidants, lipid peroxidation and DNA damage. Comp Biochem Physiol A 146: 588-600, 2007.
24. Verlecar XN, Jena KB, Chainy GBN et al. Seasonal variation of oxidative stress biomarkers in gills and digestive gland of green lipped-mussel *Perna viridis* from Arabian Sea. Estuar Coast Shelf Sci 76: 745-52, 2008.
25. Phatarpekar PV, Ansari ZA. Comparative toxicity of water soluble fractions of four oils on the growth of a microalga. Botanica Marina 43: 367-75, 2000.
26. National Institute of Oceanography. Toxicological Studies On Marine Organisms with Mineral Oil, Oil Based Mud & Mud Cutting. NIO Tech Report. NIO. Goa, India; 1988.
27. Ohkawa H, Ohisi N, Yagi K et al. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95: 351-8, 1979.
28. Wills ED. Lipid peroxide formation in microsomes. General considerations. Biochem J 113: 315-24, 1969.
29. Mecocci P, Fano G, Fulle S et al. Age-dependent increases in oxidative damage to DNA, lipids and proteins in human skeletal muscle. Free Radic Biol Med 26: 303-8, 1999.

30. Shugart LR. An alkaline unwinding assay for the detection of DNA damage in aquatic organisms. Mar Environ Res 24: 321-5, 1988.

31. Shugart LR. Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. Aquat Toxicol 13: 43-52, 1988.

32. Paoletti F, Mocali A, Aldinucci D et al. Superoxide-driven NAD(P)H oxidation induced by EDTA-manganese complex and mercaptoethanol. Chem Biol Interact 76: 3-18, 1990.

33. Aebi H. Catalase. In: Bergmeyer, HU. ed. Methods in Enzymatic Analysis, 2nd ed. Academic Press; 1974: pp. 673-8.

34. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70: 158-69, 1967.

35. Massey V, Williams CH. On the reaction mechanism of yeast glutathione reductase. J Biol Chem 240: 4470-81, 1965.

36. Habig WH, Pabst MJ, Jakoby WB et al. Glutathione-S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249: 7130-9, 1974.

37. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 82: 70-7, 1959.

38. Mitusi A, Ohata T. Photooxidative consumption and photoreductive formation of ascorbic acid in green leaves. Plant Cell Physiol 2: 31-44, 1961.

39. Pick E, Keisariy Y. Superoxide anion and hydrogen peroxide production by chemically elicited peritoneal macrophages - induction by multiple nonphagocytic stimuli. Cell Immunol 59: 301-18, 1981.

40. Lowry OH, Rosebrough NJ, Farr AL et al. Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-75, 1951.

41. Desai SR, Verlecar XN, Ansari ZA et al. Evaluation of genotoxic responses of *Chaetoceros tenuissimus* and *Skeletonema costatum* to water accommodated fraction of petroleum hydrocarbons as biomarker of exposure. Water Res 44: 2235-44, 2010.

42. Pacheco M, Santos MA. Biotransformation, endocrine and genetic response of *Anguilla anguilla* L. to petroleum distillate products and environmentally contaminated waters. Ecotoxicol Environ Saf 12: 64-75, 1991.

43. National Institute of Oceanography. Rapid EIA for Desiltation of Panaji Bank of River Mandovi from New Patto Dridge up to Bus Stop Opposite Food and Drug Control Office. NIO Technical Report No. NIO/SP-24. NIO. Goa, India; 2004.

44. National Institute of Oceanography. Environmental Monitoring for Dredging and Reclamation for Berth 5A and 6A at Mormugao Harbour. NIO Technical Report No. NIO/SP-32. NIO. Goa, India; 2004.

45. Xu L, Zheng GJ, Lam PKS et al. Relationship between tissue concentrations of polycyclic aromatic hydrocarbons and DNA adducts in green-lipped mussels *Perna viridis*. Ecotoxicol 8: 73-82, 1999.

46. Labieniec M, Gabryelak T. Response of DNA, proteins and membrane bilayer in the digestive gland cells of fresh water mussel *Unio tumidus* to tannins exposure. Toxicol in Vitro 18: 773-81, 2004.

47. Esterbauer H, Schaur RJ, Zollner H et al. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radical Bio Med 11: 81-128, 1991.

48. Marnett LJ. Lipid peroxidation-DNA damage by malondialdehyde. Mutat Res 424: 83-95, 1999.

49. Stadtman ER, Berlett BS. Reactive oxygen mediated protein oxidation in aging and disease. Drug Met Rev 30: 225-43, 1998.

50. Zusterzeel PLM, Rutten H, Roelefs HMJ et al. Protein carbonyls in decidua and placenta of preeclamptic women as markers of oxidative stress. Placenta 22: 213-9, 2001.

51. Cheung CCC, Siu WHL, Richardson BJ et al. Antioxidant responses to benzo[a]pyrene and Aroclor 1254 exposure in the green-lipped mussel, *Perna viridis*. Environ Pollut 128: 393-403, 2004.

52. Cavaletto M, Ghezzi A, Burlando B et al. Effect of hydrogen peroxide on antioxidant enzymes and metallothionine level in the digestive gland of *Mytilus galloprovincialis*. Comp Biochem Physiol 131: 447-55, 2002.

53. Fridovich I. Superoxide radical: an endogenous toxicant. Annu Rev Pharmacol Toxicol 23: 239-57, 1983.

54. Cadenas E, Davis KJA. Mitochondrial free radical generation, oxidative stress and aging. Free Radic Biol Med 29: 222-30, 2000.

55. Reid DJ, MacFarlane GR. Potential biomarkers of crude oil exposure in the gastropod mollusc, *Austrocochlea porcata*: laboratory and manipulative field studies. Environ Pollut 126: 147-55, 2003.

56. Lima I, Moreira SM, Osten JR et al. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. Chemosphere 66: 1230-42, 2007.

57. Fournier D, Bride JM, Poirie M et al. Insect glutathione S-transferases: biochemical characteristics of the major forms of houseflies susceptible and resistant to insecticides. J Biol Chem 267: 1840-5, 1992.

58. Zhang JF, Wang XR, Guo HY et al. Effects of water-soluble fractions of diesel oil on the antioxidant defenses of the goldfish, *Carassius auratus*. Ecotoxicol Environ Saf 58: 110-6, 2004.

59. Winkler BS, Orselli SM, Rex TS et al. The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. Free Radic Biol Med 17: 333-49, 1994.

60. Luchmann HK, Mattos JJ, Siebert MN et al. Biochemical biomarkers and hydrocarbon concentrations in the mangrove oyster *Crassostrea brasiliana* following exposure to diesel fuel water accommodated fraction. Aquat Toxicol 105: 652-60, 201.