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RESEARCH REPORT

An integrated approach to study the biomarker responses in marine gastropod *Nerita chamaeleon* environmentally exposed to polycyclic aromatic hydrocarbons

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Abstract

Ecological risk assessment using multiple biomarkers produce a large amount of data that is hard to interpret and the result are often contradictory. In this context, Integrated Biomarker Response (IBR) index was used to integrate the biomarkers effects to assess the impact of environmental contaminants in marine gastropod *Nerita chamaeleon* from Goa, India. Genotoxic (DNA damage as measured by comet assay and alkaline unwinding assay) and biochemical [superoxide dismutase, catalase, glutathione S-transferase, lipid peroxidation and acetylcholinesterase] biomarkers were measured in snails collected from different sites (Arambol, Anjuna, Sinquerim, Dona Paula, Velsao, Betul and Palolem). Total polycyclic aromatic hydrocarbons in snail tissue were in the range from 5.29 - 12.14 µg/g wet weight. Standardized values of biomarker response were visualized using star plots, which show unique patterns for different biomarkers. The mean IBR value was found to be highest at Dona Paula (8.07 ± 0.91) followed by Sinquerim (6.95 ± 0.91), Velsao (4.48 ± 0.68), Anjuna (3.28 ± 1.05), Palolem (2.53 ± 0.73), Arambol (1.81 ± 0.21) and Betul (0.88 ± 0.77). Additionally, the IBR values were found to be positively correlated with PAH concentration in snail tissues. These results suggest that integration of biomarkers effects using IBR along with chemical analysis can be a useful tool for the assessment of environmental pollution and to identify spatial patterns of contamination in the aquatic ecosystem.

Key Words: Integrated biomarker response; oxidative stress; genotoxic damage; polycyclic aromatic hydrocarbon; comet assay

Introduction

Polycyclic aromatic hydrocarbons (PAH) are persistent and ubiquitous environmental contaminants found in air, water, and soil. They are studied extensively due to their carcinogenic properties for human as well as animals. The International Agency for Research on Cancer (IARC) has classified PAHs as possible and probable carcinogen to human (IARC, 2010). The lipophilic and hydrophobic nature allows PAHs to accumulate in the marine organism (Mashroofeh *et al.,* 2015). The accumulation of PAHs in a marine organism can negatively affect their health (Frouin *et al.,* 2007; Grintzalis *et al.,* 2012). PAHs and their metabolites interact with DNA and form DNA adducts. PAH activation process also generates reactive oxygen species (ROS) which can induce genotoxic damage by modifying integrity of DNA (Mattson *et al.,* 2009).

Biomarkers are an important tool to detect exposure and adverse effects of human-made or natural contaminants on aquatic organisms. Some biomarkers are specific to chemicals or group of chemicals while other are non-specific and induces upon exposure to broad range of pollutants. Due to the complexity of contaminants, use of multi-biomarker has become an increasingly popular tool to study the environmental parameters as well as organism health. Comet (or single cell gel electrophoresis) assay is the most commonly used as a biomarker of DNA damage in various research areas because of it sensitive and reliable nature. DNA damage as measured by comet assay has been reported in mussels, clams and several other aquatic organisms (Martins *et al.,* 2013; Dailianis *et al.,* 2014; Sarkar *et al.,* 2014). Another technique is known as DNA-alkaline unwinding assay (DAUA) is also widely used to detect DNA damage in aquatic

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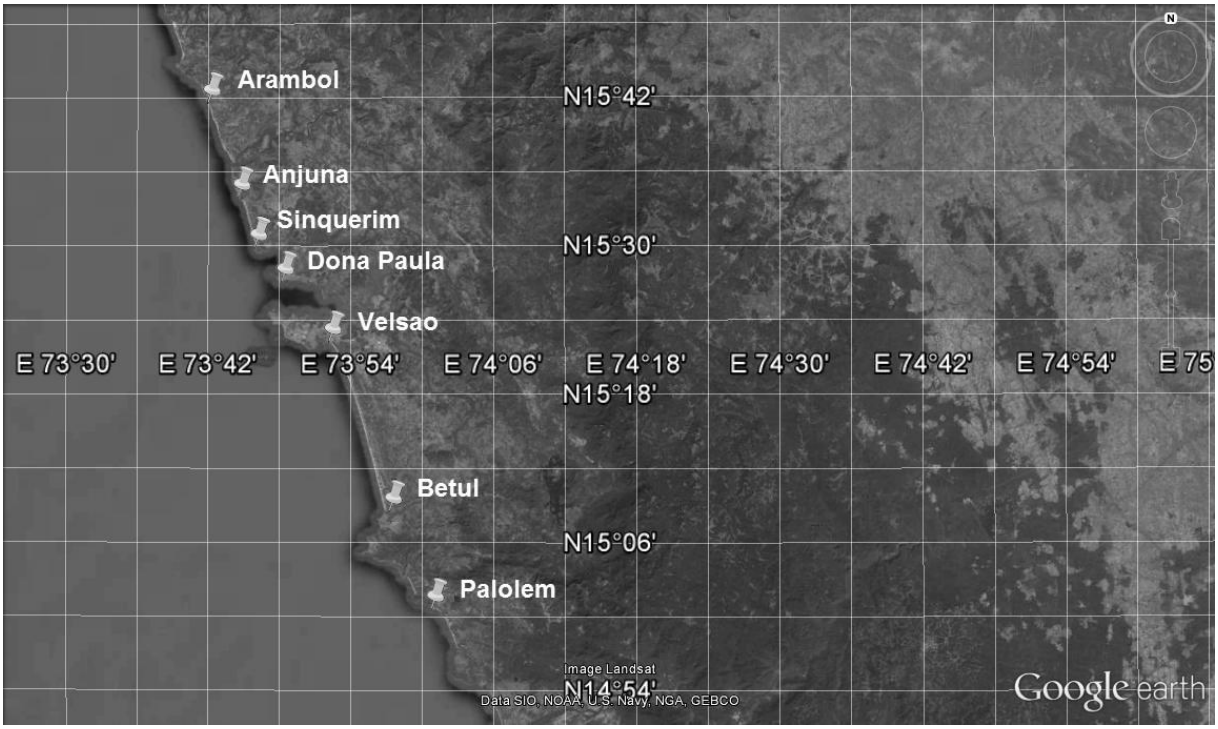

Fig. 1 Sampling site located along Goa, west coast of India.

organisms (Sarkar *et al.,* 2008, 2013; Oliveira *et al.,* 2010).

