Available online at www.sciencedirect.com
SCIENCE DIRECT®
Environmental Research 99 (2005) 387–396
Environmental Research
www.elsevier.com/locate/envres

Lysosomal responses as a diagnostic tool for the detection of chronic petroleum pollution at Todos os Santos Bay, Brazil

L.K.P. Martins[a], I.A. Nascimento[a,e,*], G. Fillmann[b], R. King[c], A.J.A. Evangelista[a], J.W. Readman[d], M.H. Depledge[c]

[a]*Lab. de Bio Marinha e Biomonitoramento, IBIO-UFBA, Campus Universitário de Ondina, 40170-290 Salvador, BA, Brazil*
[b]*Depto. de Oceanografia, Fundação Universidade Federal do Rio Grande, C.P. 474, 96201-900 Rio Grande, RS, Brazil*
[c]*PERC, University of Plymouth, Drake Circus, Plymouth PL4 8AA, UK*
[d]*Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL1 3DH, UK*
[e]*Faculdade de Tecnologia e Ciências, Av. ACM 3015 Iguatemi, 41820-021 Salvador, Bahia, Brazil*

Received 23 September 2004; received in revised form 7 January 2005; accepted 11 January 2005
Available online 11 March 2005

Abstract

Coastal marine environments, especially semienclosed systems such as bays, are under unrelenting stress caused by urban and industrial development. Biomonitoring plays a vital role in strategies to identify, assess, and control stressors. However, due to the magnitude of the challenge there is a demand for new and innovative approaches to provide timely and accessible information to environmental managers and policy makers. The present work aimed to assess hydrocarbon levels in sediments from petroleum-related industrial areas at Todos os Santos Bay (Brazil) and associate them to toxicity-induced responses (neutral red retention (NRR) assay) by the burrowing clam *Anomalocardia brasiliana*. Surface sediments collected during the dry and rainy seasons were analyzed for aliphatic and aromatic hydrocarbons. At the control site, hydrocarbon levels were low and mainly biogenic. The aliphatic hydrocarbon (''total unresolved complex mixture,'' alkanes, and isoprenoids) concentrations indicated a chronic situation with very little ''fresh'' oil contamination at the oil-related sites. The polycyclic aromatic hydrocarbons indicated sites moderately contaminated by chronic oil and some pyrolytic input. The effects of those contaminants were assessed by the lysosomal NRR assay applied to *A. brasiliana* hemocytes. Sediment toxicity at the oil-related sites was evidenced by the lowered capacity of the lysosomes to retain the neutral red dye compared to results from the control site. This research indicates that the NRR assay is a useful and efficient screening technique able to discriminate polluted from clean sites.

Keywords: Neutral red retention assay; Lysosomes; Hemocytes; PAHs; Hydrocarbon contaminants; Biomarker

1. Introduction

Brazil has a coastline of 8500 km including different ecosystems of high ecological interest such as coral reefs and mangroves (MMA, 1996). Above 50% of its human population lives along the coast, where the industries are also prevalently located. This imposes an ecological pressure onto the ecosystems of the Brazilian coast, which threatens biodiversity.

Oil exploitation started in the early 1950s in the State of Bahia (northeastern Brazil), when environmental issues were not relevant for coastal planning. Consequently a baseline environmental study does not exist; nor does a risk assessment study. Presently, the extraction, transportation, and refinement of petroleum at Todos os Santos Bay (12°42′S, 38°37′W) are the most

*Corresponding author. Faculdade de Tecnologia e Ciências, Superintendencia de Ensino, Pesquisa e Extensão, Praca da Inglaterra, 2, Ed Nobre Sala 301 Comercio, 40015-140 Salvador, Bahia, Brazil. Fax: +55 71 2359708.
E-mail address: iracema@ftc.br (I.A. Nascimento).

0013-9351/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.envres.2005.01.001

prominent industrial activities. Placed deeper into this bay is the refinery Landulfo Alves (RLAM), which processes about 10,800,000 m^3 $year^{-1}$ of oil (CRA, 2000). In addition, the extensive pipeline for the transportation of oil and its products constitutes a continuous threat to the environment. Moreover, oil processing generates waste, which contain hydrocarbons and heavy metals, and the existent treatment does not guarantee contaminant-free effluents. However, except for a few localized studies related to the determination of acute impact from oil spills (CRA, 2000) and some ecological surveys carried out during 1994–2000 (Peso-Aguiar and Almeida, 1996; Nascimento, 1996; Nascimento et al., 1998a, b, 2000a), no proper evaluation of the possible effects has been generated. Todos os Santos bay is one of the biggest (1086 km^2) in Brazil (Lessa et al., 2001) and is bordered by extensive mangrove communities. Consequently, the development of environmental diagnosis techniques and monitoring programs has become a priority.

The use of biomarkers is a promising approach for detecting in situ the early adverse effects of pollutants on organisms (Depledge, 1994; Nascimento et al., 1998b). The primary effect of toxicants is the perturbation of biochemical and molecular processes within the cell, which may alter the life functions of all exposed organisms (Bayne et al., 1988). These perturbations can also be detected at subcellular levels and can be considered early warning markers of pathological changes due to stress (Moore et al., 1996).

The lysosomal membrane liability has been indicated to be a sensitive biomarker (Moore et al., 1978; Moore and Willows, 1998) based on its capacity to respond to chemical exposure, which in turn determines contaminant-induced alterations in cell structure and function (Lowe et al., 1995a). Lowe et al. (1992) and Lowe and Pipe (1994) developed a biomarker method based on the lysosome capacity of uptake, retention, and reflux of the neutral red dye (neutral red retention (NRR) assay). The retention time of this dye is used as a determinant of effect. The application of this technique to bivalves' hemocytes is a noninvasive technique, which allows for the investigation of interactive effects of contaminants (Lowe et al., 1995b). Recent studies have demonstrated the use of this technique to identify pollution problems related to polycyclic aromatic hydrocarbons (PAHs) (Grundy et al., 1996) and other xenobiotics (Moore et al., 1996).

Especially in the case of mudflats, sediments are recognized as sinks and sources of contaminants in aquatic ecosystems (Nipper et al., 1998). In this sense, the clam *Anomalocardia brasiliana*, which lives burrowed in the mudflats, is used to show the efficiency of the NRR assay to discriminate between hydrocarbon-polluted areas and clean areas.

