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Integrated biomarker responses as environmental status descriptors of a coastal zone (São Paulo, Brazil)

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ABSTRACT

São Paulo state (Brazil) has one of the most overpopulated coastal zones in South America, where previous studies have already detected sediment and water contamination. However, biological-based monitoring considering signals of xenobiotic exposure and effects are scarce. The present study employed a battery of biomarkers under field conditions to assess the environmental quality of this coastal zone. For this purpose, the activity of CYP 450, antioxidant enzymes, DNA damage, lipid peroxidation and lysosomal membrane were analysed in caged mussels and integrated using Factorial Analysis. A representation of estimated factor scores was performed in order to confirm the factor descriptions characterizing the studied areas. Biomarker responses indicated signals of mussels' impaired health during the monitoring, which pointed to the impact of different sources of contaminants in the water quality and identified critical areas. This integrated approach produced a rapid, sensitive and cost-effective assessment, which could be incorporated as a descriptor of environmental status in future coastal zones biomonitoring.

1. Introduction

Considering the last advances on water bodies' quality criteria, ecological disturbances have been assumed as a basis of control rather than focusing on chemical agents with potential to cause adverse effects. Therefore, the assessment of water quality have been defined directly in terms of the 'functioning and structure of ecological systems' rather than based solely on chemical contamination (Vighi et al., 2006).

Within this framework, the biological–ecological quality assumes a prevailing role and the use of biomarkers has been proposed in order to detect signs of impaired health in aquatic organisms, thus providing measurable advance warning of changes in the environment (Da Ros et al., 2002). Biomarkers can be defined as biochemical, cellular, physiological or behavioural variations that can be measured in tissue or body fluid samples, or at the level of whole organisms, to provide evidence of exposure and/or effects from one or more contaminants (Depledge, 1994).

Although all biomarker types can provide useful information in terms of knowledge of exposure or effects of toxicants, certain criteria should address the selection of the most useful/relevant biomarkers to use. These include whether a biomarker is easy to measure; whether it responds in a dose- or time-dependent manner to the toxicant, whether it is sensitive and presents relevant biological significance (Hagger et al., 2006). Indeed, a suite of end points, at different levels of biological organisation, could provide a most comprehensive indication of ecosystem health (Solé, 2000).

Considering these criteria, enzymes acting in the detoxification system are among the most employed biomarker responses. The mechanism of detoxification of pollutants involving a series of enzymes is divided into Phase I (biotransformation) and Phase II (conjugation). The metabolic process of Phase I is basically

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described as oxidative reactions catalysed by the mixed-function oxygenase (MFO) dependent on a group of proteins (Cytochrome P450), while Phase II is characterised by the activity of the enzyme Glutathione-S-transferase (GST) (Stegeman and Livingstone, 1998; Dauterman, 1994).

Exposure to xenobiotics are also supposed to promote changes in the enzymatic antioxidant system, since the increased production of reactive oxygen species (ROS) is counteracted essentially by the activity of three enzymes: Superoxide Dismutase, Catalase and Glutathione Peroxidase, which play a crucial role in maintaining cellular homoeostasis. Their use has been proposed as biomarkers of exposure to contaminants in a variety of marine organisms (Winston, 1991; Bainy et al., 2000).

Xenobiotics, its metabolites or ROS may lead to impairment of biological membranes, organelles and DNA. One of the most studied effects is the lipid peroxidation, which is a complex process resulting from free radical reactions able to destroy membrane lipids (Oliveira et al., 2009). The lysosomal system is also well-known as a target site for toxic metals and organic chemicals due to its ability to accumulate xenobiotics (Da Ros et al., 2002). Furthermore, DNA alterations promoted by chemical agents include single and double strand breaks, which may also be induced by an interaction with oxygen radicals or as a consequence of apoptosis or necrosis processes (Viarengo et al., 2007).

Despite the enormous number of scientific research concerning biomarkers, they are still seldom used as ecotoxicological tools in the day to day management of the environment along the Brazilian coastal zone. Environmental monitoring and subsequently coastal management decisions have been based on chemical analysis of water and sediment matrixes. However, there are difficulties, as literature has documented, of predicting toxicological hazard for the aquatic community of a given area using only data on either water or sediment chemistry, since chemical measurements are not per se indicators of toxicity (Carballeira, 2003).

São Paulo coast (South-eastern, Brazil) presents a clear gradient of environmental contamination from the north to the central shore (CETESB, 2001, 2006). The Santos Estuarine System, located at the central shore, has been considered a critical area accumulating urban and industrial waste from the petrochemical pole, and has become one of the most polluted estuaries of the world (Sánchez-Jérez et al., 2001; CETESB, 2001). Indeed, sediments from the channel of the port of Santos are frequently dredged, propitiating their resuspension and increasing water contamination (Abessa et al., 2008). Although physical–chemical and microbiological patterns are well monitored, few studies have assessed sublethal effects of these pollutants in aquatic organisms (Abessa et al., 2005; Pereira et al., 2007), which have produced a lack of ecological interpretation for the environmental properties.

Considering the presence of multiples sources of contaminants and the incipient knowledge about their bioavailability and biological effects in this coastal zone, our study aimed to produce a more comprehensive environmental status assessment through a biomarker-based monitoring. For this purpose, mussels were deployed at different areas during the four seasons and posteriorly a battery of non-specific biomarker responses (Phase I—biotransformation and Phase II—conjugation enzymes, antioxidants, lipid peroxidation, lysosomal membrane integrity and DNA damage) were analysed. Factor Analysis was performed to identify the most representative biomarker responses, its correlations and associations with the studied areas over time.