Several antioxidant enzymes are induced to combat the excessive ROS produced as a result of PAH metabolism. Overproduction of ROS can cause oxidative damage in a cell leading to damage to proteins, molecules, and DNA. Studies on antioxidant enzymatic defenses as biomarkers of oxidative stress are well documented in marine organisms (Niyogi *et al.,* 2001a, b; Pan *et al.,* 2006). Among the oxidative stress biomarkers superoxide dismutase (SOD), catalase (CAT), Glutathione S-transferase (GST), and lipid peroxidation (LPO) have been widely used as an environmental biomarker in gastropods (Abdel-Halim *et al.,* 2013; Zheng *et al.,* 2013; Wang *et al.,* 2014). GST helps in detoxification of the reactive products produced as a result of lipid peroxidation (Olsvik *et al.,* 2010). Correlation between GST activity and PAH in the tissues has been reported in several studies on mussels *Mytilus edulis* (Gowland *et al.,* 2002). Pan *et al*. (2006) have studied lipid peroxidation in scallop *Chlamys ferreri* exposed to PAH, benzo(a)pyrene (BaP). Catalase activity was measured in clam (Frouin *et al.,* 2007) and fishes (Nahrganga *et al.,* 2009) exposed to PAH. Significant induction of lipid peroxidation was detected in mussels collected near oil spillage site (Porte *et al.,* 1991). The author also showed a strong correlation between lipid peroxidation and total body PAH in mussels. Acetylcholinesterase (AChE) activity is another very useful biomarker of neurotoxic contaminants in marine organisms (Gaitonde *et al.,* 2006; Sarkar *et al.,* 2006). AChE has been considered as specific biomarkers for organophosphate and carbamate pesticides. Recent studies also report inhibition of AChE in gastropods exposed to heavy metals and biocides (Gaitonde *et al.,* 2006; Ma *et al.,* 2014).

The combination of biomarkers yields a complicated and vast data which is hard to interpret. Integrated biomarker response (IBR) integrates results from individual biomarkers into a single index, called IBR index which provides a comparison between stations and also between biomarkers. It is widely used in aquatic organisms exposed to contaminants(Barda *et al.,* 2014; Turja *et al.,* 2014). IBR can also be summarized into a star plot where radius values are IBRs estimated at each station. Star plots provide corresponding information regarding mechanisms of biological effects of contaminants (Marigómez *et al.,* 2013). Beliaeff and Burgeot (2002) were the first to construct star plot using IBR values in flounder *Platichthys flesus* using EROD, GST, CAT, AChE enzymatic activities and DNA damage biomarkers along with PAH and PCB concentrations in tissues. PAH contamination in caged mussel *Mytilus trossulus* and *Mytilus galloprovincialis* was also studied using IBR index (Tsangaris *et al.,* 2011; Dabrowska *et al.,* 2013).

Molluscs have been widely used in environmental monitoring due to their economical and ecological importance. Gastropods have received great attention in recent years thanks to the discovery of imposex (Smith, 1981). Environment contaminants such as organotin compounds are known to cause imposex in snails that lead to a decline in pollution because of sterility and reproduction abnormality (Garaventa *et al.,*

2008). *Nerita* is among the oldest molluscan names, dating to Linnaeus and is a potential biological monitor for environment monitoring (Kumar and Devi, 1997; Kumar, 1990). In this study, a battery of biomarkers for genotoxic damage (comet assay and alkaline unwinding assay), oxidative stress (SOD, CAT, GST, LPO) and neurotoxicity (AChE) were considered. IBR integrates the biological response of multiple biomarkers and provides a single value; hence, in this study, IBR was applied to study the spatial variation in biomarkers in *N. chamaeleon*. PAH content in the tissues of snail was also determined to relate to the variability of the biomarker response.

Materials and Methods

Chemicals

1-chloro-2, 4-dinitrobenzene (CDNB), acetylcholine bromide, bromothymol blue, calf thymus DNA, epinephrine, ethidium bromide, ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), guaiacol glycerol ether, hydrogen peroxide (H_2O_2), L-glutathione reduced, trypan blue and Sephadex G-50 were purchased from Sigma-AldrichPvt. Ltd, India. Frosted slides and cover slips were supplied by Himedia, Goa, India, Dimethyl sulfoxide was obtained from Qualigens, Goa. Tris buffer and triton X-100 were obtained from Merck, Goa.

Sampling site and gastropods

Nerita chamaeleon (around 30 snails from each site) were collected during post-monsoon (2011) period from the intertidal rocks along the seven sites (Anjuna, Arambol, Sinquerim, Dona Paula, Velsao, Betul and Palolem) in Goa, west coast of India (Fig. 1). Arambol is situated at the northern tip of Goa near Tiracol estuary and is chosen as reference site because of its comparatively uncontaminated environment (Sarkar *et al.,* 2014). Anjuna and Sinquerim are most popularly known for their touristic activities around the world and hold a large number of restaurants, resorts, and shacks in the close proximity of the beach. Discharge of contaminated water from the shacks and restaurant might contribute largely towards the pollution at these sites. It should be noted that MV River Princess has been grounded on Sinquerim-Candolim beach since 2000. The grounding of the ship for such a long time release of huge amount of petroleum hydrocarbon from the ship might also contribute to coastal pollution (Ingole *et al.,* 2006). Dona Paula is situated between Mandovi and Zuari estuaries, just opposite to Mormugao harbor. Excessive shipping activities such as tourist boats, cargo ships, fishing trawlers, casinos, and barges carrying iron ores from the mines and water scooters are major contributors to the rapid increasing water pollution at this site. Velsao beach lies in the vicinity of one of the leading agrochemical industries (Zuari Agrochemicals) of India in Verna industrial state region. During the sampling at Velsao, the prevalence of pungent smell from the region of discharge outlet clearly indicated the prevailing state of coastal pollution. Betul and Palolem are situated in the southern part of Goa.

The collected snails were identified using the certified reference sample from Zoological Survey of India; Kolkata, India (Subba Rao *et al.,* 1992). Snails of similar size (around 18 - 30 mm) irrespective of their sex were used in this study. Snails were transported in a plastic container to the lab within 3 h of collection. The collected snails were acclimatized in 4 liters plastic aquaria for 48 h in aerated seawater at room temperature. The shells of the gastropods were gently broken and whole body tissue was carefully excised out. Soft tissues from three to four snails were pooled together (1 gram) and used for biochemical, alkaline unwinding assay and comet assay. All the measurements were carried out in triplicate.

Measurement of physicochemical parameters

Physicochemical parameters such as pH of the water from the sampling site were measured using pH analyzer ELICO Model LI-612, whereas turbidity and conductivity were measured using turbidimeter (systronics type 132) and conductivity meter (systronics digital direct Model: 304). Nutrients (nitrate, nitrite, and phosphate) were measured spectrophotometrically using Shimadzu UV 1800 spectrophotometer whereas dissolved oxygen (DO) and biochemical oxygen demand (BOD) were determined following the standard methods of Grasshoff *et al*. (1983).

