

DNA damage and oxidative stress in marine gastropod *Morula granulata* exposed to phenanthrene

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Abstract

DNA damage and oxidative stress in marine gastropod *Morula granulata* was measured after *in vivo* exposure to four different concentrations (10, 25, 50 and 100 µg/L) of phenanthrene. Comet assay was used for measurement of DNA damage, whereas oxidative stress was assessed using a battery of biomarkers such as glutathione-S-transferase (GST), catalase (CAT) and lipid peroxidation (LPO). Our data showed concentration-dependent increase in percentage DNA in tail (TDNA), LPO and GST activity in gastropods exposed to phenanthrene. CAT activity in gastropods was not found to be consistent with the phenanthrene concentrations. Significant increase in TDNA was observed at all concentrations above 10 µg/L of phenanthrene. Positive correlations were observed among oxidative stress biomarker and TDNA. Integrated biomarker response (IBR) analysis showed that among the four biomarkers, LPO and DNA damage (TDNA) were the most sensitive in response to phenanthrene exposure. Our results clearly showed that phenanthrene is genotoxic to gastropods and also causes oxidative stress.

Keywords: Gastropods; genotoxicity; biomarker; antioxidant enzyme; comet assay

1. Introduction

During the last decades, rapid increase in industrial, agricultural and urban activities has led to an unprecedented increase in anthropogenic contaminants in the environment. Among the environmental pollutants, polycyclic aromatic hydrocarbons (PAHs) are of great concern due to their pervasive nature. They are ubiquitous in the environment and are included in the list of persistent organic pollutant of United Nations Environment Program (UNEP, 1999). The International Agency for Research on Cancer (IARC) has classified PAHs as possible and probable carcinogen to human (IARC, 2010). PAHs tend to accumulate in marine organism because of their lipophilic and hydrophobic nature (Mashroofeh et al., 2015). Phenanthrene occurs as the major component of PAH and is among 16 PAHs that are on United States Environmental Protection Agency (USEPA) priority pollutant list (US, EPA, 2009). Phenanthrene has shown to cause cytotoxicity (Schirmer et al., 1998), genotoxicity (Machado et al., 2014), neurotoxicity (Martyniuk et al., 2009), oxidative stress (Giannapas et al., 2012) and mutagenic effects (Wood et al., 1979).

In last few years, oxidative stress has received great attention from aquatic toxicologists around the world (Souid et al., 2013; Regoli and Giuliani, 2014). Antioxidant enzymatic defences are useful biomarkers of pollution by PAH that generate oxidative stress in marine organisms (Niyogi et al., 2001a, b; Pan et al., 2006). Phenanthrene and its metabolites enhance the production of reactive oxygen species (ROS) which further leads to activation of variety of antioxidant enzymes (Martins et al., 2013). Among the antioxidant enzymes, Glutathione-S-transferase (GST) plays a significant role in the detoxification of the reactive products from lipid peroxidation (Olsvik et al., 2010; Fernandez et al., 2012). An increase of GST activity has been reported in mussels *M. edulis* exposed to PAH (Gowland et al., 2002). ROS produced as a result of PAH metabolized products has been shown to induce lipid peroxidation (LPO) (Giannapas et al., 2012). LPO in scallop *Chlamys ferrari* exposed to PAH, benzo(a)pyrene (BaP) has been studied (Pan et al., 2006). Catalase (CAT) is another very important antioxidant enzyme that plays a key role in antioxidant mechanism by converting hydrogen peroxide (H_2O_2) to water. Catalase activity (CAT) was measured in clam (Frouin et al., 2007), fish (Kopecka-Pilarczyk and Correia, 2009) exposed to PAH. ROS can induce genotoxic damage by modifying integrity of DNA (Gauthier et al., 2014). The integrity of DNA can be greatly affected by genotoxic agents due to DNA strand breaks, loss of methylation and formation of DNA adducts (Pisoni et al., 2004). The relationship between DNA damage and the exposure of gastropods to environmental contaminants is well documented (Sarkar et al., 2008, 2011; An et al., 2012; Bhagat et al., 2012). Comet assay has been extensively used for the evaluation of DNA damage in aquatic organisms because of its rapid, versatile and reliable nature (Frenzilli et al., 2009; de Lapuente et al.,

2015). Comet assay has been used for the measurement of DNA damage by phenanthrene in flounder *Paralichthys olivaceus* (Woo et al., 2006), clam *Ruditapes decussatus* (Martin et al., 2013) and mussel *Mytilus galloprovincialis* (Dailianis et al., 2014). *In vivo* studies of mussel (Perez-Cadahia et al. 2004; Banni et al., 2010) exposed to PAH have been studied using comet assay. DNA damage as measured by comet assay has been reported in mussel (Dailianis et al., 2014), clam (Martins et al., 2013) and Flounder (Woo et al., 2006). Few reports are available in gastropods using comet assay (Angeletti et al., 2013; Almamoori et al., 2013; Sarkar et al., 2013, 2014; Ali et al., 2015).