2. Material and methods

Sampling stations in the northeast of Todos os Santos bay were selected based on the availability of *A. brasiliana*, currents, and location of major oil-related activities, such as a refinery (RLAM, 12°43′S, 38°34′W), an oil transportation lane (Madre de Deus, 12°45′S, 32°37′W), and an oil extraction site (Ilha das Fontes, 12°43′S, 32°38′W) (Fig. 1). A control station (Barra dos Carvalhos, 13°39′S, 38°57′W) was chosen in another estuarine area located outside the Bay (coastal area south of the state of Bahia) (Fig. 1).

Surface sediments (2 cm depth) were sampled twice (dry and rainy seasons) during low tide at the intertidal zone. Composite sediment samples were collected from each station along a horizontal transect (0.5 km long), at every 100 m, by using an SS spatula and transported to the laboratory in iceboxes (4 ± 2 °C). Each sediment sample was well mixed and kept frozen until analysis.

Specimens of *A. brasiliana* ($n = 30$) were also collected from the superficial sediment (1−5 cm) at each sampling site and immediately transported to the laboratory, where they were maintained under aeration in clean and filtered (20 μm) sea water (S‰ = 28; T °C = 25 ± 2) for 24 h. Fifteen organisms from each station were randomly taken to be tested by the NRR assay following the methodology described by Lowe et al. (1995a). Hemolymph (0.1 mL) was withdrawn, using a hypodermic syringe containing 0.1 mL physiological saline, from the anterior adductor muscle and transferred to presiliconized microcentrifuge tubes. Hemolymph/physiological saline mixture (40 μL) was pipetted onto each slide, and the cells were treated by neutral red dye. The slides were placed in a lightproof humidity chamber and incubated for 15 min. Systematically the slides were examined under a light microscope every 15 min up to the first hour and then at each 30-min interval. The whole slide was scanned and replaced in the chamber as quickly as possible (ideally 1 min per slide maximum); the end point was taken to be when 50% or more of the cells exhibited lysosomal leakage or showed abnormalities.

Aliphatic and aromatic hydrocarbon analyses were carried out using a method modified from Readman et al. (2002). Briefly, the sediments were freeze-dried and dry/wet ratios were determined. Each sediment sample (10–20 g) was spiked with internal standard: C_{18-1} and 9,10-dihydroanthracene. The samples were Soxhlet-extracted into 250 mL of hexane/dichloromethane (50:50) for 16 h and concentrated using rotary evaporation followed by nitrogen "blow down." Sulfur was removed by shaking the extracts with activated copper. Extractable organic matter was gravimetrically determined. Cleanup and fractionation was performed by passing the extract through a silica/alumina column. Silica and alumina were both activated at 200 °C for 4 h

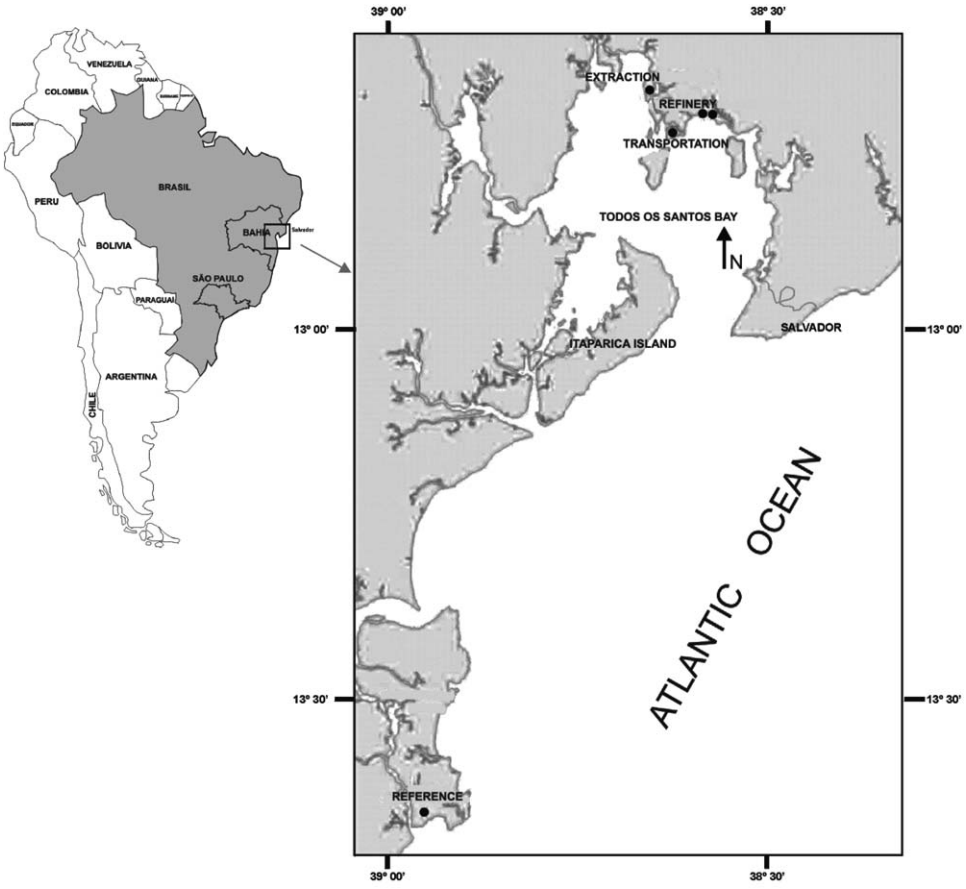

Fig. 1. Sampling sites at Todos os Santos Bay (oil related) and at the reference (control) site.

and then partially deactivated with 5% water. Elution was performed using hexane to yield the first fraction (containing the aliphatic hydrocarbons) and then hexane/dichloromethane (90:10) followed by hexane/dichloromethane (50:50) that, combined, contain the PAHs.

Fractions were then analyzed by gas chromatography (GC) using a Hewlett–Packard HP5890 series II with a flame ionization detector. The capillary column used was a DB-5MS (5% phenyl/95% dimethylpolysiloxane, 30 m length, 0.25-mm ID, 0.25-μm film thickness). The temperature was programmed from 40 to 60 °C at 40 °C min^{-1}, from 60 to 300 °C at 5 °C min^{-1}, and maintained at 300 °C for 20 min. Injector and detector temperatures were set at 40 and 300 °C, respectively. Confirmation of peak identification was obtained for selected extracts using GC/MS (Hewlett–Packard Model 5890 II Plus GC and a 5972 mass selective detector). Appropriate blanks were analyzed and reference material IAEA-357 was analyzed simultaneously with each batch.

Total organic carbon (TOC) was determined using a Carlo Erba NA-1500 Elemental Analyzer following methodology described by Verardo et al. (1990).