Measurement of DNA Damage

DNA damage in snails has been evaluated by comet assay and alkaline unwinding assay. Comet assay was performed in *N. chameleon* using the methods as described in our previous studies (Sarkar *et al.,* 2015). The DNA integrity was measured in *N. chameleon* using partial alkaline unwinding assay following the methods of Sarkar *et al*. (2013).

Biochemical assays

For extraction of SOD, one gram of tissue was homogenized (using Ultra-turrax T 25 basic 1KA Werke homogenizer) in 1 ml of 0.1 M Phosphate buffer (pH 7.4). Following that 0.2 ml of ice cold chloroform, 0.15 ml of ice-cold ethanol, and 1 ml of distilled water was added and the whole solution was shaken thoroughly. The mixture was then centrifuged at 3000 rpm for 10 min at 4 °C (using eltek refrigerated centrifuge RC 4100D). SOD activity was determined by the rate of auto-oxidation of epinephrine to adrenochrome (Misra and Fridovich, 1972). The reaction volume (1 ml) contained 10 mM epinephrine, 50 mM sodium carbonate buffer (pH 10.2) and 10 mM EDTA. SOD activity is reported in per milligram of protein (U mg^{-1}); where 1 U of SOD is defined as the amount of sample causing 50 % of inhibition of epinephrine auto-oxidation.

For catalase, one gram of tissue was homogenized with 4 ml of phosphate buffer (0.1M, pH- 7.4) using high-speed Ultra Turrax homogenizer for 1 min and centrifuged at 18,000 rpm, 1 h, 4 ºC. Catalase activity was measured following the methods of Sinha *et al*. (1972). 1 ml of sodium phosphate buffer (0.01 M, pH 7.0), 0.5 ml of 0.2 M hydrogen peroxide (H_2O_2), and 0.4 ml distilled water

were mixed to make the reaction mixture. The reaction was stopped by pouring 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. After the mixture cools down, the absorbance 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.

Glutathione S-transferase (GST) activity was measured according to the method of Habig *et al*. (1974). It is based on conjugation of 1-Chloro-2,4-Dinitrobenzene (CDNB) solution (30 mM) and Reduced Glutathione (GSH) solution (30mM) in reaction buffer (0.1 M K_2HPO_4, EDTA-Na_2, pH 6.5). The change in absorbance was measured at 340 nm for every 30 seconds for 5 min using a UV-vis spectrophotometer. The activity of GST was determined using extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB. GST activity was expressed as nM/min/mg of protein.

Lipid peroxidation was measured by the 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 min.0.2 ml of 8 % SDS, 1.5 ml of 20 % acetic acid and 1.5 ml of 0.8 % TBA was added to 0.2 ml of the tissue homogenate. It was then made up to 4 ml using distilled water and heated at 95 ˚C for 60 min. It was then cooled and centrifuged at 3,000 rpm for 10 min. Following that, the absorbance was read at 532 nm. LPO value was measured as malondialdehyde (MDA) equivalent and expressed as nM of MDA min^{-1} mg^{-1}.

Extraction of AChE from tissue was performed in phosphate buffer (100 mM; pH 7.4) mixed with sucrose solution (250 mM) (1 ml of both per gram of tissue) spiked with 100µL TritonX-100 in order to break the tissue. The homogenate was centrifuged at 14,000 rpm at 4° C for 45 min. AChE activity in snails was measured using the ∆-pH-metric method as described by Sarkar *et al*. (1992) and Gaitonde *et al*. (2006). Briefly, 0.1 ml of sample enzyme was incubated with 0.2 ml of substrate (acetylcholine bromide) in phosphate buffer (0.01M, pH-8.0 ±0.10) with Bromothymol blue as an indicator. The changes in pH (∆-pH) was measured at an interval of 10 min over a period of 1 h of incubation corresponding to the amount of acetic acid liberated due to interaction with the acetylcholinesterase enzyme. The unit of activity of AChE was expressed as micromoles of acetic acid liberated per minute per mg of protein. Proteins were determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Estimation of polycyclic aromatic hydrocarbons (PAH)

PAH were extracted by homogenization of tissues of snails with bi-distilled hexane using Ultra Turrax homogenizer. The moisture content in the solvent extracts was removed by anhydrous sodium sulphate and the solvent extracts were concentrated to 1 ml using Kuderna Danish evaporator followed by purification of aliquots through alumina (10 % deactivated) column using bi-distilled hexane (Grasshoff *et al.,* 1983). The final 10 ml of aliquots thus obtained were measured by a spectrofluorometer (Shimadzu RF-5301 PC) with excitation at 310 nm and emission at 360 nm (Burns, 1993; Sarkar *et al.,* 2008, 2014). Kuwait oil was used as the standard. Total PAH in tissues was represented as µg/g wet weight.

Statistical Analysis

The data were expressed as mean ± standard deviation. Results from biochemical assays and genotoxic damage were analyzed by 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. Biomarker values were compared with the reference site, Arambol. Three levels were significance is reported: (a) $p < 0.05$, (b) $p < 0.01$, and (c) $p < 0.001$. All statistical comparisons were performed using OriginPro 8.5.0. Principal component analysis (PCA) was conducted to determine physicochemical water parameters association with biochemical using XLSTAT.

Integrative Biomarker Response (IBR)

Integrated biomarker response (IBR) was calculated as described by Beliaeff and Burgeot (2002) with modification by Guerlet *et al.* (2010) and Devin *et al.* (2014). Briefly, the biomarker response data for each site was standardized using the formulae $Y_i = (X_i\text{-}m)/s$ 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 for all sites respectively. The mean of standardized biomarker response (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 at all station was also calculated from the standardized biomarker response. The scores for the biomarker was computed as $S_i = Z_i+|Min_i|$. Individual areas Ai connecting the i^{th} and the $(i + 1)^{th}$ radius coordinates of the star plot were obtained according to the formulae $Ai = S_i * S_{i+1} * \sin(2\pi/k)/2$, where S_i and S_{i+1} represent the individual biomarker scores (calculated from standardized 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 as follows: percentage tail DNA (TDNA), DNA integrity value (DNA-F), SOD, CAT, GST, LPO and AChE (Serafim *et al.,* 2012).And the IBR value is calculated as follow: IBR= $\sum_{i=0}^{n} Ai$, 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.