Aquatic ecosystem is an important source of food and plays an essential role in human health. Consumption of aquatic organisms exposed to toxicants can cause health risk to human. In this context, biomonitoring of marine organisms has become necessary in studying the effect of these toxic pollutants. Molluscs are extensively used as a sentinel organism in areas affected by pollution. Gastropods account for eighty percent of all molluscs, and are excellent sentinel organism for environmental monitoring due to their sedentary life style. Gastropods have been known for their large accumulation of toxic chemicals into their system due to their feeding behaviour (Zhang et al., 2012; Boshoff et al., 2013). Genotoxicity studies with gastropods represent an important effort in determining the potential adverse effects of PAH. The marine gastropod, *Morula granulata* has been previously used as a bioindicator in ecotoxicological studies (Reitsema and Spickett, 1999; Bech, 2002; Afsar et al., 2012). The current study was carried out to evaluate the effect of phenanthrene on the DNA damage in marine gastropod *Morula granulata*. In addition, enzymatic and non-enzymatic biomarkers GST, CAT and LPO were measured to study the possible antioxidant defence mechanism. Use of multiple biomarkers produces large amount of data which is hard to interpret. In this context of integrated biomarker response (IBR) is widely used in aquatic organisms exposed to contaminants (Barda et al., 2014; Liu et al., 2014; Pain-Devin et al., 2014; Turja et al., 2014). IBR simplifies the response interpretation of multiple biomarkers by providing a single value; hence in this study IBR was applied to study the effect of phenanthrene in *M. granulata*.

2. Material and Methods

2.1. Chemicals

1-chloro-2,4-dinitrobenzene (CDNB), ethidium bromide, L-glutathione reduced, ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), guaiacol glycerol ether, hydrogen peroxide (H₂O₂), phenanthrene, trypan blue were purchased from Sigma Aldrich Pvt. Ltd. Frosted slides and cover slips were supplied by Himedia Pvt. Ltd. Dimethyl sulfoxide was obtained from Qualigens, India. Tris buffer and triton X-100 were obtained from Merck Ltd India.

2.2. Sampling site

For this study, *M. granulata* were collected during low tide from intertidal rocks scattered along Arambol beach, Goa, India (Supplementary Fig. 1 and 2). This site was chosen as reference site because of its serene environment (Sarkar et al., 2014). Gastropods were identified using the certified reference sample from Zoological Survey of India; Kolkata, India (Subba Rao et al., 1992). The size of the gastropods ranged from 2 cm to 3 cm. They were transported in plastic container to the lab within 3 h of collection. The collected snails were acclimatized in 4 litres plastic aquaria for 96 h in aerated seawater at room temperature before the beginning of the experiment. After acclimatization period, around 300 gastropods were equally divided in five experimental groups: control group and four treatment groups with different concentrations of phenanthrene (10, 25, 50 and 100 µg/L). Phenanthrene was first diluted in DMSO and then mixed with seawater to make the stock solution (2500 µg/ml). The final phenanthrene concentration for treatment groups were achieved by diluting stock solution in seawater. The final concentration of dimethyl sulfoxide (DMSO) in all treatment groups was less than 0.01%. Control gastropods were incubated in seawater containing 0.01% of DMSO. The concentration of phenanthrene used in this experiment were in accordance with studies in golden grey mullet *Liza aurata* (Oliveira et al., 2007) and tilapia *Oreochromis niloticus* × *O. aureus* (Xu et al., 2009). Snails were exposed to phenanthrene for 24 h in aerated seawater at room temperature. They were not fed during the exposure.