2. Material and methods

2.1. Study area

The study area comprises 200 km of the São Paulo coast and it extends from Caraguatatuba to Santos bay, including the São Sebastião Channel. In São Paulo coast (Southeast Brazil) is located one of the most economically important coastal zones of South America, which possesses the biggest South American commercial harbour, a major petrochemical and metallurgical industrial pole with more than 1.100 industries (Martins et al., 2008) and the largest Brazilian oil terminal (DTCS).

Studied areas were established to comprise zones influenced by different contamination sources. In the São Paulo North Shore it was selected the reference area (Cocanha Beach), a mussel farm where the specimens of brown mussel *Perna perna* were acquired to be caged along the study area. Cocanha beach (Co) was selected as reference station based on the results of the environmental monitoring performed by the São Paulo Environmental Agency, which has detected aquatic contaminants below threshold levels (CETESB, 2006). Mussels were transplanted to four possibly contaminated areas: (1) Ilhabela (Ib), where marinas and submarine domestic sewage outfalls are present; (2) DTCS Oil Terminal (Ot), where it is located

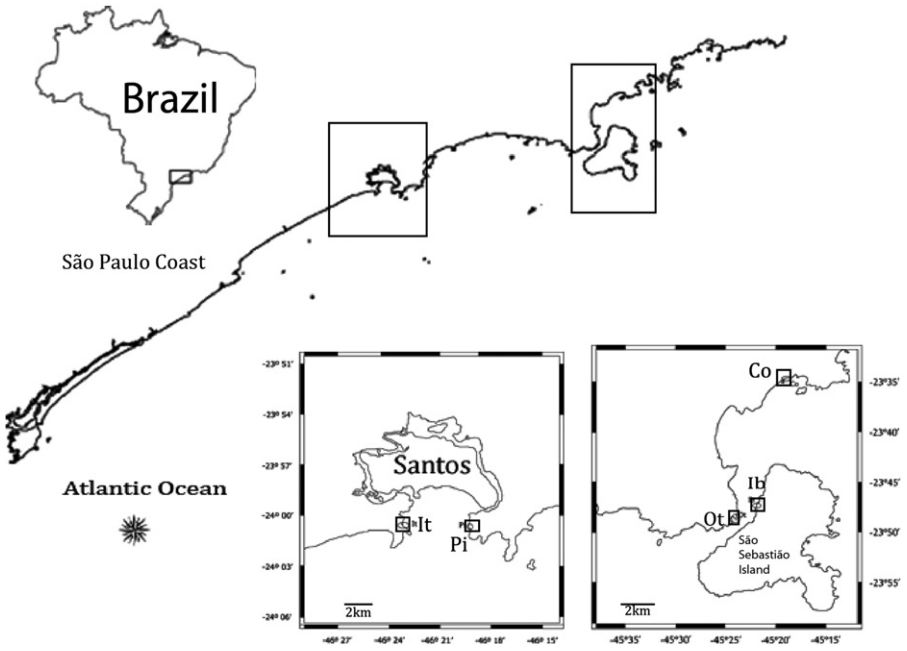

Fig. 1. Study area with the location of the Cocanha farming zone (Co) and the transplanted sites: Ilhabela (Ib), Oil terminal (Ot), Palmas island (Pi) and Itaipu (It) (extracted from NOAA/NGDC).

the biggest Brazilian oil terminal; (3) Palmas Island (Pi) in the Santos Estuary mouth, where the Port of Santos is located; (4) Itaipu (It), in the São Vicente Estuary mouth, where three submarine domestic sewage outfalls are located (Fig. 1).

2.2. Biological material and study design

About 1000 individuals of brown mussel *P. perna*, divided between five ropes containing about 200 organisms per rope, were obtained from a mussel farm (Cocanha beach) at the beginning of each season (winter and spring 2005, summer and autumn 2006). The mussels were transplanted to the studied areas, where they remained during the exposure period of three months in each season. One rope was always kept in the mussel farm to be used as a reference. At the end of each exposure period ropes were collected from each study site and mussels of approximately 60 mm in length were analysed. Ten mussels were used for the biomarker analyses in gill tissues and thirty to developing the Neutral Red Neutral Retention Assay (NRRT).

2.3. Biomarkers

Gills of ten mussels were dissected and immediately frozen in liquid nitrogen. In the laboratory, the samples were homogenised separately, following the procedure developed by Lafontaine et al. (2000). Once samples were homogenised in buffer (NaCl 100 mM, Hepes-NaOH 25 mM, EDTA 0.1 mM, DTT 0.1 mM, pH 7.5) those for enzymatic activity determination (EROD, DBF, CAT, GPx and GR) were centrifuged at 15 000 *g* for 20 min at 4 °C, and the supernatant (S_{15}) was extracted for the determination of enzyme activities. Samples obtained to determine DNA damage and lipid peroxidation were not centrifuged, and the homogenised fraction was analysed. Total protein contents in S_{15} and homogenised fraction were analysed according the dye-binding principle (Bradford, 1976). The same ten samples of mussels' gill tissues were employed for all biomarker analyses described above. To develop the NRRT assay, haemolymph was withdrawn from the posterior adductor muscle of thirty living mussels few hours (from 6 to 12 h) after collecting in each transplanted site. During this period, mussels were kept in 300 L aquariums with controlled salinity (34 ± 2) and temperature (22 ± 2 °C).