Results

Water quality parameters

Physico-chemical water parameters varied significantly across all the sampling sites along the coast of Goa (Table 1). pH of the seawater was in the

Table 1 Physico-chemical properties of water sampled at different sites along the coast of Goa, India

Sites	pH	Temp. (˚C)	Turbidity (NTU)	Conductivity mS/cm	Nitrite µM/L	Nitrate µM/L	Phosphate µM/L	DO (mg/L)	B.O.D (mg/L)
Arambol	7.97±0.01	31.75±0.35	5.60±0.14	40.50±0.71	0.47±0.12	1.66±0.06	0.54±0.14	4.29±0.00	1.19±0.08
Anjuna	**8.37±0.02*****	33.00±0.00***	2.35±0.49***	41.50±0.71	0.34±0.05	1.69±0.06	0.38±0.08	**5.93±0.08*****	1.58±0.08**
Sinquerim	8.09±0.04*	30.00±0.35***	5.15±0.49	36.50±0.71*	0.91±0.04**	9.14±0.20***	0.73±0.15	5.87±0.32***	1.69±0.08***
Dona Paula	7.75±0.01***	30.00±0.35***	**10.05±0.35*****	40.00±1.41	1.11±0.01***	11.08±0.02***	0.80±0.02	4.40±0.16	0.56±0.02***
Velsao	8.06±0.01	**33.00±0.25*****	2.25±0.35***	**64.50±0.71*****	**9.64±0.01*****	**32.76±0.80*****	**3.48±0.05*****	4.12±0.08	2.20±0.04***
Betul	8.21±0.02**	32.00±0.25	1.24±0.02***	39.50±0.71	0.23±0.03*	1.44±0.10	0.28±0.02	5.14±0.08**	**4.01±0.02*****
Palolem	7.90±0.04	31.00±0.00**	4.15±0.07*	42.50±0.71	0.43±0.04	0.07±0.02	0.42±0.00	4.52±0.00	3.95±0.05***

Values are represented as means± standard deviation. Maximum values were shown in bold. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ significantly different from the reference site Arambol (ANOVA, Tukey HSD post-test).

range from 7.75 to 8.37. Maximum values for seawater temperature was observed at Anjuna (33 ˚C), and minimum at Palolem (31 ˚C). The highest turbidity was found at Dona Paula (10.05 ± 0.35 NTU) and the least at Betul (1.24 ± 0.02 NTU). Seawater from Velsao showed significant variation in nutrients as compared to all other stations ($p < 0.001$). Velsao also showed the maximum values for conductivity (64.50 ± 0.71 mS/cm). The values for Nitrite, nitrate, and total phosphate at Velsao were found to be 9.64 ± 0.01 µM/L, 32.76 ± 0.80 µM/L and 3.48 ± 0.05 µM/L respectively. Dissolved oxygen (DO) also varied significantly between the sites, with maximum values observed at Anjuna (5.93 ± 0.08 mg/L). The highest Biological Oxygen Demand (BOD) was measured at Betul (4.01 ± 0.02 mg/L) and the least at Dona Paula (0.56 ± 0.02 mg/L).

Biomarker Responses

DNA damage

The impairment of DNA in whole body tissue of marine gastropods is clearly indicated by the decrease in the integrity of DNA in *N. chamaeleon* exposed to various types of genotoxic contaminants prevalent at different sites along the coast of Goa (Fig. 2). The highest value of TDNA was observed at Sinquerim (55.86 ± 4.09). A significant difference in TDNA was observed between Sinquerim and Arambol ($p < 0.01$) and Sinquerim and Anjuna ($p < 0.01$). The DNA integrity at the reference site Arambol was found to be relatively quite high (0.71 ± 0.03) as compared to the other sampling sites. Except for Betul and Palolem, all the sampling sites showed a significant decrease in DNA integrity as compared to Arambol. The mean value of DNA integrity in snails was found to be 0.48.

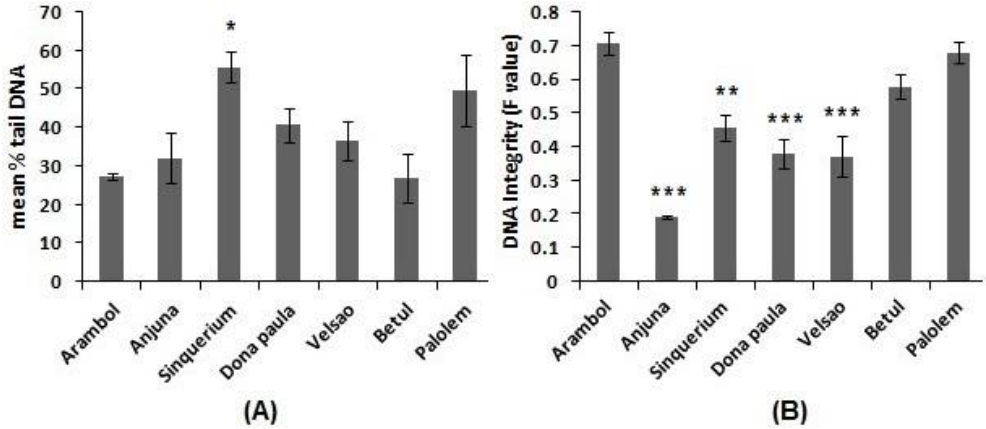

Fig. 2 (A) Mean percentage DNA in the tail (TDNA) and (B) DNA integrity (F value) in marine gastropod *Nerita chamaeleon* collected from different sites along the coast of Goa, India. Values are means ± standard deviation (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ significantly different from the reference site, Arambol (ANOVA, Tukey HSD post-test).