2.3. Cell harvesting

The shells of the gastropods were gently broken and whole body tissues were excised out carefully. Soft tissues from four to five animals measuring one gram were pooled together for comet assay as well as biochemical analysis. For comet assay soft tissues were chopped into small pieces in cold extrusion buffer (71.2 mM NaCl, 5 mM EGTA, 50.4 mM guaiacol glycerol ether, pH 7.5). It was then centrifuged at 5000 rpm for 3 min. The pellet was then washed with phosphate buffer saline (1.2 M NaCl, 0.027 M KCl, 11.5 mM K₂HPO₄, 0.08 M Na₂HPO₄, pH 7.3) and suspended in the same buffer. Cell viability was determined using 0.4% Trypan blue exclusion test (Anderson et al., 1994). The cell viability was found to be greater than 85% in all the experiments.

2.4. Biochemical Assays

Glutathione-S-transferase activity was determined following the methods of Habig et al., (1974) and expressed in nM of CDNB conjugate per min per mg of total protein. The assay tube contains reaction buffer (0.1 M K₂HPO₄, EDTA-Na₂, pH 6.5), 0.1 ml of GSH, 0.1 ml of CDNB and 100 µl of cytosolic fraction. The change in absorbance was recorded at 340 nm for every 30 seconds for 5 min.

The activity of GST was determined using extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ for CDNB. The activity of GST was expressed as nM/min/mg of protein.

Catalase activity was measured according to method of Sinha et al., (1972). The reaction mixture contains 0.5 ml of 0.2 M hydrogen peroxide (H_2O_2), 1 ml of sodium phosphate buffer (0.01 M, pH 7.0) and 0.4 ml distilled water. The reaction was stopped by 2 ml of dichromate-acetic acid reagent (containing potassium dichromate 1 part and glacial acetic acid 3 parts). It was then heated for 10 min and allowed to cool; the green colour developed was read at 583 nm against blank on a spectrophotometer. The activity of CAT was expressed as mM of H_2O_2 consumed/min/mg protein.

Lipid peroxidation was measured by method adapted from Ohkawa et al., (1979). Briefly, 1 g of soft tissue was homogenized with 9 ml of 0.25 M sucrose using ultra turrax homogenizer for 1 minute. 0.2 ml of the tissue homogenate was then mixed with 0.2 ml of 8% SDS, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA. The mixture was made up to 4 ml using distilled water and heated at 95°C for 60 min. It was then cooled and centrifuged at 3000 rpm for 10 min. The absorbance was read at 532 nm. LPO value was measured as malondialdehyde (MDA) equivalent and expressed as nM of MDA mg^{-1} of protein.

Proteins were determined according to the method of Lowry et al., (1951), using bovine serum albumin as standard.

2.5. *In vitro* exposure to hydrogen peroxide

To validate the comet assay protocol, freshly dissociated cells from soft tissues were treated with H_2O_2 (1; 10; 25 and 50 μM) in PBS at room temperature (25°C) for 30 min in dark. The control cells were incubated in PBS without H_2O_2 . Three replicates per condition were performed.

2.6. Comet assay

The alkaline version of comet assay was performed following the protocol described by Singh et al., (1988) with minor modifications. About 20 μl of cell suspension was mixed with 100 μl of low melting agarose and poured over frosted glass slide pre-coated with 100 μl 1% normal melting agarose. Cells were lysed in lysis buffer (2.5 M NaCl, 0.1 M di-sodium EDTA, 0.01 M Tris Buffer, 0.2 M NaOH with 1% Triton X-100, and 10% DMSO added just before use) for 1 hour at 4°C in dark. Afterwards, the DNA was allowed to unwind for 15 min in electrophoretic buffer (300 mM NaOH, 1 mM EDTA, pH 13.0). The electrophoresis was carried out in the same buffer at 20 V ($\sim 300 \text{ mA}$) for 15 min. Following electrophoresis, the cells were neutralized using neutralizing buffer (0.4 M Tris, pH 7.5) drop wise for four times at an interval of 5 min each. The cells were then

stained with ethidium bromide. All the steps for slide preparation were performed under yellow light to prevent additional DNA damage.

The slides were examined with a fluorescent microscope (DM100 Leica, Leica Microsystems, Germany) equipped with appropriate filters (excitation wavelength of 515–560 nm and emission wavelength of 590 nm). The comets were observed at a magnification of 40X and photographed using charge-coupled device (CCD) camera attached to the microscope. Comet scoring was carried out using image analysis system Komet 6.0 (Kinetic Imaging, UK). 100 comets per test (2 slides) were randomly selected, scored and analyzed. All experiments were carried out in triplicate to take into account of the possible variations between different cell preparations. TDNA was scored from each slide and expressed in terms of means \pm standard deviation for each treatment group (Anderson et al., 1994).