2.3.1. Ethoxyresorufin O-deethylase (EROD) activity

The mixed-function oxidase activity (MFO) for the 7-hydroxyresorufin substrate was measured using the adapted EROD assay (Gagné and Blaise, 1993). 50 µl of S_{15} was added to 10 µM 7-ethoxyresorufin and 10 mM reduced NADPH in 100 mM KH_2PO_4 buffer (pH 7.4). The reaction was initiated by the addition of NADPH, allowed to proceed for 60 min at 30 °C, and stopped by the addition of 100 µl of 0.1 M NaOH. 7-Hydroxyresorufin was determined fluorometrically using 485 nm (excitation) and 580 nm (emission) filters. Determination of 7-hydroxyresorufin in the samples was carried out using a standard calibration curve of 7-hydroxyresorufin concentration. Results were expressed as nmol min^{-1} mg^{-1} total protein.

2.3.2. Dibenzylfluorescein (DBF) activity

The mixed-function oxidase activity (MFO) for the dibenzylfluorescein substrate was determined as described before (Stresser et al., 2000; Gagné et al., 2007). The S15 of the gill (100 µg/mL) was incubated with 10 µM of substrate in 100 mM NaCl containing 10 mM Hepes-NaOH, pH 7.4. The reaction was started by the addition of 50 µM NADPH (blanks consisted of the reaction mixture without the substrate) and incubated for 0, 15, 30, 45 and 60 min at 30 °C. Fluorescence for fluorescein was measured at 485 nm excitation/516 nm emission. Results were expressed as nmol min^{-1} mg^{-1} total protein.

2.3.3. Catalase (CAT) activity

Catalase activity was determined using the method described by Beutler (1975), which quantifies the rate of decomposition of hydrogen peroxide (H_2O_2) by the enzyme through decrease of absorbance at 240 nm, using the molar extinction coefficient (ε) of 0.04 M. The values of CAT activity were expressed as U/mg protein. One unit of CAT is the amount of enzyme that hydrolyses 1 µmol of $H_2O_2^{-1}$ min^{-1} mg^{-1} total protein.

2.3.4. Glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was determined using the method described by McFarland et al. (1999). GPx activities were measured spectrophotometrically at 340 nm every 2 min for 10 min, using 1 mM cumene hydroperoxide as the substrate. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP was indicative of GPx activity. Results were expressed as nmol min^{-1} mg^{-1} total protein.

2.3.5. Glutathione-S-transferase (GST) activity

The procedure utilised for the determination of GST activity was adapted from McFarland et al. (1999). The activity was analysed using 42 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM GSH as substrates and measured spectrophotometrically at 340 nm every 30 s for 3 min. Results were expressed as nmol min^{-1} mg^{-1} total protein.

2.3.6. DNA damage

DNA damage was assessed by an alkaline precipitation assay (Olive, 1998) based on the K-SDS precipitation of DNA–protein crosslink. It was used fluorescence to quantify DNA strand breaks (Gagné et al., 1995). DNA quantitation was achieved using Hoescht dye at a concentration of 100 nM in 200 mM Tris–HCl, pH 8.5, containing 300 mM NaCl and 4 mM sodium cholate. Salmon sperm DNA standards were used for calibration and fluorescence readings were taken at 360 nm excitation and 460 nm emission. The results were expressed as µg of DNA · mg^{-1} total protein.

2.3.7. Lipid peroxidation (LPO)

Lipid peroxidation was measured according to Wills (1987). LPO was determined in gill homogenates by the thiobarbituric acid. Thiobarbituric acid reactants (TBARS) were determined by fluorescence at 530 nm for excitation and 630 nm for emission using a fluorescence microplate reader. Because the reagent could react with other aldehydes, the results were expressed as µg of TBARS · mg^{-1} total protein.

2.3.8. Neutral red retention time assay (NRRT)

NRRT assay is based on the principle that only lysosomes in healthy cells take up and retain the vital dye neutral red. Lysosomal membranes' damage caused by the impact of xenobiotics can decrease the NRRT times by inducing the leaking of lysosomal components (Dailianis et al., 2003). NRRT assay was carried out following the method described by Lowe et al. (1995). This non-destructive method employed haemolymph withdrawn from the posterior adductor muscle of living mussels. The haemolymph was mixed to physiological saline solution (pH 7.3 containing 4.77 g/l HEPES, 25.48 g/l NaCl, 13.06 g/l MgSO4, 0.75 g/l KCl, 1.47 g/l $CaCl_2$), spread on slides and transferred to a lightproof chamber, where it remained 15 min to allow cells attachment. Excess liquid was removed and 40 µL of the Neutral Red (NR) dye were added to the cell monolayer. A cover slip was added. After a 15-min incubation period, slides were examined every 15 min by optical microscopy (400 ×) for both structural abnormalities and NR dye loss from the lysosomes to the cytosol. The same analyst carried out the assessment for all slides during the study. The endpoint was considered the time when at least 50% of the examined cells exhibited these characteristics and a NRRT mean value was calculated for each group.