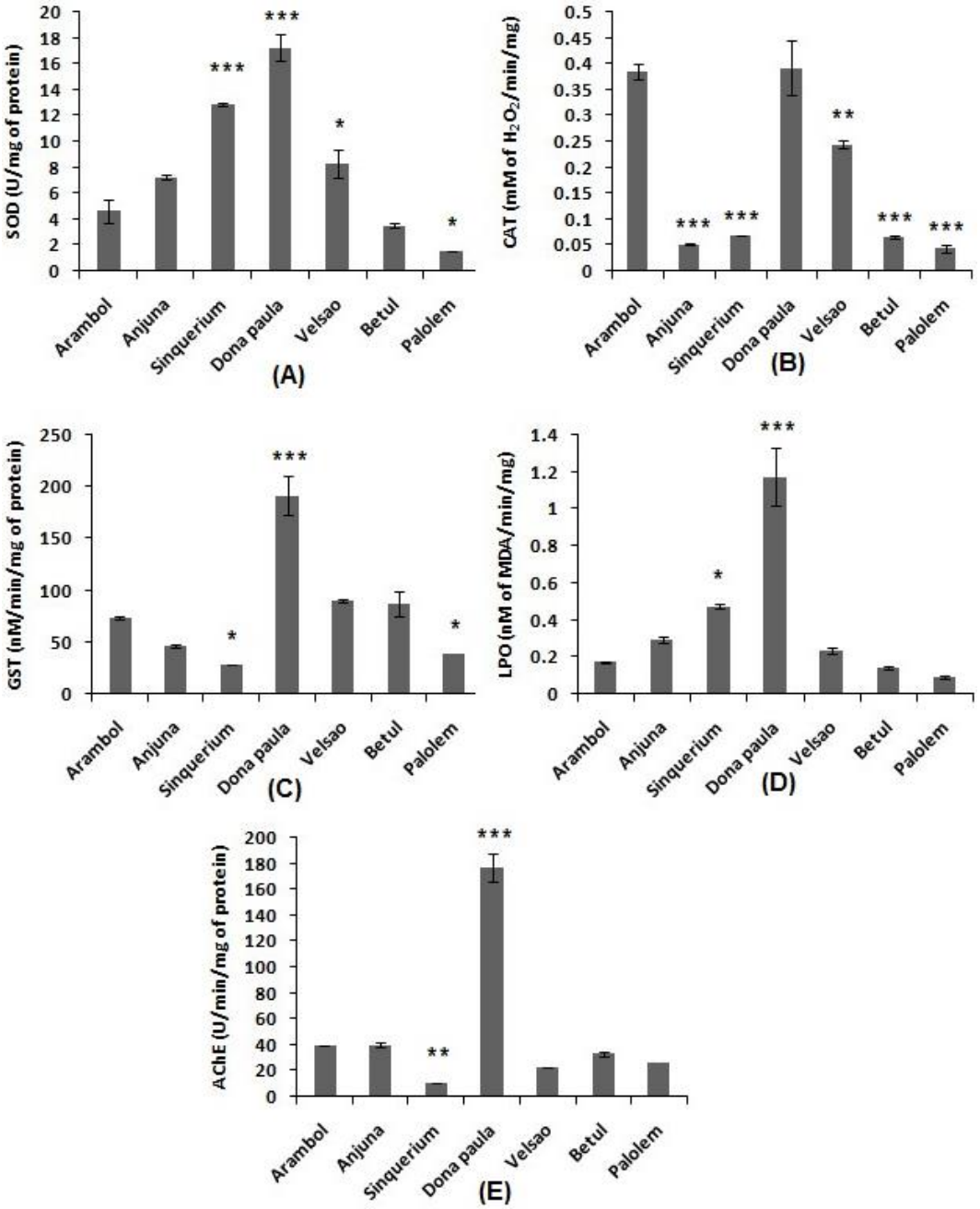

Fig. 3 (A) Superoxide dismutase (SOD), (B) catalase (CAT), (C) glutathione S-transferase (GST), (D) acetylcholinesterase (AChE) activities, and (E) lipid peroxidation (LPO) level in marine gastropod *Nerita chamaeleon* collected from different sites along the coast of Goa, India. Values are means± standard deviation (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ significantly different from the reference site, Arambol (ANOVA, Tukey HSD post-test).

Biochemical assays

All biomarkers varied across sites. SOD activity in whole body tissue of snails was in the range from 1.57 to 17.27 U/mg of protein (Fig. 3). Significant differences in SOD activity was observed for Sinquerim, Dona Paula, Velsao and Palolem as compared to the reference site Arambol. CAT activity also showed significant variations among the sampling sites. The highest CAT activity was observed at Dona Paula (0.39 ± 0.05 mM/min/mg) and the least at Palolem (0.04 ± 0.01 mM/min/mg). All the other site except Dona Paula, showed significant decrease in CAT activity as compared to Arambol. CAT activity between Sinquerim and Palolem were not significant, however, a significant change was observed between Palolem and Dona Paula ($p < 0.001$). GST activity showed maximum value recorded at Dona Paula (191.54 ± 18.7 nM/min/mg) and minimum at Sinquerim (28.11 ± 0.06 nM/min/mg). GST activity at Dona Paula was significantly ($p < 0.001$) higher than Arambol (74.21 ± 1.47 nM/min/mg).The lowest LPO value (0.09 ±

0.01 mM MDA/min/mg) was observed in snails collected at Palolem whereas Dona Paula exhibited the highest value (1.18 ± 0.16 mM MDA/min/mg). AChE activity were significantly lower ($p < 0.01$) in snails from the Sinquerim (10.44 ± 0.07 U/min/mg) in comparison to Arambol (39.68 ± 0.18 U/min/mg). PCA analysis showed that nutrients (phosphate, nitrate and nitrate) group together with conductivity. A strong positive correlation between LPO and turbidity (r = 0.84), and AChE and turbidity (r = 0.801) was observed. Dissolve oxygen showed negative correlation with DNA integrity value, whereas pH showed negative correlation with all the biomarkers.

Measurement of PAH

PAH concentrations in tissue of snail showed wide variation among the sites. Sinquerim showed the highest value for total PAH (12.14 ± 0.27 µg/g wet weight) in snail tissues whereas it was found to be least at Arambol (5.29 ± 0.67 µg/g w.w.). The entire sampling site except Anjuna showed significant variations in the PAH content in snails (Fig. 4). Table 2 shows the PAH content in molluscs from a different part of the world.

Relationship between biomarker response and PAH content

A moderate positive correlation was observed between TDNA and PAH (r = 0.626, $p = 0.017$) contents in snails (Table 3). SOD activity also showed positive correlation with PAH (r = 0.604, $p = 0.022$) while AChE activity was found to be negatively correlated with PAH (r = 0.542, $p = 0.045$). Strong positive correlation between GST activity and AChE activity (r = 0.534, $p = 0.049$), GST and CAT activity (r = 0.534, $p = 0.047$) were also observed. Similar trend was observed between CAT and SOD activity (r = 0.613, $p = 0.20$,) and LPO value and SOD activity (r = 0.942, $p = 0.022$). In contrast, DNA-F was shown to be negatively correlated with SOD activity (r = -0.617, $p = 0.019$) and LPO value (r = 0.679, $p = 0.008$).