2.7. Integrative Biomarker Response (IBR)

Integrated biomarker response (IBR) was calculated as described by Beliaeff and Burgeot (2002) with modification by Devin et al., (2014).

Briefly the biomarker response data was standardized using the formulae

$$Y_i = (X_i - m) / s$$

where Y_i is the standardized biomarker response, X_i is response value of each biomarker, m and s are mean value and standard deviation respectively.

Z_i was then calculated using the formulae as $Z_i = Y_i$ or $Z_i = -Y_i$ for biomarker responding to contamination by induction or inhibition, respectively. The minimum value for each biomarker was also calculated from the standardised biomarker response.

The scores for the biomarker was computed as $S_i = Z_i + |\text{Min}_i|$

Individual areas A_i connecting the i^{th} and the $(i + 1)^{\text{th}}$ radius coordinates of the star plot were obtained according to the formula:

$$A_i = S_i * S_{i+1} * \sin(2\pi/k) / 2$$

where S_i and S_{i+1} represent the individual biomarker scores (calculated from standardised data) and their successive star plot radius coordinates and k represent the number of radii corresponding to the biomarkers used in the survey.

Biomarkers were ranged clockwise from sub-cellular level to individual level as follows: TDNA, CAT, GST, LPO (Serafim et al., 2012).

And the IBR value is calculated as follow:

$$IBR = \sum_{i=0}^n A_i$$

Where A_i is the triangular area represented by two consecutive biomarker scores on the star plot, and n is the number of biomarkers used in the IBR calculation.

2.8. Statistics Analysis

Data on DNA damage and oxidative stress parameters were presented as arithmetical mean \pm standard deviation. Statistical differences between the control and the treated cells and also among treated cells of different concentrations were analyzed using analysis of variance (ANOVA) followed by Tukey HSD post-test. Kolmogorov–Smirnov test for normality of distribution was used prior to ANOVA. Spearman correlation matrix was also calculated to study the relationships between the different biomarkers measured. Three levels of significance are reported: (a) $p < 0.05$, (b) $p < 0.01$, and (c) $p < 0.001$. All statistical analysis was performed using OriginPro 8.5.0.

3. Results

3.1. Biochemical Assays

GST activity increased significantly at all the exposed concentrations with respect to control, indicating oxidative damage in treated gastropods (Fig. 1a). Overall GST activity showed concentration-dependent increase in gastropods exposed to phenanthrene. GST activity increased from 23.88 ± 1.12 nM/min/mg in control to 82.33 ± 5.62 nM/min/mg at 100 $\mu\text{g/L}$ ($P < 0.01$) of phenanthrene. There were significant differences in GST activity at 10 $\mu\text{g/L}$ and 25 $\mu\text{g/L}$ ($P < 0.01$), 50 $\mu\text{g/L}$ ($P < 0.001$) and 100 $\mu\text{g/L}$ ($P < 0.001$).

There was no consistent change in CAT activity in exposed snails. CAT showed significant decrease at 25 $\mu\text{g/L}$ with respect to control ($p < 0.01$) (Fig. 1b). However a significant difference in CAT activity was observed when 25 $\mu\text{g/L}$ and 50 $\mu\text{g/L}$ were compared ($p < 0.001$).

These results showed that LPO were higher in gastropods exposed to phenanthrene as compared to control gastropods (Fig. 1c). LPO in control was found to be 0.21 nM MDA/mg, and on exposure to phenanthrene, increased to 0.26 ± 0.03 nM MDA/mg at 10 $\mu\text{g/L}$ ($p < 0.05$) and 0.37 ± 0.03 nM MDA/mg at 25 $\mu\text{g/L}$ ($p < 0.05$). However, there was decrease in LPO observed from 0.49 ± 0.04 nM MDA/mg at 50 $\mu\text{g/L}$ to 0.39 ± 0.06 nM MDA/mg at 100 $\mu\text{g/L}$. When LPO between 25 $\mu\text{g/L}$ and 100 $\mu\text{g/L}$ were compared no significant difference was observed. Moreover, the 50 $\mu\text{g/L}$ displayed a significant increase with all the other groups.

DNA damage- comet assay

3.2. In vitro exposure to hydrogen peroxide

Fig. 2 shows significant increase in TDNA in *M. granulata* exposed to hydrogen peroxide. H₂O₂ at 50 µM showed 2.3 fold increase in TDNA compared with the control (p<0.001). H₂O₂ induced a concentration-dependent increase in TDNA.