2.4. Statistical analysis

Initially, the data were submitted to Chi-square and Hartley's tests to verify for normality and homogeneity of variance, respectively. The results obtained in each season were compared by one-way analysis of variance ANOVA followed by Dunnett's test ($p < 0.05$) to verify significant differences on biomarker responses of mussels kept in the farming area and mussels caged in different studied stations. Thereafter, the original data set of each season including all biomarker responses (means) were analysed through an integrative approach by Factor Analysis, employing Principal Components Analysis as the extraction procedure. The data set was rearranged in a correlation matrix and three factors (or new variables) were extracted considering eigenvalues higher than 1.0 (Kaiser's criteria). For the Factor Analysis, the variables were auto scaled (Varimax normalised) to be treated with equal importance. The criteria for consideration of a variable as being associated with a particular factor was defined as its having a loading of 0.6 or higher, based on the suggestion by Tabachnic and Fidell (1996) that component loading greater than 0.45 be considered fair or better. Besides the analysis of the variables aggregated by PCA, a representation of estimated factor scores from each studied area to the centroid of all cases for the original data was performed in order to confirm the factor descriptions and to characterize the studied stations. All analyses were performed using the PCA option of the multivariate exploratory techniques procedure, followed by the basic set-up for Factor Analysis procedure from the STATISTICA software tool (Stat Soft, Inc., 2001; version 6.0).

3. Results

3.1. Winter survey

Mussels transplanted to Ilhabela exhibited significantly higher ($p < 0.05$) activities of GPx and decrease on NRRT, whereas organisms transplanted to the Oil terminal showed significantly higher DBF, GPX, and GST activities, DNA damage, LPO and decrease on NRRT than mussels kept in the farming zone (Co). Mussels transplanted to Palma island exhibited significant

($p < 0.05$) increase in GPX activity, besides the decrease on NRRT, whereas mussels transplanted to Itaipu exhibited DBF, GPx and GST activities, besides DNA damage, LPO and decrease on NRRT significantly higher ($p < 0.05$) than the group kept in the farming zone. Data are represented in Table 1.

3.2. Spring survey

The organisms transplanted to Ilhabela exhibited GST activity, LPO levels and decrease on NRRT significantly different ($p < 0.05$) than mussels kept in the farming zone, whereas organisms transplanted to Oil terminal only exhibited significantly higher DNA damage. Mussels transplanted to Palmas island presented a significant decrease ($p < 0.05$) on NRRT, whereas organisms transplanted to Itaipu exhibited significantly higher ($p < 0.05$) activities of EROD, DBF and GPx, LPO levels and reduced N RRT when compared to mussels kept in the farming zone (Co). Data are represented in Table 2.

3.3. Summer survey

Mussels transplanted to Ilhabela exhibited significant difference ($p < 0.05$) on DNA damage, LPO and NRRT, whereas organisms transplanted to the Oil terminal showed significantly ($p < 0.05$) higher DBF, GPx, and GST activities and reduced NRRT comparatively to animals kept in the farming zone. Significant biomarker responses were not observed between mussels transplanted at Palmas Island and the reference mussels (Co). The organisms transplanted to Itaipu were lost during the exposure period and data could not be presented. Results are represented in Table 3.

3.4. Autumn survey

Mussels transplanted to Ilhabela exhibited induction of EROD, DBF and GST activities, whereas organisms transplanted to the Oil terminal showed significantly higher ($p < 0.05$) DBF activity, elevated LPO and decrease on NRRT comparatively to mussels kept in the farming zone. High DBF and CAT activities were observed in mussels transplanted to Palmas Island, whereas organisms transplanted to Itaipu showed significant ($p < 0.05$) higher EROD, DBF, CAT, GPX and GST activities, beside decrease on NRRT compared to the organisms kept in the farming area. Data are presented in Table 4.

3.5. Integrated analysis of the biomarker responses

To provide a integrated view of entire monitoring data set, a PCA-Factor Analysis was performed employing all the biomarker responses (means) showed in Tables 1–4 (winter, spring, summer and autumn, respectively). The application of Principal Components Analysis indicated that the original set of variables could be narrowed down to three new variables or factors, those explained 73.6% of total variance (Table 5).

The first principal factor (F1) accounted for 42.57% of the variance. Factor 1 accounts for the relationship between Phase I CYP 450 (EROD and DBF) and Phase II GST induction with the activity of the antioxidant enzyme GPX, indicating a defence mechanism to organic contamination exposure which could be generating oxidative stress. The second factor (F2) accounts for 17.20% of the variance. It represents the relationship between DNA damage and decrease on NRRT, which act as effect biomarkers of contamination. The third factor (F3) accounts for 13.82% of

Table 1
Biomarker results of the winter survey (mean ± SD). An asterisk indicates a significant difference from the reference station Cocanha (ANOVA—Dunnetts test, $p < 0.05$).

Stations	EROD (nmol/min/mgprot.)	DBF (nmol/min/mgprot.)	CAT (U/mgprot.)	GPx (nmol/min/mgprot.)	GST (nmol/min/mgprot.)	DNA (μg/mgprot.)	LPO (μMTBARs/mgprot.)	NRRT (min.)
Cocanha	0.04 ± 0.01	0.07 ± 0.03	26.9 ± 7.8	32.3 ± 14.5	182.5 ± 59.64	611.7 ± 272.6	0.32 ± 0.04	62.9 ± 19.9
Ilhabela	0.05 ± 0.02	0.09 ± 0.04	16.3 ± 5.1	138.8 ± 87.3*	227.7 ± 40.10	642.3 ± 310.2	0.40 ± 0.14	34.0 ± 15.0*
Oil terminal	0.04 ± 0.02	0.23 ± 0.06*	23.6 ± 8.9	166.8 ± 27.0*	231.9 ± 57.71*	920.0 ± 372.0*	0.49 ± 0.18*	29.5 ± 15.3*
Palmas island	0.03 ± 0.01	0.09 ± 0.04	23.6 ± 9.0	246.8 ± 90.1*	210.1 ± 43.5	628.5 ± 280.4	0.47 ± 0.21	24.6 ± 12.4*
Itaipu	0.03 ± 0.01	0.35 ± 0.13*	18.2 ± 4.6	413.5 ± 153.5*	267.7 ± 73.5*	1076.5 ± 200.6*	0.47 ± 0.12*	15.0 ± 7.5*

Table 2
Biomarker results of the spring survey (mean ± SD). An asterisk indicates a significant difference from the reference station Cocanha (ANOVA—Dunnetts test, $p < 0.05$).