Integrative Biomarker Response (IBR)

IBR values for Dona Paula showed the maximum value for TDNA (9.31 ± 0.91) as measured by comet assay and the minimum value was observed at Palolem (0.25 ± 0.063) (Fig. 5). The IBR values were significant for DNA-F when sites Arambol and Dona Paula were compared ($p < 0.001$). A very similar pattern was observed in star plots for all the oxidative stress biomarkers. The maximum IBR values for SOD were measured at Dona Paula (7.17 ± 0.63) and the minimum values occurred at Betul (0.91 ± 0.87). The IBR values for LPO at Sinquerim and Dona Paula were significantly different from those of Arambol ($p < 0.001$). However, no significant differences existed in any biomarker when IBR values for Anjuna, Betul and Palolem were compared with Arambol. Sinquerim and Dona Paula showed significant differences in all the biomarkers studied with compared to Arambol. The mean IBR values calculated from seven biomarkers was found to be highest at Dona Paula (8.07 ± 0.91) followed by Sinquerim (6.95 ± 0.91), Velsao (4.48 ± 0.68), Anjuna (3.28 ± 1.05), Palolem (2.53 ± 0.73), Arambol (1.81 ± 0.21) and Betul (0.88 ± 0.77). IBR values for genotoxic (TDNA and DNA-F), oxidative stress (sum of SOD, CAT, GST and SOD) biomarker and total PAH concentration in tissues were represented as star plot in Figure 6. Comparison of IBR for genotoxic, oxidative biomarkers and PAH concentration shows that along with PAH there might be other genotoxicants which are responsible for the impairment of DNA in snails from different sites.

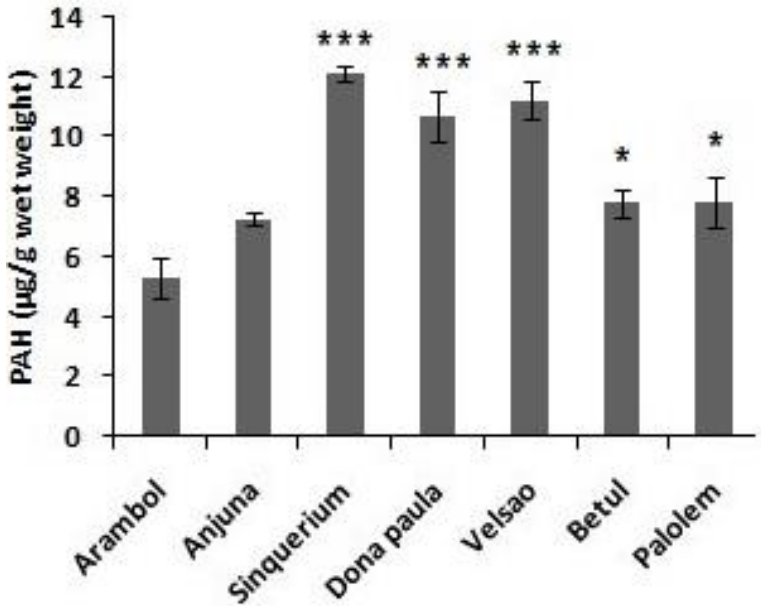

Fig. 4 Polycyclic aromatic hydrocarbon (PAH) content in marine gastropod *Nerita chamaeleon* collected from different sites along the coast of Goa, India. Values are means± standard deviation (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ significantly different from the reference site, Arambol (ANOVA, Tukey HSD post-test).

Table 2 Polycyclic aromatic hydrocarbon (PAH) concentrations in molluscs reported from different parts of the world

Organisms	Group	Location	Tissues	No. of PAH	Wet weight (µg/g)	References
Nerita chamaeleon	Gastropod	Goa Coast, India	Whole body tissues	Total PAH	5.29-12.14	This study
Cronia contracta	Gastropod	Goa Coast, India	Muscle tissue	Total PAH	22.32–53.78	Sarkar *et al*., 2008
Turbo cornutus	Gastropod	Japan Coast, Japan	Soft tissue	8	0.044	Koyama *et al*., 2004
Sunetta scripta	Clam	Cochin Harbor, India	Soft tissue	Total PAH	13.35-21.49	Menon & Menon, 1999
Donax trunculus	Clam	Comunidad Valenciana Coast	Soft tissue	8	0.43-10.09	Bouzas *et al*., 2011
Donax trunculus	Clam	Abu Qir Bay, Egypt	Soft tissue	17	1137.07	EL-Deeb et al., 2007
Saccostrea cucullata	Oyster	Hooghly Estuary, India	Soft tissue	Total PAH	0.8-12.5	Niyogi *et al*., 2001a
Mytilus galloprovincialis	Mussel	Marmara sea, Izmit Bay	Soft tissue	16	5.67-14.81	Telli-Karakoç *et al*., 2002
Mitylus galloprovincialis	Mussel	Adriatic Sea, Italy	Soft tissue	13	0.034	Perugini *et al*., 2007
Mytilus galloprovincialis	Mussel	Gulf of Rijeka, Croatia.	Soft tissue	10	0.049-0.134	Bihari *et al*., 2007
Mytilus galloprovincialis	Mussel	Gulf of Trieste	Soft tissue	-	644-685	Notar *et al*., 2001
Mytilus galloprovincialis	Mussel	Comunidad Valenciana Coast	Soft tissue	8	0.21-8.95	Bouzas *et al*., 2011
Mytilus edulis	Mussel	Baltic Sea, Poland	Soft tissue	14	8.64–29.7	Potrykus *et al*., 2003
Palaeomonetes sp.	Shrimp	Norco, USA	Soft tissue	Total PAH	7.18-10.86	Oberdorster *et al.,* 1999
Penaeus japonicus	Shrimp	Gulf of Suez	Muscle tissue	16	2.01	Ali *et al*., 2014
Sepia species	Cuttlefish	Gulf of Suez	Muscle tissue	16	4.09	Ali *et a*l., 2014
Portunus pelagicus	Crab	Gulf of Suez	Muscle tissue	16	8.10	Ali *et al*., 2014
Nephrops norvegicus	Lobster	Adriatic Sea, Italy	Soft tissue	13	0.015	Perugini *et* al., 2007

Discussion

In this study, seven biomarkers were measured in marine snail *N. chamaeleon* collected from Goa coast and the obtained results show differences in individual biomarker responses. Significant variations in genotoxic as well as biochemical biomarkers were observed and this can be associated with pollutant exposure. Biomarker responses in snails showed clear spatial variations. The range of PAH in snails in this study lies within the same range as reported in clams (Menon and Menon, 1999) and oysters (Niyogi *et al.,* 2001a) in Indian coastal waters. However, higher values of PAH were reported marine gastropods *Croniacontracta* by Sarkar *et al*. (2008) (Table 2). Total PAH concentrations in snails from Goa region range from the lowest value at Arambol (5.29 ± 0.67 µg/g wet weight) to highest value at Sinquerim (12.14 ± 0.27 µg/g wet weight). Sinquerim also showed the highest values for TDNA (55.86 ± 4.09) and lower DNA-F (0.46 ± 0.04). 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). Sarkar *et al*. (2008) studied the seasonal variation of PAH in *Cronia contracta* collected from six sites along the Goa coast and reported a positive correlation between the PAH and DNA damage. Such a huge impairment in DNA integrity in the gastropod at Sinquerim can be attributed to genotoxic pollutants like polycyclic aromatic hydrocarbons being discharged extensively from various types shipping activities such as cargo ships, research vessel, tourist vessel, motor boats, fishing trawler, water scooters, barges sailing through this site as well as accidental oil