3.3. In vivo exposure of gastropods to phenanthrene

TDNA as measured by comet assay in *M. granulata* exposed to phenanthrene also showed significant increase in DNA damage (Fig. 2). A significantly increase in TDNA was observed at 50 µg/L (28.60±1.23) (p < 0.001) and 100 µg/L (32.6±0.19) (p < 0.001) with respect to control (18.23±0.47). It has been observed that TDNA increased significantly at 100 µg/L as compared to those in 10 µg/L (p < 0.001) and 25 µg/L (p < 0.001).

3.4. Relationship between oxidative stress parameters and comet assay

There were significant correlations between GST and LPO (r = 0.86, P < 0.001) as well as between GST activity and TDNA (r = 0.96, P < 0.001). A similar pattern between TDNA and LPO (r = 0.82, P < 0.001) was revealed by spearman's correlation. There was no correlation observed between CAT and any of the parameters tested.

3.5. Integrated Biomarker Response (IBR)

The IBR values for four biomarkers were standardized and computed as star plot (Table 1, Fig. 3). All the four biomarkers were responsive to phenanthrene exposure and showed similar pattern in star plots. The IBR values increased with increase in exposure concentrations of phenanthrene. The IBR value ranged from 0.70±0.07 to 45.74±1.45. The lowest value for IBR was observed in control samples whereas the highest value was observed at 100 µg/L of phenanthrene. There was no significant variations observed when IBR values for 10 µg/L were compared with control. At 50 µg/L and 100 µg/L concentrations, all the biomarker showed significant increase in IBR values when compared to control (p < 0.001).

4. Discussion

Hydrophobic contaminants are efficiently taken up inside the cell because of its lipophilic nature. Phenanthrene has been shown to be accumulated in oyster (Sanders, 1994), mussels (Namiesnik et al., 2008), clams (Tian et al., 2014), scallop (Hannam et al., 2010 a,b), amphipod (Landrum et al., 1994) and fishes (Bandowe et al., 2014). Studies have reported high concentrations of phenanthrene in tissues of gastropods (Rostad and Pereira, 1987). In our study exposure to phenanthrene in *M.*

granulata has resulted in induction of antioxidant enzymes to combat the ROS produced. Results of this study indicate concentration-dependent increase in GST activity in phenanthrene exposed snails. Increase in GST activity in exposed snails may be a physiological adaptation for the elimination of ROS produced due to phenanthrene and it demonstrates activation of detoxification mechanism. GST is a phase II multifunctional enzyme and plays a critical role in conjugation of electrophilic compounds (phase I metabolites) on one hand, and in the defense against oxidative damage and peroxidative products of DNA and lipids (Oost et al., 2003) on the other hand. GSTs are involved in the metabolic activation and deactivation of PAH metabolites. An increase in GST activity indicates increase in the production of hydroxyl radical in liver of *C. auratus* (Sun et al., 2006) and impairment in oxidative system in oyster *Crassostrea brasiliana* (Luchman et al., 2014). Similar findings were also reported in clam, *Venerupis philippinarum* when exposed to 0-50 µg/L of phenanthrene (Zhang et al., 2014). Other studies have shown induction of GST activity in molluscs *Pecten maximus* exposed *in vitro* to PAH in crude oil (Pennec and Pennec, 2003). Silva et al., (2005) has also observed a significant increase in GST activity in oyster *Crassostrea rhizophorae* exposed to diesel oil.

We have not observed any consistent pattern of change in CAT activity in phenanthrene exposed snails. CAT enzyme, along with glutathione peroxidase (GPx), is essential for removing the hydrogen peroxide formed during oxidation reactions. CAT converts the hydrogen peroxide to water and its activity is dependent on various factors such as level of H₂O₂ in the cell, presence of oxygen, activity of superoxide dismutase and GPx. Similar findings were reported in estuarine guppy *Poecilia vivipara* exposed to phenanthrene (10, 20 and 200 µg/l) (Machado et al., 2014). Increase, decrease or unaltered activity of CAT in aquatic organism exposed to PAH has been reported by various studies (Cheung et al., 2001; Niyogi et al., 2001a, b; Pichaud et al., 2008). Frouin et al., (2007) also reported unaltered CAT activity in clams *Mya arenaria* exposed to PAH. B[a]P exposure to *E. fetida* (Saint-Denis et al., 1999) and *Lateolabrax japonicus* (Jifa et al., 2006) has also shown unaltered CAT activities. Wu et al., (2011) has also found that CAT activity in *Eisenia fetida* was unaltered suggesting that PAH exposure does not induce increased CAT activity.