Stations	EROD (nmol/min/mgprot)	DBF (nmol/min/mgprot)	CAT (U/mgprot.)	GPx (nmol/min/mgprot.)	GST (nmol/min/mgprot.)	DNA (μg/mgprot.)	LPO (μMTBARs/mgprot.)	NRRT (min.)
Cocanha	0.04 ± 0.02	0.12 ± 0.06	22.0 ± 7.0	1342.7 ± 420.4	197.2 ± 59.3	229.9 ± 42.9	0.17 ± 0.07	60.6 ± 18.0
Ilhabela	0.06 ± 0.02	0.21 ± 0.11	23.8 ± 9.4	1323.9 ± 583.9	449.2 ± 117.6*	223.1 ± 90.0	0.47 ± 0.19*	39.2 ± 11.3*
Oil terminal	0.07 ± 0.04	0.10 ± 0.05	23.8 ± 9.0	1288.7 ± 302.9	385.6 ± 134.6*	403.3 ± 177.3*	1.03 ± 0.43*	31.8 ± 10.9*
Palmas	0.04 ± 0.01	0.10 ± 0.06	26.0 ± 8.1	1079.1 ± 302.9	179.7 ± 62.6	139.1 ± 47.6	0.28 ± 0.11	35.8 ± 7.6*
Itaipu	0.07 ± 0.02*	0.36 ± 0.13*	24.3 ± 7.3	477.9 ± 125.9*	236.7 ± 57.5	257.4 ± 109.9	0.89 ± 0.36*	31.9 ± 10.8*

Table 3
Biomarker results of the summer survey (mean ± SD). An asterisk indicates a significant difference from the reference station Cocanha (ANOVA—Dunnetts test, $p < 0.05$).

Stations	EROD (nmol/min/mgprot.)	DBF (nmol/min/mgprot.)	CAT (U/mgprot.)	GPx (nmol/min/mgprot.)	GST (nmol/min/mgprot.)	DNA (μg/mgprot.)	LPO (μMTBARs/mgprot.)	NRRT (min.)
Cocanha	0.06 ± 0.02	0.21 ± 0.08	16.9 ± 5.0	425.1 ± 250.1	212.25 ± 57.9	268.4 ± 88.9	1.22 ± 0.4	53.6 ± 13.5
Ilhabela	0.07 ± 0.02	0.30 ± 0.09	21.2 ± 7.3	437.7 ± 166.5	287.3 ± 78.8	489.3 ± 99.0*	1.87 ± 0.4*	38.1 ± 13.3*
Oil terminal	0.06 ± 0.02	0.43 ± 0.11*	14.7 ± 3.4	556.9 ± 117.1*	410.3 ± 108.8*	223.2 ± 49.2	0.92 ± 0.3	21.3 ± 7.6*
Palmas	0.07 ± 0.02	0.20 ± 0.13	17.4 ± 3.8	293.1 ± 87.2	203.2 ± 70.4	282.5 ± 73.5	1.25 ± 0.46	45.6 ± 15.6

Table 4
Biomarker results of the autumn survey (mean ± SD). An asterisk indicates a significant difference from the reference station Cocanha (ANOVA—Dunnetts test, $p < 0.05$).

Stations	EROD (nmol/min/mgprot.)	DBF (nmol/min/mgprot.)	CAT (U/mgprot.)	GPx (nmol/min/mgprot.)	GST (nmol/min/mgprot.)	DNA (μg/mgprot.)	LPO (μMTBARs/mgprot.)	NRRT (min.)
Cocanha	0.12 ± 0.03	0.07 ± 0.02	15.4 ± 3.54	587.7 ± 187.4	350.7 ± 46.88	203.7 ± 63.7	0.15 ± 0.4	48.4 ± 16.6
Ilhabela	0.19 ± 0.05*	0.28 ± 0.09*	17.9 ± 3.3	912.1 ± 395.5	1073.3 ± 137.7*	211.1 ± 45.0	0.20 ± 0.6	35.2 ± 17.3
Oil terminal	0.07 ± 0.02	0.12 ± 0.03*	18.8 ± 4.9	389.2 ± 36.9	300.0 ± 106.0	250.3 ± 87.9	0.26 ± 0.7*	24.7 ± 9.1*
Palmas	0.07 ± 0.02	0.25 ± 0.08*	26.1 ± 3.43*	577.1 ± 307.7	669.8 ± 248.2	127.3 ± 46.2	0.09 ± 0.02	35.2 ± 13.1
Itaipu	0.29 ± 0.06*	0.97 ± 0.34*	20.95 ± 3.24*	1762.7 ± 386.2*	1458.9 ± 485.7*	128.1 ± 27.3	0.05 ± 0.01	27.3 ± 13.2*

Table 5
Sorted rotated factor loadings of the original variables on the three principal factors for the all year long monitoring data set. (Presented loadings are ≥ 0.60.)