3. Results

The results of the NRR assay ($n = 15$/site) for the dry season (Fig. 2) showed that the hemocyte lysosomes from the control area had, in average ($\pm$SD), the capacity to retain the dye for 86 ($\pm$19.2) min. Those from the transportation site retained the dye for 33 ($\pm$8.4) min, while 21 ($\pm$9.5) min was the retention time for hemocyte lysosomes from the oil extraction site. At the refinery, hemocyte lysosomes lost the dye to the cytosol in an average time of 20 ($\pm$7.3) min. One-way analysis of variance (Sokal and Rohlf, 1981) and a multiple means comparison test confirmed that the control site NRR average value was significantly higher ($P < 0.05$) than those obtained for other sites.

A similar trend for the rainy season can be seen (Fig. 3). The lysosomes of the control site clams presented the highest NR average retention time (46 ± 30.7 min). However, this average value did not differ significantly ($P > 0.05$) from the values obtained for transportation (40 ± 24.5 min), extraction (34 ± 18.3 min), and refinery (33 ± 15.1 min) sites. The sediments in the study sites were mostly muddy or sand muddy (silt and clay fraction varying from 37.5% to

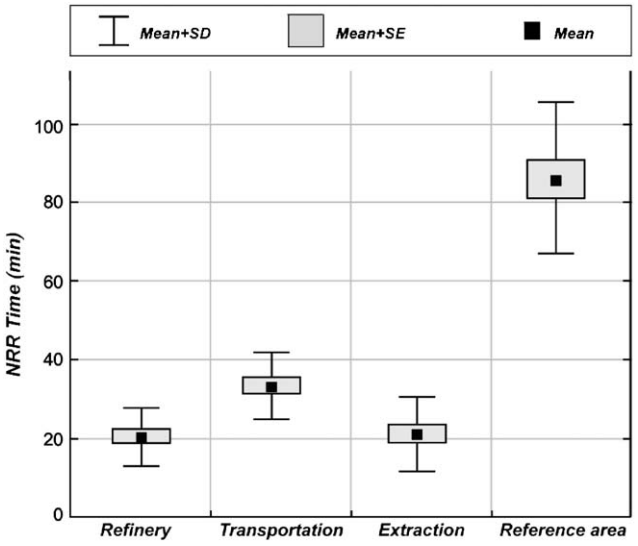

Fig. 2. Retention time of neutral red in hemocytes of *Anomalocardia brasiliana* during the dry season.

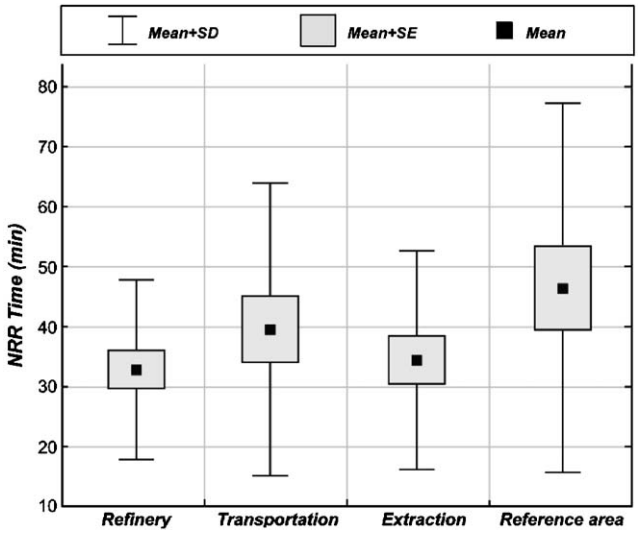

Fig. 3. Retention time of neutral red in hemocytes of *Anomalocardia brasiliana* during the rainy season.

77.7% for the oil-related sites and 71.8% at the control site). The total organic carbon (TOC) varied from 0.3% to 1.2% for the oil-related sites and from 3.6% to 5.2% for the control site (Table 1).

With regard to "total" aliphatic hydrocarbons, the most contaminated stations were refinery ($400\,\mu g\,g^{-1}$ dry wt for the rainy season) and extraction (185 and $92\,\mu g\,g^{-1}$ dry wt for the dry and rainy season, respectively), while samples taken from the control (37 and $43\,\mu g\,g^{-1}$ dry wt for the dry and rainy season, respectively) and transportation (34 and $59\,\mu g\,g^{-1}$ dry wt for the dry and rainy season, respectively) sites showed comparatively little contamination (Table 1). Organic-rich marine sediments may contain up to $100\,\mu g\,g^{-1}$ of total aliphatic hydrocarbons, but concentrations higher than these are usually associated with petroleum inputs (Volkman et al., 1992; Bouloubassi and Saliot, 1993). Compared to other regions of the world (Readman et al., 2002), the lower levels are shown to be comparable to those encountered in places where hydrocarbon contamination was considered relatively low, whereas the higher levels registered for sediments are, however, comparable to those reported for locations known to be chronically contaminated by oil (e.g., Odessa, Black Sea ($110–310\,\mu g\,g^{-1}$ dry wt); the Gulf ($11–6900\,\mu g\,g^{-1}$ dry wt); Hong Kong ($60–646\,\mu g\,g^{-1}$ dry wt); New York Bight ($35–2900\,\mu g\,g^{-1}$ dry wt)).

In general, the presence of an unresolved complex mixture (UCM) in aliphatic hydrocarbon chromatograms is considered to be associated with degraded or weathered petroleum residues (Farrington and Tripp, 1977; Le Dréau et al., 1997). Ratios of unresolved to resolved (U/R) > 4 confirm the widespread presence of important petroleum-related residues (Mazurek and Simoneit, 1984; Lipiatou and Saliot, 1991). In the oil-related sites, the UCM was by far the major component of the total sedimentary aliphatic hydrocarbons. UCM concentrations varied from 32 to $386\,\mu g\,g^{-1}$ dry wt, which accounted for 84–97% of the total aliphatic hydrocarbons (Table 1). The U/R ratio ranged from 5.4 to 27 (Table 1), indicating chronic petroleum contributions to these sediments, particularly for the refinery site. On the other hand, in the control site the UCM concentrations were 14 and $23\,\mu g\,g^{-1}$ (dry wt), which accounted for 38% and 53% of the total aliphatic hydrocarbons (dry and rainy season, respectively) (Table 1). The U/R ratio was indicative of low chronic petroleum contributions to these sediments.