In our studies we have reported consistent increase in LPO in *M. granulata* from control to 50 µg/L of phenanthrene. Free radicals produced as a result of metabolic activities can react with polyunsaturated fatty acids in cell membrane, resulting in increase of lipid peroxidation (Livingstone et al., 2003). Previous studies have also confirmed that short-term exposure to phenanthrene induces LPO in gill cells of fish *Liza aurata* (Oliveira et al., 2008). Phenanthrene exposure induces a similar increase in LPO in fish *Sparus aurata* (Kopecka-Pilarczyk and Correia, 2009) and in scallop *Pecten*

maximus (Hannam et al., 2010a). Increase in lipid peroxidation in *Sparus aurata* exposed to phenanthrene was also reported by Correia et al., (2007). LPO results in production of MDA that can react with DNA and form DNA adduct. Increased formation of DNA adduct in gill cells of *Mytilus galloprovincialis* after exposure to PAH, BaP have been reported by Venier and Canova, (1996). We have also observed significant correlation between LPO and GST in marine gastropod ($r = 0.86$, $P < 0.001$). Therefore antioxidant enzyme activities and LPO levels in *M. granulata* can be the reflection of the whole ability of detoxification of the organism as well as the damage extent caused by the oxidative stress.

The results of comet assay in *M. granulata* suggested that phenanthrene induces DNA damage in snail. In our study significant differences in TDNA were observed among the groups. TDNA in tail increases with exposure to phenanthrene with their highest value recorded at 100 $\mu\text{g/L}$. It should be noted that at low concentration (10 $\mu\text{g/L}$) of phenanthrene, percent DNA in tail was not found to be significant with respect to control. Above 10 $\mu\text{g/L}$, all the groups showed significant increase ($P < 0.001$) in TDNA as compared to control. The DNA damage occurred in this study could be due to various reasons such as DNA single strand breaks, DNA double strand breaks, DNA adduct formations, DNA–DNA, and DNA–protein cross-links (Mitchelmore et al., 1998) resulting from the interaction of PAH or their metabolites with DNA. Accumulation of PAH has been linked to DNA damage through production of ROS (Jarvis et al., 2013). The ROS produced as a result of PAH exposure can cause single or double strand breakage in the DNA (Kaloyianni et al., 2009). Khan et al., (2013) has reported strong correlation between phenanthrene concentration and DNA damage as measured by comet assay in *E. feotida*. A recent work carried out with mussel *Mytilus galloprovincialis*, showed elevated levels of DNA damage in phenanthrene treated haemocytes (Dailianis et al., 2014). A higher comet score was observed in the erythrocytes of fish, *Poecilia vivipara* exposed to 10 $\mu\text{g/L}$ of phenanthrene (Machado et al., 2014).

In our study, the lowest mean values of IBR for the four biomarkers were observed in control samples. The IBR results demonstrated that LPO and TDNA were the most responsive in gastropods exposed to phenanthrene. IBR index has been used to study the PAH contamination in caged mussel *Mytilus trossulus*, *Mytilus galloprovincialis* (Tsangaris et al., 2011; Dabrowska et al., 2013). Campillo et al., (2013) have used integrated biomarker response index to assess the agricultural inputs using a battery of biomarkers such as AChE, GST, CAT and LPO in caged clam *Ruditapes decussatus*. IBR index was successfully used in *Cyprinus carpio* to study toxicological effects of perfluorinated organic compounds (Kim et al., 2010). IBR has been used to construct star plot with PAH and PCB concentrations in tissues along with early warning signals (EROD, GST, CAT, AChE

enzymatic activities) and adverse effects (DNA adducts) biomarkers in flounder *Platichthys flesus* (Beliaeff and Burgeot, 2002).

In conclusion our study demonstrates *M. granulata* as a good candidate to be used as a sentinel organism to detect PAH pollution in marine environments. This study demonstrated that phenanthrene at 10-100 µg/L concentration level can induce oxidative stress in *M. granulata* leading to DNA damage. The results suggest that LPO and GST activity can be used as indicator of oxidative stress in snails exposed to phenanthrene. IBR approach was successfully applied to investigate the adverse effects of phenanthrene in gastropods.

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Figure captions:

Supplementary Fig. 1. Sampling site at Arambol, Goa, India.