	Factor 1	Factor 2	Factor 3
% Variance	*42.57*	*17.20*	*13.82*
EROD	0.85	–	–
DBF	0.78	–	–
CAT	–	–	0.79
GPX	0.87	–	–
GST	0.89	–	–
DNA	–	0.64	–
LPO	–	–	–
NRR	–	0.90	–

the total variance. This factor is related to the induction of CAT activity, possibly due to a complementary defence biomarker against oxidative stress.

A graphical representation of the estimated factor values corresponding to each case (studied site) is presented in order to confirm the descriptions of these new factors. Values corresponding to Factors 1, 2 and 3 are described in Fig. 2. Factor 1 values indicated the induction of CYP 450 (EROD and DBF) and glutathione peroxidase activity of mussels transplanted to Cocanha, Ilhabela and Palmas in spring, to Oil terminal in summer and to Cocanha, Ilhabela, Palmas and Itaipu in autumn (Fig. 2A). Meanwhile, Factor 2 values are related to the effects on DNA and lysosomal membranes, and positive values for this factor were found for mussels transplanted to Ilhabela, Oil terminal, Palmas and Itaipu in winter, to Oil terminal in summer and Ilhabela, Oil terminal and Itaipu in autumn (Fig. 2B). Factor 3 represents the induction of CAT activity indicating complementary defence against oxidative stress and this factor showed positive scores to Cocanha, Oil terminal, Palmas, Itaipu in winter; Cocanha, Ilhabela, Oil terminal, Palmas and Itaipu in spring and Palmas and Itaipu in autumn (Fig. 2C).

The scores of the first and second factors were plotted in a two-dimensional way aiming to produce an integrated view of the studied areas during the monitoring. This representation allows distinguishing four groups of stations, based on the biomarkers associated by their loadings in each one of the two factors (Fig. 3).

A first group of stations was positioned with negative values for both factors. The farming station Cocanha in winter, Oil terminal and Itaipu in spring and Cocanha, Ilhabela and Palmas in summer are located in this group. The second group was formed by the stations with positive values to Factor 1 and negative values for Factor 2; the stations Cocanha, Ilhabela and Palmas in spring and Cocanha and Palmas in autumn are located in this group. The third group was constituted by negative values to Factor 1 and positive values to Factor 2, including the stations Ilhabela, Oil terminal, Palmas and Itaipu in winter and Oil terminal in autumn and a fourth group represented the stations with positive values for both factors, where are located the station

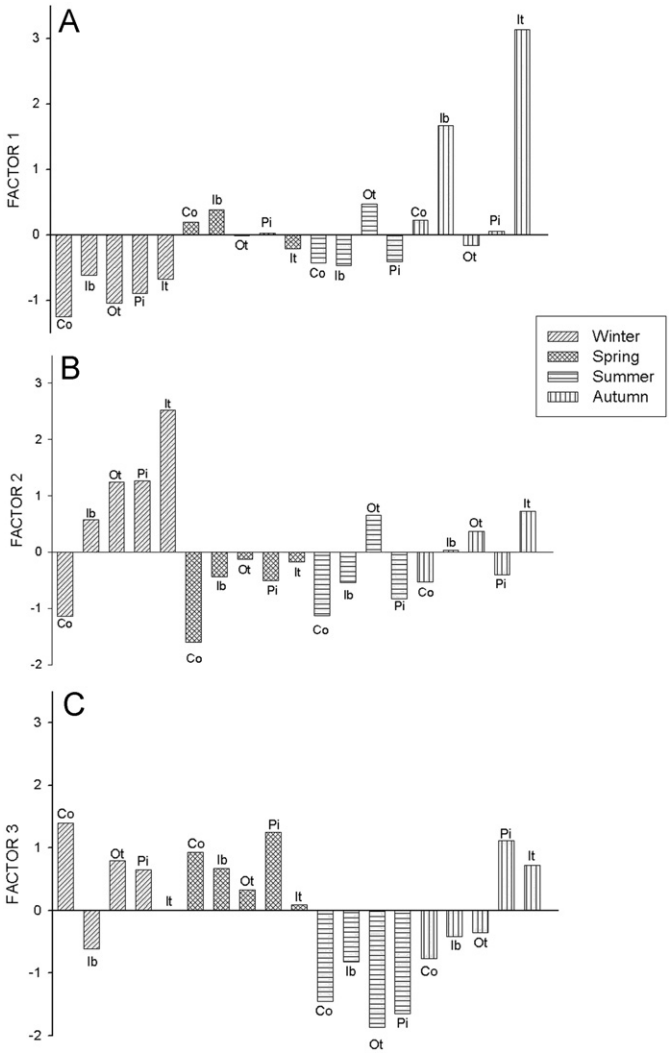

Fig. 2. Representation of Factor 1 (A), Factor 2 (B) and Factor 3 (C) scores estimation for each of the 19 cases evaluated employing biomarker responses after multivariate analysis.

Oil terminal in summer and Ilhabela and Itaipu in autumn with positive scores for both factors.