Although biogenic hydrocarbons from recent sources can dominate chromatograms in uncontaminated samples (UNEP/IOC/IAEA, 1992), Σn-C_{14} to n-C_{35} can provide a good indication of "fresh" oil inputs (Readman et al., 2002). n-Alkanes (Σn-C_{14} to n-C_{35}) concentrations in the sediments ranged from 1.3 to $16.3\,\mu g\,g^{-1}$ (dry wt) for the dry season and from 2.3 to $18.6\,\mu g\,g^{-1}$ (dry wt) for the rainy season (Table 1). The lowest concentrations were reported for the oil sites indicating less fresh oil inputs at this location. The refinery site, however, has shown no odd-even predominance, which is often an indication of recent inputs of oil (Table 1 and Fig. 4a). Conversely, alkane profiles for the control site, which contained the highest alkane concentrations, showed a well-defined odd-even predominance (>3.9) that is consistent with a major source in terrestrial, higher plant material (odd carbon alkanes from n-C_{23} to n-C_{33}, with a maximum at n-C_{27}, n-C_{29}, or n-C_{31}) (Eglinton and Hamilton, 1967) (Fig. 4b). The transportation and extraction sites have also shown small biogenic inputs. Algae-related n-C17 appears as a prominent peak in the control (Fig. 4b) and extraction

Table 1
Concentrations of aliphatic hydrocarbons (μg g^{-1} dry wt) and total organic carbon (%) in the sediments from oil industry and control sites during the dry and rainy season

Aliphatic hydrocarbon (μg g^{-1} dry wt)	Dry season (November 1998)				Rainy season (July 1999)			
	Refinery	Transport	Extraction	Control	Refinery	Transport	Extraction	Control
C12	ND	ND	ND	ND	ND	ND	ND	ND
C13	ND	ND	ND	ND	0.01	ND	ND	0.01
C14	0.01	ND	0.05	0.01	0.01	0.01	0.01	0.02
C15	0.08	0.04	1.72	0.20	0.09	0.03	0.03	0.08
C16	0.03	0.04	0.24	0.07	0.03	0.01	0.01	0.02
C17	0.32	0.23	0.95	3.01	0.15	0.16	0.11	0.23
Pristane	0.05	0.01	0.19	0.15	0.09	0.02	0.01	0.62
C18	0.08	0.03	0.13	0.09	0.30	0.02	0.04	0.03
Phytane	0.05	0.02	0.07	0.03	0.17	0.03	0.03	0.03
C19	0.09	0.01	0.15	0.11	0.21	0.02	0.04	0.14
C20	0.07	0.01	0.09	0.03	0.13	0.02	0.06	0.06
C21	0.08	0.01	0.19	0.29	0.14	0.04	0.07	0.38
C22	0.09	0.01	0.15	0.08	0.20	0.04	0.08	0.12
C23	0.09	0.02	0.27	0.29	0.30	0.05	0.14	0.45
C24	0.09	0.02	0.18	0.19	0.24	0.04	0.06	0.19
C25	0.11	0.05	0.65	0.81	0.50	0.12	0.28	1.11
C26	0.08	0.03	0.35	0.37	0.23	0.09	0.15	0.51
C27	0.10	0.11	0.91	1.77	0.44	0.18	0.36	2.51
C28	0.07	0.05	0.46	0.81	0.19	0.10	0.21	1.18
C29	0.15	0.28	2.20	4.11	0.81	0.53	0.78	5.91
C30	0.07	0.04	0.41	0.80	0.21	0.16	0.15	1.18
C31	0.09	0.14	1.23	2.27	0.54	0.30	0.50	3.29
C32	0.04	0.03	0.21	0.24	0.87	0.07	0.10	0.35
C33	0.05	0.07	0.60	0.54	0.17	0.15	0.24	0.55
C34	0.03	0.02	0.21	0.05	0.21	0.04	0.06	0.11
C35	0.03	0.02	0.20	0.12	0.10	0.09	0.09	0.16
C36	0.02	ND	0.04	0.05	ND	0.07	0.04	0.07
Sum *n*-C14–35	1.9	1.3	11.5	16.3	6.1	2.3	3.5	18.6
Total aliphatic	43.8	33.4	184.6	37.0	399.6	58.9	91.5	43.0
UCM	40.8	31.5	155.7	14.2	385.6	55.9	84.9	22.7
Resolved aliphatics	3.1	1.9	28.9	22.8	14.1	3.0	6.6	20.3
%UCM	93.0	94.3	84.3	38.4	96.5	94.9	92.8	52.7
Unresolved/resolved	13.3	16.6	5.4	0.6	27.4	18.7	12.9	1.1
Odd/even	1.8	3.7	3.6	4.9	1.3	2.5	2.8	3.9
Pristane/phytane	1.2	0.7	2.5	5.2	0.5	0.7	0.5	22.5
Total organic carbon (%)	0.3	0.3	1.23	3.60	0.4	0.4	0.7	5.2

ND, below detection limit.

sites from the dry season, indicating an important contribution of algae productivity to these samples (Meyers and Ishiwatari, 1993).

Pristane (C_{19}) and phytane (C_{20}) are common isoprenoids detected in coastal marine sediments. They are often considered good indicators of petroleum contamination. As a rule, a high ratio of pristane to phytane or the predominance of a single isoprenoid (such as pristane) indicates a biogenic source (UNEP/IOC/IAEA, 1992). The pristane to phytane ratios in the sediments were $\leqslant 1$ in most of the oil-related sites (except for extraction from the dry season), reflecting contamination originated from petroleum. Whereas, highest ratios of pristane to phytane (>2.0) were recorded in the control site, reflecting biogenic origin.

Concentrations of total PAHs, (the sum of 24 parental and alkylated compounds) in sediments of the oil-related sites varied from 440 to 777 ng g^{-1} (dry wt) and from 96 to 310 ng g^{-1} (dry wt) for the dry and rainy season, respectively (Table 2). These concentrations were comparable to moderately polluted locations worldwide (Readman et al., 2002). Conversely, the concentration observed for the control site (rainy season) was lower than 50 ng g^{-1} (dry wt), which is a typical concentration for locations distant from extensive anthropogenic activities (Baumard et al., 1998b).