Table 3 Spearman's correlation matrix on all the biomarkers and polycyclic aromatic hydrocarbon (PAH) content in marine gastropod, *Nerita chamaeleon*

	T-DNA	DNA-F	SOD	CAT	GST	LPO	AChE
DNA-F	-0.029						
SOD	0.332	-0.617*					
CAT	-0.143	-0.007	0.613*				
GST	-0.398	-0.244	0.257	0.538*			
LPO	0.279	-0.679*	0.942*	0.495	0.182		
AChE	-0.442	-0.152	0.099	0.327	0.534*	0.200	
PAH	0.626*	-0.345	0.604*	0.095	-0.002	0.516	-0.542*

(*) $p < 0.05$ significantly different(ANOVA, Tukey HSD post-test)

spills, etc. (Desai, *et al*, 2010). Ingole *et al*. (2006) have reported a high level of total petroleum hydrocarbon at Sinquerim-Candolim beach due to the grounding of MV River Princess. In Velsao, the PAH concentrations increase by two-fold as compared to Arambol, such an increase in PAH in snails at Velsao revealed the severity of pollution in Velsao. During sampling at Velsao pungent smell from the discharge outlet from the industry has been observed, that indicates the severity of pollution at this site. There were no significant differences between PAH concentration at Velsao, Sinquerim and Dona Paula.

Oxidative stress biomarker in *N. chamaeleon* showed spatial variability, with significant induction in SOD activity at Dona Paula and Sinquerim.SOD activity at both of these sites is strongly correlated with the high values of PAH measured in snails. Numerous studies have also documented significant relationships between antioxidant activity and heavy metal/PAH body burdens (Giguere *et al.,* 2003; Manduzio *et al.,* 2003). Dona Paula has been previously reported for heavy metal and PAH contamination (Sarkar *et al.,* 2008). High values of TBT and other organotin compounds were also reported in Dona Paula (Meena *et al.,* 2009). Catalase is well known to play an important role in scavenging H_20_2 (Di Giulio and Meyer, 2008), which is produced as a result of scavenging of superoxide radicals by SOD. Positive relationships between CAT activity and PAH levels were observed in oyster (Niyogi *et al.,* 2001a), barnacle (Niyogi *et al.,* 2001b), and in the gills of the mussel (Cheung *et al.,* 2001). In this study, a significant positive correlation was observed between SOD and CAT activity. The increase in SOD and CAT activity may be due to increasing in cellular ROS produced due to exposure to PAH (Au *et al.,* 1999). Niyogi *et al.,* (2001a) has also reported a positive correlation of CAT and SOD activity with PAH tissue content in

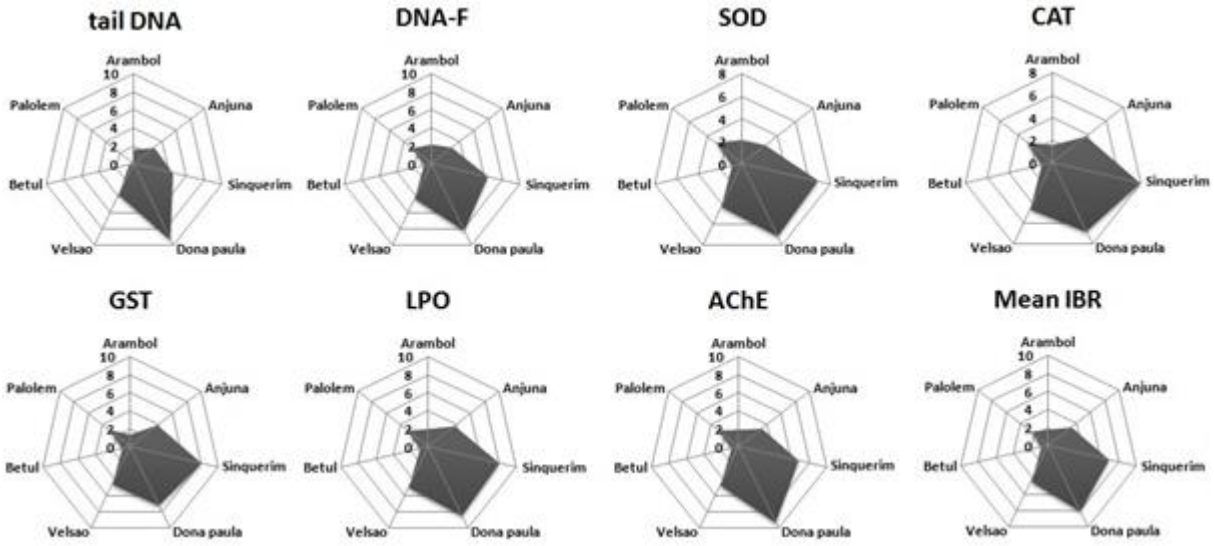

Fig. 5 Integrated biomarker response (IBR) represented by star plots for each sampling sites.

Fig. 6 Comparison of IBR index for genotoxic (DNA damage as measured by comet assay and alkaline unwinding assay) and oxidative stress [superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and lipid peroxidation (LPO)] biomarkers with average polycyclic aromatic hydrocarbon (PAH) value in snails from different sites of Goa, India.

S. cucullata. Such a relationship has also been reported for different bivalve species exposed to hydrocarbons (Richardson *et al.,* 2008) and suggests that hydrocarbons induce oxidative stress by producing ROS such as O_2^-. The activity of CAT is dependent on the level of H_2O_2 in the cell. Another enzyme, GPx (glutathione peroxidase) eliminates H_2O_2 while carrying out GSH to GSSG conversion. Increase in GPx activity might lead to reduction of H2O2 that in turn can affect the activity of catalase. In our study high level of CAT activity was observed at the reference site. In spite of high amount of PAH in the other sites, a decreasing trend in CAT activity was observed. 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. Jifa *et al.* (2006) also reported unaltered changes in *Lateolabrax japonicus* CAT activity after (B[a]P) exposure. GST is a phase II metabolizing enzyme and catalyzes the conjugation of reduced glutathione (GSH) with PAH derivatives. The activity of GST depends on the availability of GSH. Contrarily to other antioxidant enzymes, GST showed a negative correlation with PAH in this study. Decrease in GST activity after long exposure to PAH can be due to the reduction of GSH levels. GSH can also be converted to its oxidized state (GSSG) by Glutathione peroxidase (GPx). Decrease in GSH has been reported in molluscs exposed to PAHs (Grintzalis *et al.,* 2012). Studies with GST in aquatic organisms exposed to environmental or anthropogenic contaminants has shown increase (Zheng *et al.,* 2013; Cabecinhas *et al.,* 2014), unaltered (Bianco *et al.,* 2013; Rivadeneira *et al.,* 2013) or decrease (Ma *et al.,* 2014; Ali *et al.,* 2015) enzyme activities.