4. Discussion

Our study has shown spatial and seasonal differences concerning on biomarker responses. Mixed-function oxidase activity (MFO) was evaluated through EROD and DBF measurements, which are substrates employed to assess CYP 450 activities in vertebrate species. They have been also reported in many species

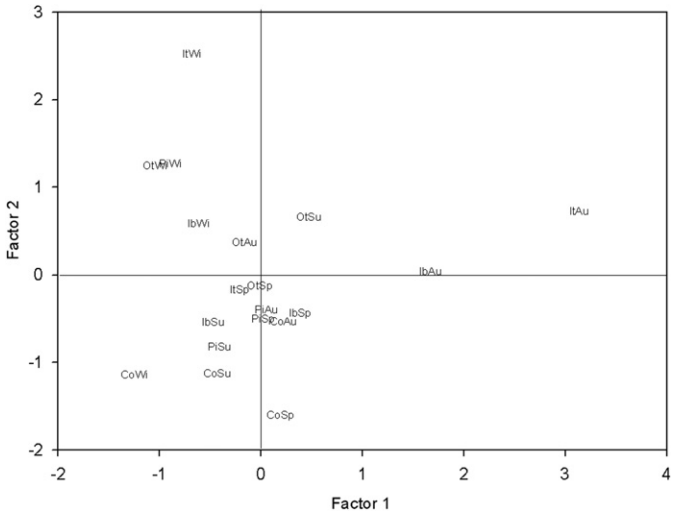

Fig. 3. Two-dimensional representation of Factors 1 and 2 scores for each of the 19 cases evaluated using the biomarker responses after multivariate analysis (Wi: winter survey; Sp: spring survey; Su: summer survey; Au: autumn survey).

of invertebrates as well, following exposure to organic pollutants, such as PAHs, PCBs and pharmaceuticals (Lafontaine et al., 2000; Quinn et al., 2004). EROD and DBF activities exhibited a significant increase in mussels transplanted to Ilhabela (autumn), Oil terminal (winter, summer, autumn), Palmas (autumn) and Itaipu (winter, spring, autumn), indicating exposure to organic compounds. The presence of organic xenobiotics in these zones could be related to industrial and municipal submarine effluents outfalls located especially nearby the Oil terminal and Itaipu, respectively. Previous studies showed high PAH body burden in native mussels from the Oil terminal (Pereira et al., 2007) and Santos Estuary (CETESB, 2001), where the station Itaipu is located. The possible influence of pharmaceuticals on mussels' DBF activity, according described by Gagné et al. (2007), should be also further investigated, since no data are available about the contamination of these compounds in Brazilian coastal zones.

Phase II detoxification system (conjugation) was evaluated in the present study by GST activity. Glutathione transferases are a family of enzymes that utilise glutathione (GSH) as a substrate in reactions, which permit the biotransformation and disposal of a wide range of exogenous compounds (Contreras-Vergara et al., 2004). These compounds may be xenobiotics, drugs or products of oxidative stress, but are mainly polar organic compounds (Martín-Díaz et al., 2008). Mussels caged on Ilhabela (spring, autumn), Oil terminal (winter, spring, summer) and Itaipu (winter, autumn) exhibited induced GST activity when compared to organisms kept in the farm zone. The correlation between MFO enzymes and GST activities observed especially in mussels transplanted to Oil terminal and Itaipu denotes a physiological adaptation to organic contamination.

The enzymatic antioxidant system was evaluated through GPx and CAT activities in gills of mussels. CAT and GPx are known to protect the cell by reducing H_2O_2 to H_2O. Hydrogen peroxide is the main cellular precursor of the hydroxyl radical, the most toxic ROS; due to the relative poor efficiency of antioxidants towards this ROS, H_2O_2 removal has been indicated as an important strategy for counteracting the toxicity of hydroxyl radicals (Oliveira et al., 2009). Many studies have demonstrated the relationship between the enzymatic antioxidant system and exposure to xenobiotics in aquatic invertebrates (Cajaraville et al., 2000).

Our results showed GPx activity increased in mussels caged in Ilhabela (winter), Oil terminal (winter, summer), Palmas (winter) and Itaipu (winter, autumn), indicating a defence mechanism against oxidative stress. CAT activity was significantly higher only in the mussels transplanted to Itaipu in the autumn survey. Regarding oxidative stress promoted by increased ROS generation, mussels transplanted to Ilhabela (spring and summer), Oil terminal (winter, spring and autumn) and Itaipu (winter and spring) showed signals of lipid peroxidation through a significant increase in the levels of end products such as malondialdehyde, which were assayed with thiobarbituric acid and expressed as TBARs levels.

Lysosomal stability in mussels' haemocytes constitutes a very useful index of cellular damage. Lysosomal activity is directly related to immunoreactivity in bivalves since lysosomes play a central role in the degradation of phagocytised materials. Alterations in lysosomes may result in immunity impairment that may compromise the entire physiology (Grundy et al., 1996). Indeed, damage to lysosomal membranes in bivalves may also reduce growth and reproductive potential (Moore and Viarengo, 1987). In the present study, mussels transplanted to Ilhabela (winter, spring, summer) Oil terminal (winter, spring, summer, autumn) Palmas (winter, spring) and Itaipu (winter, spring, autumn) exhibited significantly reduced NRRT when compared with the mussels kept in the farming zone, which can be interpreted as an indication of physiological stress.