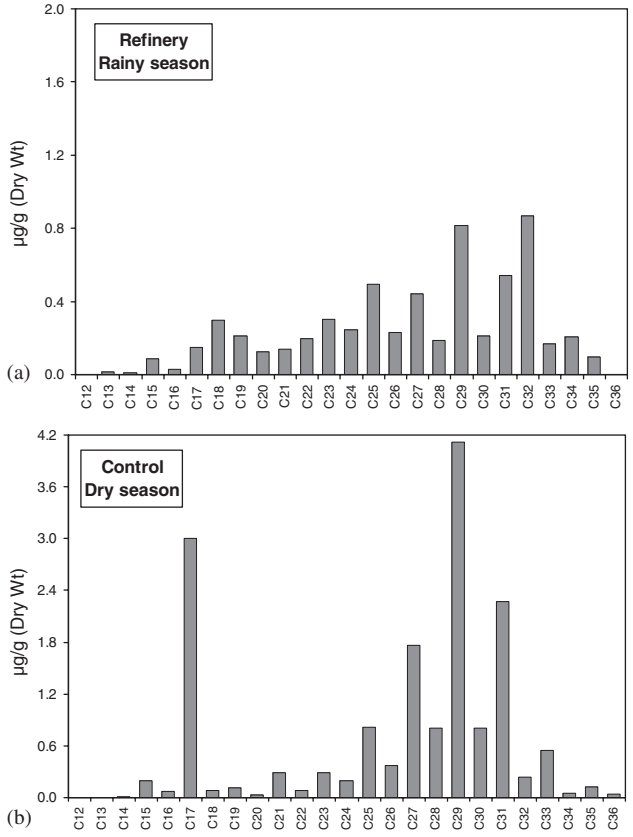

Fig. 4. Individual alkane profile (n-C_{12}–n-C_{36}) for (a) refinery (rainy season) and (b) control (dry season) sites.

The dry season, however, showed a slightly higher value (83 ng g^{-1} dry wt) (Table 2).

Concentrations of aromatic UCM ranged from 4518 to 63,719 ng g^{-1} (dry wt) and from 7528 to 55,167 ng g^{-1} (dry wt) for the dry and rainy season, respectively, which accounted for 73–96% of the total aromatic hydrocarbons (Table 2) and 11–38% of the total (aromatic + aliphatic) hydrocarbons. Conversely, values were smaller than 3250 ng g^{-1} (dry wt) at the control site, which accounted for less than 62% of the total aromatic hydrocarbons (Table 2) and 0.6 (dry) and 6.7 (rainy) of the total (aromatic + aliphatic) hydrocarbons.

The individual PAHs profile (Fig. 5a) and the molecular indices based on ratios of selected PAH concentrations (Table 2) indicates that the PAHs in the sediments from the oil-related sites originated from both petrogenic and pyrolytic sources. It is likely that combustion-derived PAHs will have atmospheric components to their transport mechanisms whereas petrogenic PAHs will be predominantly from the oil industrial activities. The predominance of higher-molecular-weight PAHs indicates (together with the aliphatic hydrocarbon results) chronic oil inputs since these molecules are more stable in the sediment in comparison to the lower-molecular-weight compounds (Neff, 1979). A small contribution of petrogenic and pyrolytic PAHs can be also observed in the control sites (Fig. 5b).

4. Discussion

Among various chemical contaminants, the pollution caused by hydrocarbons is of greatest concern. This is due to the carcinogenicity and therathogenicity of the highest-molecular-weight compounds and the toxicity of the most soluble compounds (Baumard et al., 1998a, b).

The significance of sediment hydrocarbons contamination, considering the hydrophobic character of most of these compounds, has been recognized (Neff, 1979; Hellow et al., 1993; Djomo et al., 1996; Baumard et al., 1999a, b). Due to their characteristic of being bound to organic substrates, it is already demonstrated that hydrocarbons may cause adverse biological effects even though quality criteria are not exceeded (NRC, 1989).

There has been much evidence worldwide of acute hydrocarbon contamination in coastal areas (Botello et al., 1998, Readman et al., 1986, 2002; Baumard et al., 1999a; Soclo et al., 2000). However, the chronic effects of these contaminants on biota are more difficult to detect. Even though the main reason for the interest in chemical concentrations is their connection with biological effects (Kalakkis and O'Connor, 1985), most of the research on this topic considers compound concentrations as indirect evidence of effects (Baumard et al., 1999a).

Working with a database containing simultaneously measured chemical concentrations and biological effects of estuarine and marine sediments, Long et al. (1995) selected the 10th and 50th percentile chemical concentration ranked values as being, respectively, effects range-low (ER-L) and effects range-median (ER-M) values. Total PAH sediment concentrations below ER-L value (4022 ng g^{-1} dry wt) were rarely associated with biological effects. Concentrations in the range between ER-L and ER-M (44,792 ng g^{-1} dry wt) were found to occasionally cooccur with effects. Biological effects were often found to cooccur with sediment concentrations above the ER-M value (44,792 ng g^{-1} dry wt).

When analyzed according to the sediment quality guidelines proposed by Long et al. (1995), values for total PAH (sum of 24 resolved compounds) as evidenced by the present research for all the study areas and periods were below the level which may cause effects to biota (ER-L). These results, however, did not consider the aromatic UCM. Rowland et al. (2001) confirmed that this UCM includes alkyl benzenes and C-ring monoaromatic steroids linked to toxicity responses and adverse health effects in exposed mussels. When UCM values were included in the analysis, the total aromatic sediment concentrations at the refinery site were above ER-M (biological effect often found) in both study

Table 2
Concentrations of aromatic hydrocarbons (ng g^{-1} dry wt) and total hydrocarbons (μg g^{-1} dry wt) in sediment from oil industry sites and control sites during the dry and rainy season