Free radicals produced as a result of metabolic activities can react with polyunsaturated fatty acids in the cell membrane, resulting in an increase of lipid peroxidation (Livingstone *et al.,* 2001). LPO is well known oxidative stress biomarkers in bivalves exposed to PAH (Kaloyianni *et al.,* 2009). LPO results in production of MDA which can react with DNA and form DNA adduct. Increased formation of DNA adduct in gill cells of *Mytilus galloprovincialis* after exposure to PAH, benzo(a)pyrene have been reported by Venier and Canova (1996). Enhancement of lipid peroxidation and inhibition of AChE were detected in gills of mussels *M.galloprovincialis* exposed to phenanthrene (Grintzalis *et al.,* 2012). Elevated levels of CAT, LPO and SOD were observed in mussels (*Mytilus galloprovincialis*) collected from sites affected by the oil spill (Sureda *et al.,* 2011). In this study significant decrease in AChE activity was observed in Sinquerim. This may be due to the prevalence of hydrocarbon pollution as observed by high amount of PAH reported in the gastropods. Presence of high amount of PAH in snails from Sinquerim may be due to extensive shipping activities as well as accidental oil spills (Desai *et al.,* 2010). Enhancement of lipid peroxidation by-products and inhibition of AChE in mussels exposed to phenanthrene and/or anthracene were reported by Grintzalis *et al.* (2012). PCA indicated that biochemical biomarkers tend to be increased under condition of higher turbidity and lower pH. PCA analysis provided an important association between the physicochemical parameters and biomarker response. The fact that the biomarkers assessed were moderately associated with the environmental variables suggest that other contaminants, besides those measured here, were also contributing to the lower health status of the snails.

IBR with star plots showed higher biomarker response in snails from Dona Paula, Sinquerim, and Velsao, and moderate at Anjuna and Palolem. Thus higher values of PAH and oxidative stress biomarkers at Velsao, Sinquerim and Dona Paula shows critical unbalance ROS formation and the severity of pollution at these sites. Several authors have reported higher values of IBR in contaminated sites as compared to the reference site (Tankoua *et al.,* 2013; Turja *et al.,* 2014). In this study elevated the level of PAH as well as oxidative stress biomarkers response was observed in snails from Sinquerim. Vega-López *et al.* (2013) has investigated the relation between oxidative stress and

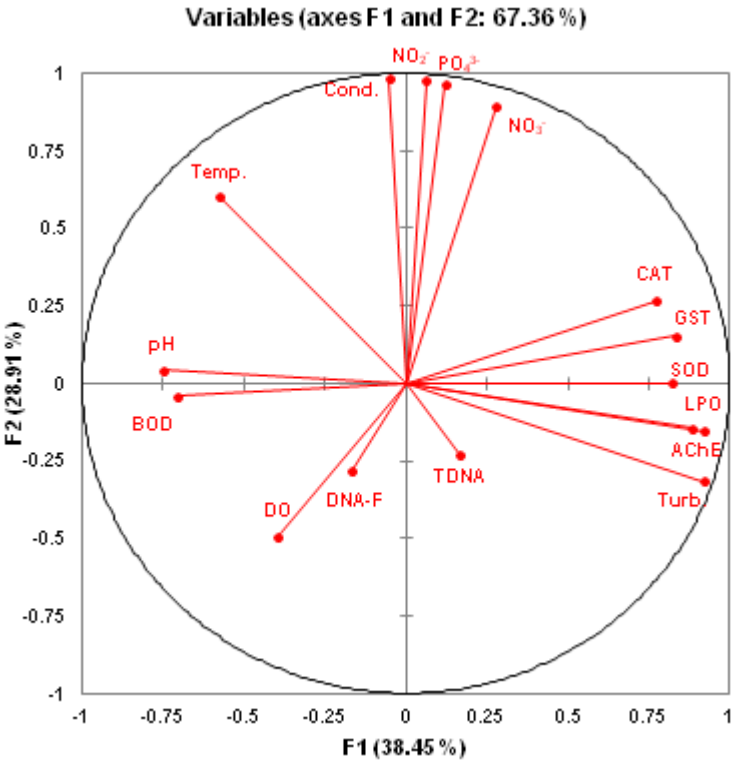

Fig. 7 Principal component analysis (PCA) on the data set with physicochemical water parameters [pH, temperature (temp.), turbidity (turb.), conductivity (cond.), nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), dissolve oxygen (DO), biological oxygen demand (BOD)] and biomarkers response [DNA integrity (DNA-F), percentage tail DNA (TDNA), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), acetylcholinesterase (AChE) activities, and lipid peroxidation (LPO)] in *Nerita chamaeleon.*

antioxidant defenses in phytoplankton with heavy metal and PAH. The author stated that oxidative damage was related with PAH (benzo[b]fluoranthene) using IBR. PAH are suspected of having an oxidative damage leading to damage in the genetic material and some of the PAH such as Benzo(a)pyrene, Indeno[1,2,3-c,d]pyrene, Benzo[g,h, i]perylene etc. are well known for such actions (Perez-Cadahia *et al.,* 2004; Woo *et al.,* 2006). There is strong evidence that some of them are carcinogenic (Diguilio *et al.,* 1995) with the capacity to cause various types of oxidative stress and DNA damage. These results suggest that integration of genotoxic and biochemical biomarker can serve as a useful tool in environmental monitoring programs.

Conclusion

The present study showed that TDNA, DNA-F, SOD, CAT, GST, LPO and AChE levels in snails varied along different sites. Integrated biomarker response index based on a battery of biomarkers proved a useful tool for visualization of biological responses in snails, facilitating comparisons between different sites. Increased value of IBR index at Sinquerim and Dona Paula can be attributed to exposure of snails to contaminants prevalent at these sites. Our study demonstrated, the sensitivity of marine snail *N. chamaeleon* as a good candidate species for PAH contamination. This study demonstrates the usefulness of multi-biomarker approach in the coastal bio-monitoring program.

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