Our results presented NRRT means of mussels from reference station ranging from 50 to 60 min, which could be considered a sign of stress, according criteria established by OSPAR (2007). However, these results are in agreement with previous studies on NRRT of *P. perna* populations from Brazilian coast. Francioni et al. (2007) observed NRRT means of 60 min in mussels from a non-polluted area where PAHs body burden were analysed and tissue concentrations were considered between normal ranges. Similar results were observed in our previous study (Pereira et al., 2007), which native *P. perna* mussels were collected in an uncontaminated area from São Paulo coast. Low levels of contaminants (metals and PAHs) were observed in mussels' tissues, but NRRT means did not exceed 45 min. Abessa et al. (2005) analysing NRRT in mussels from an environmental protection area of São Paulo coast (Ponta Grossa—Ubatuba) demonstrated NRRT means no higher than 40 min. Based on these studies, we can hypothesise that healthy mussels from tropical warmer waters present lower NRRT than healthy mussels from temperate regions. This variation in lysosomal membrane stability could occur in response to temperature, as already proposed by previous studies (Regoli, 1992; Tremblay et al., 1998; Nicholson, 2001), which could lead to erroneous interpretations of tropical mussels' health through direct application of physiological condition criteria established to organisms from temperate zones.

Finally, DNA damage can lead to reproductive impairment, abnormal development, cancer, lethal mutations, as well as to an increase or decrease of the genetic variability (Dickmann et al., 2004). DNA strand breaks evaluated in mussels' gills exhibited significant higher occurrence in mussels transplanted to Ilhabela (summer), Oil terminal (winter, spring) and Itaipu (winter), indicating exposure and effects of xenobiotics with potential genotoxicity. Previous studies had already detected bioaccumulation of PAHs in native mussels from the Oil terminal (Pereira et al., 2007) and bioaccumulation of trace metals (Cr and Pb) in mussels from Itaipu (Catharino et al., 2008).

The multivariate analysis (PCA-Factor Analysis) applied to the complete biomarker data set was used firstly to identify a subset of biomarkers that summarises most of the responses and its correlations (Factor loadings), and subsequently to improve the discriminatory power of the adopted multi-biomarker strategy characterizing the studied stations over time (Factor scores).

In the context of biomonitoring and in order to categorise biomarkers according to their type of response, at this point we find appropriate to refer to them as biomarkers of 'defence' (e.g. the induction of MFO or antioxidant enzymes is a defence reaction by

an organism exposed to some contaminants) or as biomarker of 'damage' (e.g. DNA strand breaks, lipid peroxidation or reduced stability of lysosomal membranes are direct alterations caused by toxic contaminants to the organisms), according to Lafontaine et al. (2000). In this sense, Factor 1 can be considered as the association of the biomarkers of defence (EROD, DBF, GPx or GST), while Factor 2 represents the association of the biomarkers of damage (DNA damage and reduced NRRT).

Thus, analysing Fig. 3, it is possible to distinguish a first group of studied stations positioned with negative values for both factors and it could be understood as the group where defence and damage biomarker responses were not induced, considering all data set integrated. The farming area Cocanha (winter and summer), Ilhabela (summer), Oil terminal (spring), Palmas (summer) and Itaipu (spring) are located in this group. A second group formed by studied stations presented positive values to Factor 1 and negative values for Factor 2 could be interpreted as an exposure to xenobiotics without observed effects, suggesting that defence mechanisms have been effective. Another possibility is the interference of the reproductive event, typical in the spring and autumn to *P. perna* mussels in Brazil (Lunetta, 1969; Galvão et al., 2006), especially on the GPx and GST baseline activities. Similar results were observed to *Mytilus galloprovivialis* from reference areas by Bochetti and Regoli (2006) and to *P. perna* from Southern Brazilian coast by Wilhelm Filho et al. (2001), probably related to the intense gametogenesis period and water temperature increasing. Cocanha (spring and autumn), Ilhabela (spring) and Palmas (spring and autumn) are located in this group. A third group constituted by the stations with negative values to Factor 1 and positive values to Factor 2, indicates the occurrence of effects not associated to the biomarkers of defence analysed. According to Oliveira et al. (2009), the lack of measurable defence in concomitance with effects could be a result of an environmental condition insufficient to stimulate protection though pernicious enough to cause damage. Mussels transplanted to Ilhabela (winter), Oil terminal (winter and autumn), Palmas (winter) and Itaipu (winter) were located in this group. Finally, a fourth group represented the sampling stations presenting positive values for both factors, indicating the relationship between exposure to possibly organic contaminants, considering Phase I and Phase II association, generating ROS and oxidative stress considering GPx induction with effects as DNA damage and reduced integrity of lysosomal membrane. Ilhabela (autumn), Oil terminal (summer) and Itaipu (autumn) showed positive scores for both factors.

Considering this approach, studied stations with positive values to Factor 2 can be considered as the most threatened and the biomarker responses could be understood as early warnings of effects in higher biological levels, since DNA damage and reduced lysosomal membrane integrity has been linked to impairments at individual and population level. The Stations Oil terminal and Itaipu, except in the spring survey, presented Factor 2 positive scores all year long, suggesting exposure to contaminants leading to physiological stress and biological effects. This interpretation is supported by previous studies (Zanardi et al., 1999; Medeiros and Bícego, 2004; Cesar et al., 2007; Melo and Nipper, 2007; Abessa et al., 2008), which detected contamination and/or acute toxicity in sediment samples from São Sebastião Channel and Santos Bay, where are located the study stations Oil terminal and Itaipu, respectively.

5. Conclusion

Mussels transplanted to the Oil terminal and Itaipu presented early warning signals of impaired health during the monitoring, which points to the impact of different sources of contaminants, such as the industrial and domestic effluent discharges, in the water quality. The integrated approach based on multi-biomarker responses of caged mussels under field conditions showed satisfactory results producing a rapid, sensitive and cost-effective assessment, which could be incorporated as a descriptor of environmental status in future coastal zones biomonitoring.

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