Aromatic hydrocarbon (ng g^{-1} dry wt)	Dry season (November 1998)				Rainy season (July 1999)			
	Refinery	Transport	Extraction	Control	Refinery	Transport	Extraction	Control
Naphthalene	4.3	ND	6.3	5.8	ND	ND	ND	ND
2-Methyl naphthalene	ND	ND	2.4	8.4	ND	ND	ND	ND
1-Methyl naphthalene	ND	ND	2.7	ND	ND	ND	ND	ND
Biphenyl	ND	ND	ND	ND	ND	ND	ND	ND
2,6-Dimethyl naphthalene	ND	ND	ND	ND	ND	ND	ND	ND
Acenaphthylene	ND	ND	ND	ND	ND	ND	ND	ND
Acenaphthene	ND	ND	ND	ND	5.1	ND	ND	ND
2,3,5-Trimethyl naphthalene	ND	ND	ND	ND	1.0	ND	ND	ND
Fluorene	ND	ND	ND	ND	ND	ND	ND	ND
Phenanthrene	14.0	21.7	21.7	ND	13.8	18.7	4.0	2.1
Anthracene	13.0	12.9	11.4	ND	ND	ND	ND	ND
1-Methyl phenanthrene	ND	2.9	4.0	ND	6.3	2.2	ND	ND
Fluoranthene	73.5	64.2	84.0	ND	7.5	40.8	8.8	14.7
Pyrene	8.6	58.3	84.7	ND	43.9	41.0	8.1	11.1
Benzo(a)anthracene	ND	6.4	27.9	2.8	ND	ND	ND	ND
Chrysene	23.5	36.9	49.4	8.5	6.3	24.2	ND	ND
Benzo(b)fluoranthene	70.6	73.0	115.0	9.0	33.7	49.4	19.0	15.7
Benzo(k)fluoranthene	30.9	27.7	36.5	ND	9.2	17.4	7.2	7.5
Benzo(e)pyrene	34.9	43.4	84.5	3.3	44.4	27.3	12.8	6.2
Benzo(a)pyrene	44.5	41.6	51.5	ND	9.1	27.6	8.0	6.5
Perylene	21.4	10.2	29.8	ND	5.7	6.2	1.4	6.0
Indeno(1,2,3-cd)pyrene	45.7	36.7	60.2	ND	15.3	22.1	7.6	4.6
Dibenzo(a,h)anthracene	6.5	18.1	12.7	2.9	24.5	5.3	4.7	1.8
Benzo(g,h,i)perylene	48.7	40.6	92.0	3.6	84.7	27.5	14.5	6.7
Sum PAHs (24 compounds)	440.2	494.5	776.6	44.3	310.5	309.7	96.1	83.1
Total aromatic	46,356	6110	66,575	389	59,054	9003	14,000	5373
UCM	33,873	4518	63,719	240	55,167	7528	13,482	3248
Resolved aromatics	12,483	1592	2856	149	3887	1476	518	2125
%UCM	73.1	73.9	95.7	61.8	93.4	83.6	96.3	60.5
Unresolved/resolved	2.7	2.8	22.3	1.6	14.2	5.1	26.0	1.5
Phenanthrene/anthracene	1.1	1.7	1.9					
Fluoranthene/pyrene	8.5	1.1	1.0		0.2	1.0	1.1	1.3
Benzo(a)anthracene/chrysene		0.2	0.6	0.3				
Phe/Ant//Flth/Pyr	0.1	1.5	1.9					
Total hydrocarbon (μg g^{-1})	70.2	39.5	251.1	37.3	458.7	67.9	105.5	48.4

ND, below detection limit.

periods (dry and rainy seasons). The ER-M level was also exceeded for the dry season samples from the extraction site. For all the other oil-related sites and periods the total PAH + UCM concentrations were above ER-L, but below ER-M (biological effects occasionally occurring). For the reference (control) station those compounds presented significantly ($P<0.05$) lower values (below ER-L during the dry season and a little above this level during the rainy season).

Kalakkis and O'Connor, 1985, applying Long et al. (1995) criteria to the database (COSED) of chemical concentrations in US coastal and estuarine sediments, concluded that chemical concentrations are not particularly strong predictions of toxicity, since concentrations in the lowest range (below ER-L) still test as toxic in about 15% of the cases, while concentrations in the highest range (above ER-M) do not correspond to more than about a 50% frequency of toxicity. This accounts for the differences on bioavailability.

Predictions with regard to the bioavailability (exposure) of chemical compounds can be made based on the early detection of the adverse effects of pollutants on organisms in situ (Depledge, 1994). This can be achieved by identifying biomarkers of declining health which have much greater ecological relevance (Sanders, 1990; Depledge et al., 1993). Liability and damage of the lysosomal membrane has been indicated to be a sensitive biomarker based on its capacity to respond to chemical challenges (Moore et al., 1978, 1996). Lysosomal

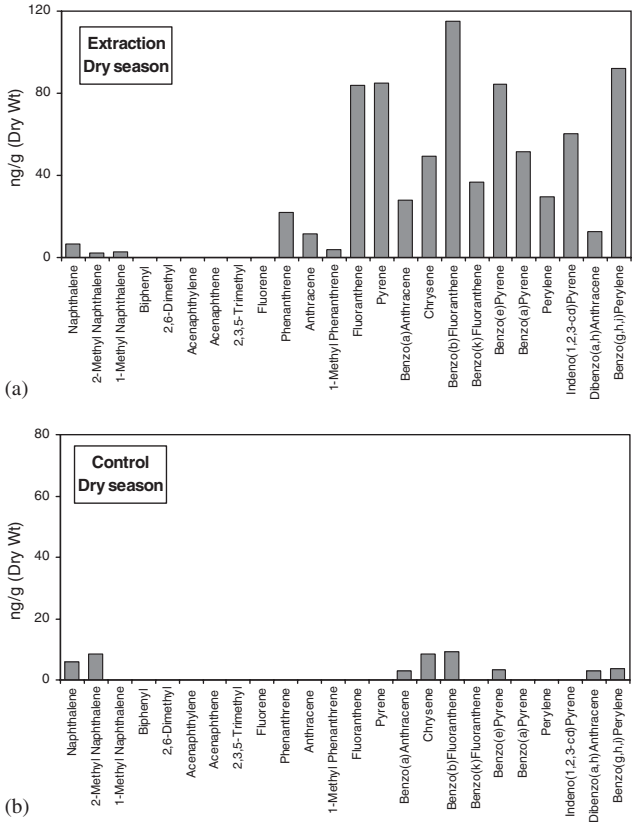

Fig. 5. Individual PAHs profile for (a) extraction and (b) control (reference) sites during dry season.

activity is directly related to immunoreactivity in bivalves and, since lysosomes play a central role in the degradation of phagocytosed materials, their integrity is essential to guarantee the animal's resistance to disease. Alterations in lysosomes may result in immunity impairment that may compromise the entire physiology (Grundy et al., 1996; Nascimento et al., 2000b). Considering that damage to lysosomal membranes does not appear to be transient, increased lysosomal membrane fragility in bivalves may also reduce growth and reproductive potential (Moore and Viarengo, 1987), impairments which have considerable ecological impact (Moriarty, 1983).

The present research shows that the NRR assay data for the rainy season were not significantly different ($P>0.05$) among specimens from all the study sites. However, for the dry season the hemocyte lysosomes of *A. brasiliana* collected from oil-related sites showed a dye retention capacity significantly ($P<0.05$) lower than those from specimens collected at the control site. The total aromatic concentrations in sediment samples during this period may explain the higher lysosomal fragility verified in the hemocytes from local bivalves. Similar results have also been obtained by Cheung et al. (1998) who found correlation between lysosomal retention time and contaminant concentrations along the pollution gradient in Tolo Harbor, Hong Kong. There were significant differences between mussels collected from stations on offshore islands and those collected from inner harbor sites.