Supplementary Fig. 2. *Morula granulata* spread over the oyster belt in the intertidal rocks scattered along Arambol, Goa, India

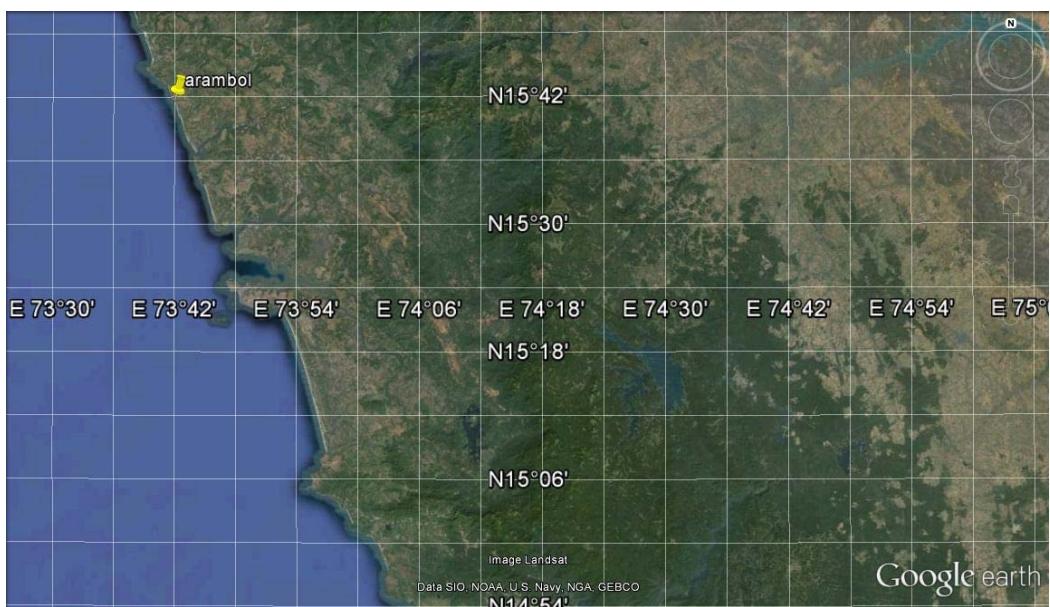
Fig. 1. Glutathione-S-transferase (GST), catalase (CAT) and lipid peroxidation (LPO) value, depicted by mean±standard deviation in marine gastropod *Morula granulata* exposed to phenanthrene. (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$ significantly different from the control (ANOVA, Tukey HSD post-test).

Fig. 2. Comet parameter (DNA strand breakage) in *Morula granulata* exposed to hydrogen peroxide and phenanthrene. Values are means± standard deviation, (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$ significantly different from the control (ANOVA, Tukey HSD post-test).

Fig. 3. Star plots for standardized biomarker response in *Morula granulata* exposed to different concentrations of phenanthrene.

Tables :

Table 1. Integrated biomarker response (IBR) in gastropod *Morula granulata* exposed to different concentrations of phenanthrene.