Previous studies in the Bay of Todos os Santos (Nascimento, 1996; Tavares, 1996; da Silva et al., 1997; Nascimento et al., 1998b, 2000a) identified a moderate to low pollution potential in all areas of the petroleum industry, except for the refinery site, where the sediment toxicity was classified as high (Nascimento et al., 1998a, 2000a).

This research shows that the NRR assay is a useful technique that may discriminate areas under oil pollution, even though the concentrations of total (resolved) PAH is below the effect level. This technique can be used to indicate declining health of organisms exposed to low contamination and may act as an early warning tool for pollution assessment.

5. Conclusions

The aliphatic and aromatic hydrocarbons analysis on the surficial sediment from the petroleum industry sites at Todos os Santos Bay showed an area moderately contaminated by chronic petroleum hydrocarbon, while at the Control site the biogenic origin of the hydrocarbon was clearly prevalent.

Damaging effects of the petroleum industry wastes were shown during the dry season. The neutral red retention times for hemocyte lysosomes of *A. brasiliana* from all the oil-related sites were lower than those from the control area. Significant differences ($P>0.05$) were not found, however, between these sites during the rainy season.

The clam *A. brasiliana*, which lives burrowed in the mudflats, has been shown to be a good indicator in the appraisal of chronic toxicity assessed by the neutral red retention assay. This technique was sensible enough to discriminate between oil-impacted and clean areas.

References

Baumard, P., Budzinski, H., Garrigues, P., Sorbe, J.C., Burgeot, T., Bellocq, J., 1998a. Concentrations of PAHs (polycyclic aromatic hydrocarbons) in various marine organisms in relation to those in sediments and to trophic level. Mar. Pollut. Bull. 36, 951–960.

Baumard, P., Budzinski, H., Mchin, Q., Garrigues, P., Burgeot, T., Bellocq, J., 1998b. Origin and bioavailability of PAHs in the Mediterranean Sea from mussel and sediment records. Estuarine Coastal Shelf Sci. 47, 77–90.

Baumard, P., Budzinski, H., Garrigues, P., Narbonne, J.F., Burgeot, T., Michel, X., Bellocq, J., 1999a. Polycyclic aromatic hydrocarbon (PAH) burden of mussels (*Mytilus* sp.) in different marine environments in relation with sediment PAH contamination, and biovailability. Mar. Environ. Res. 47, 415–439.

Baumard, P., Budzinski, H., Garrigues, P., Dizer, H., Hansen, P.D., 1999b. Polycyclic aromatic hydrocarbons in recent sediments and mussels (*Mytilus edulis*) from the Western Baltic Sea: occurrence, bioavailability and seasonal variations. Mar. Environ. Res. 47, 17–47.

Bayne, B.L., Addison, R.F., Capuzzo, J.M., Clarke, K.R., Gay, J.J., Moore, M.N., Warwick, R.M., 1988. An overview of the GEEP workshop. Mar. Ecol. Prog. Ser. 46, 235–243.

Botello, A.V., Villanueva, S.F., Diaz, G.G., Escobar-Briones, E., 1998. Polycyclic aromatic hydrocarbons in sediments from Salina Cruz Harbour and coastal areas, Oaxaca, Mexico. Mar. Pollu. Bull. 36 (7), 554–558.

Bouloubassi, I., Saliot, A., 1993. Investigation of anthropogenic and natural organic inputs in estuarine sediments using hydrocarbon markers (NAH, LAB, PAH). Oceanol. Acta 16, 145–161.

Cheung, V.V., Wedderburn, R.J., Depledge, M.H., 1998. Molluscan lysosomal responses as a diagnostic tool for the detection of a pollution gradient in Tolo Harbour, Hong Kong. Mar. Environ. Res. 46 (1), 1–5.

CRA (Centro de Recursos Ambientais), 2000. Relatório de diagnóstico da qualidade das águas da Baía de Todos os Santos. Consórcio Hidros e CH2MHill. CD-ROOM.

da Silva, E.M., Peso-Aguiar, M.C., Navarro, M.F.T., Chastinet, C.B.A., 1997. Impact of petroleum pollution on aquatic coastal ecosystems in Brazil. Environ. Toxicol. Chem. 16 (1), 102–118.

Depledge, M.H., 1994. The rational basis for the use of biomarkers as ecotoxicological tools. In: Fossi, M.C., Leonzio, C. (Eds.), Non destructive Biomarkers in Vertebrates. Lewis Publishers, Boca Raton, FL, pp. 271–295.

Depledge, M.H., Amaral-Mendes, J.J., David, B., Hallbrook, P., Kloepper-Sams, A., Moore, M.N., Peakal, D.B., 1993. The conceptual basis of the biomarker approach. In: Peakal, D.B. (Ed.), Biomarker. Springer, Berlin, Heildelburg, pp. 15–30 (Chapter II).

Djomo, J.E., Garrigues, P., Narbonne, J.F., 1996. Uptake and deouration of polycyclic aromatic hydrocarbons from sedimento by the zebrafish (*Bracydanio rerio*). Environ. Toxicol. Chem. 15, 1177–1181.

Eglinton, G., Hamilton, R.J., 1967. Leaf epicuticular waxes. Science 156, 1322–1335.

Farrington, J.W., Tripp, B.W., 1977. Hydrocarbons in western North Atlantic surface sediments. Geochim. Cosmochim. Acta 41, 1627–1641.

Grundy, M.M., Ratcliffe, N.A., Moore, M.N., 1996. Immune inhibition in marine mussels by polycyclic aromatic hydrocarbons. Mar. Environ. Res. 42 (1–4), 187–190.

Hellow, J., Upshall, C., Payne, J.F., Naidu, S., Paranjape, M.M., 1993. Total unsaturated compounds and polycyclic aromatic hydrocarbons in molluscs collected from waters around Newfoundland. Arch. Environ. Contam. Toxicol. 24, 249–257.

Kalakkis, K.D., O'Connor, T.P., 1985. Distribution of chemical concentrations in US Coastal and Estuarine sediment. Mar. Environ. Res. 40 (4), 381–398.

Le Dréau, Y., Jacquot, F., Doumenq, P., Guiliano, M., Bertrand, J.C., Mille, G., 1997. Hydrocarbon balance of a site which had been highly and chronically contaminated by petroleum wastes of a refinery (from 1956 to 1992). Mar. Pollu. Bull. 34, 456–468.

Lessa, G.C., Dominguez, J.M.L., Bittenourt, A.C.S.P., Brichta, A., 2001. The tides and tidal of Todos os Santos Bay, Northeast Brazil: a general characterization. An. Acad. Bras. Cienc. 73 (2), 245–261.