Supplementary Fig. 1



Supplementary Figure 2

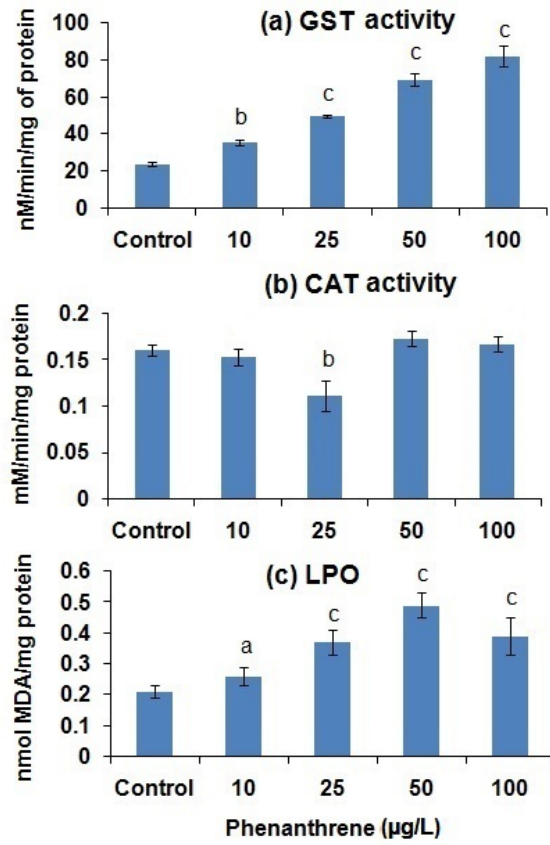


Fig 1

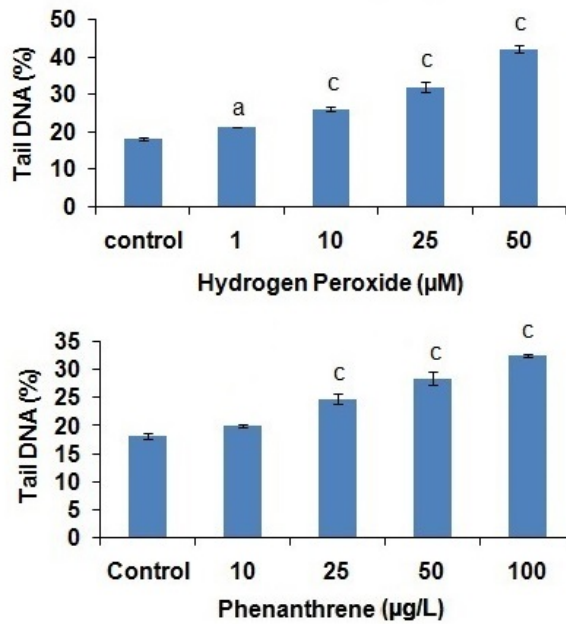


Fig 2

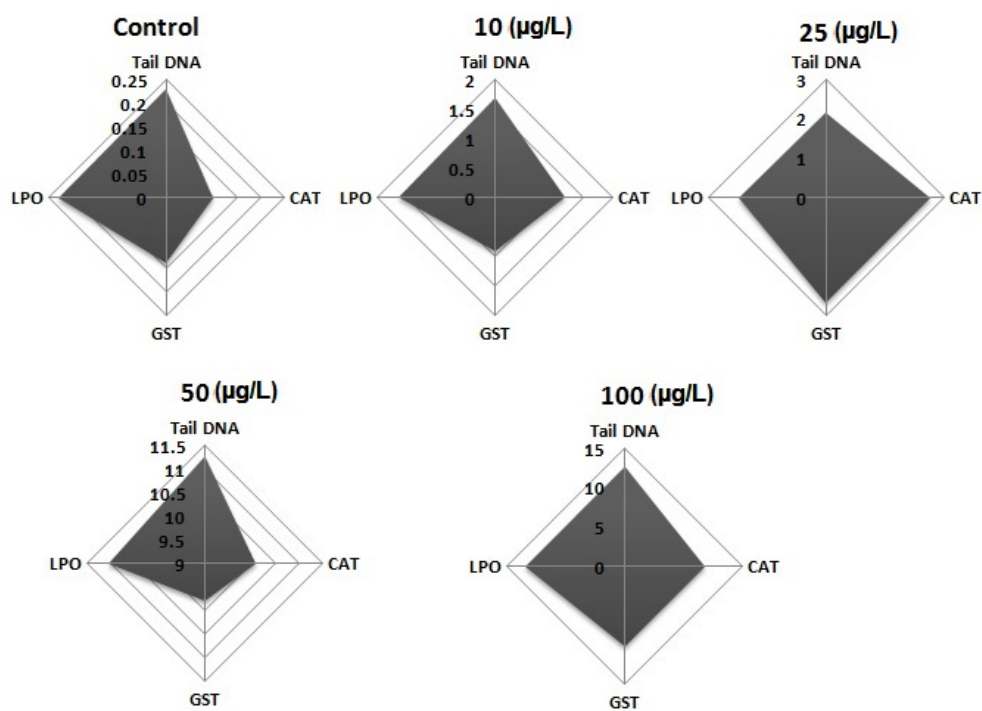


Fig 3

Table 1

Exposure concentrations (µg/L)	Score of Biomarkers				IBR value
	Tail DNA	CAT	GST	LPO	
Control	0.23±0.07	0.10±0.09	0.14±0.15	0.23±0.07	0.70±0.07
10	1.69±0.20	1.19±0.16	0.92±0.19	1.64±0.19	5.44±0.37
25	2.16±1.01	2.67±0.49 ^a	2.66±0.48 ^b	2.22±0.90	9.71±0.28
50	11.29±0.64 ^c	10.08±0.29 ^c	9.79±0.50 ^c	11.05±0.98 ^c	42.21±0.73 ^c
100	12.70±1.98 ^c	10.20±1.70 ^c	10.16±1.17 ^c	12.68±1.72 ^c	45.74±1.45 ^c

^ap<0.05

^bp<0.01

^cp<0.001