Lipiatou, E., Saliot, A., 1991. Hydrocarbon contamination of the Rhone delta and western Mediterranean. Mar. Pollu. Bull. 22, 297–304.

Long, E.R., Macdonald, D.D., Smith, S.L., Calder, F.D., 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. Environ. Manage. 19 (1), 81–97.

Lowe, D.M., Pipe, R.K., 1994. Contaminant induced lysossomal membrane damage in marine mussel digestive cells: an in vitro study. Aquat. Toxicol. 30, 357–365.

Lowe, D.M., Moore, M.N., Evans, B.M., 1992. Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. Mar. Ecol. Prog. Ser. 91, 135–140.

Lowe, D.M., Fossato, V.U., Depledge, M.H., 1995a. Contaminant-induced lysossomal membrane in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an in vitro study. Mar. Ecol. Prog. Ser. 129, 189–196.

Lowe, D.M., Soverchia, C., Moore, M.N., 1995b. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. Aquat. Toxicol. 33, 105–112.

Mazurek, M.A., Simoneit, B.R.T., 1984. Characterization of biogenic and petroleum-derived organic matter in aerosols over remote rural and urban areas. In: Keith, L.H. (Ed.), Identification and Analysis of Organic Pollutants in Air. Ann Arbor Science/Butterworth, Boston, pp. 353–370.

Meyers, P.A., Ishiwatari, R., 1993. Lacustrine organic geochemistry—an overview of indicators of organic matter sources and diagenesis in lake sediments. Org. Geochem. 20, 867–900.

MMA (Ministério do Meio Ambiente, dos Recursos Hídricos e da Amazônia Legal), 1996. Macrodiagnóstico da Zona Costeira do Brasil. Na Escala da União. 277pp.

Moore, M.N., Viarengo, A., 1987. Lysosomal membrane fragility and catabolism of cytosolic proteins evidence for a relationship. Experientia 43, 320–323.

Moore, M.N., Willows, R.I., 1998. A model for cellular uptake and intracellular behavior of particulate-bound micropollutants. Mar. Environ. Res. 46 (1–5), 509–514.

Moore, M.N., Livingstone, D.R., Widdows, J., Lowe, D.M., Pipe, R.K., 1978. Molecular, cellular and physiological effects of oil-derived hydrocarbons on molluscs and their use in impact assessment. Philos. Trans. R. Soc. London 316, 603–623.

Moore, M.N., Wedderburn, R.J., Lowe, D.M., Depledge, M.H., 1996. Lysosomal reaction to xenobiotics in mussel haemocytes using BODIPY-FL-Verapamil. Mar. Environ. Res. 42 (1–4), 99–105.

Moriarty, F., 1983. Ecotoxicology: The Study of Pollutants in Ecosystems. Academic Press, London 233pp.

Nascimento, I.A., 1996. Relatório setorial final do sub-projeto "Testes toxicológicos" do Programa de monitoramento da Baía de Todos os Santos, Março. Convênio Petrobrás/UFBA, p. 80

Nascimento, I.A., Smith, D.H., Pereira, S.A., Leite, M.B.N.L., Sansone, G., 1998a. The use of different organisms and biological levels to determine water and sediment quality at industrial sites. Cuad. Invest. Biol. 20 (1), 339–349.

Nascimento, I.A., Leite, M.B.N.L., Sansone, G., Pereira, S.A., Smith, D.H., 1998b. Stress protein accumulation as an indicator of impact by the petroleum industry in Todos os Santos Bay, Brazil. J. Aquat. Ecosyst. Health Manage. 1 (1), 101–108.

Nascimento, I.A., Smith, D.H., Pereira, S.A., Araujo, M.M.S., Silva, M.A., Mariani, A.M., 2000a. Integration of varying responses of different organisms to water and sediment quality at sites impacted and not impacted by the petroleum industry. Aquat. Ecosyst. Health Manage. 3, 449–458.

Nascimento, I.A., Sansone, G., Martino, D., Pauls, E., Pereira, S.A., 2000b. Field and laboratory applications of the lysosomal responses in *Mytilus galloprovincialis* as biomarkers of environmental stress. Aqua 2000. World Aquaculture Society. Nice, France Special Publications, no. 28, p. 207.

Neff, J.M., 1979. In: Policyclic Aromatic Hydrocarbons in the Aquatic Environment. Sources, Fates and Biological Effects. London, Applied Science Publishers, pp. 1–262.

Nipper, M.G., Roper, D.S., Williams, E.K., Martin, M.L., Van Dam, L.F., Mills, G.N., 1998. Sediment toxicity and benthic communities and mildly contaminated mudflats. Environ. Toxicol. Chem. 17 (3), 502–510.

NRC, 1989. Contaminated Marine sediments—Assessment and Remediation. National Research Council. National Academic Press, Washington, DC 493pp.

Peso-Aguiar, M.C., Almeida, V.G., 1996. Relatório gerencial final do Programa de monitoramento dos ecossistemas ao Norte da Baía de Todos os Santos. Convênio Petrobrás/UFBA/FAPEX, Salvador, Bahia 30pp.

Readman, J.W., Mantoura, R.F.C., Llewellyn, C.A., Preston, M.R., Reeves, A.D, 1986. The use of pollutants and biogenic markers as source discriminants of organic inputs to estuarine sediments. Int. J. Environ. Anal. Chem. 27, 29–54.

Readman, J.W., Fillmann, G., Tolosa, I., Bartocci, J., Villeneuve, J.P., Catinni, C., Mee, L.D., 2002. Hydrocarbons residues in sediments from the Black Sea. Mar. Pollut. Bull. 44 (1), 48–62.

Rowland, S., Donkin, P., Smith, E., Wraige, E., 2001. Aromatic hydrocarbon "humps" in the marine environment: unrecognized toxins? Environ. Sci. Technol. 35, 2640–2644.

Sanders, B.M., 1990. Stress proteins: potential as multitiered biomarkers. In: McCarthy, J., Shugart, L. (Eds.), Biological Markers of Environmental Contamination. CRC Publishers, Boca Raton, Florida, pp. 135–161.

Soclo, H.H., Garrigues, P., Ewald, M., 2000. Origin of polycyclic Aromatic Hydrocarbons (PAHs) in coastal marine sediments: case studies in Coonou (Benin) and Aquitaine (France) areas. Mar. Pollut. Bull. 40, 387–396.

Sokal, R.R., Rohlf, F.J., 1981. Biometry. The Principles and Practice of Statistics in Biological Research, second ed. W. H. Freeman and Company, New York 859pp.

Tavares, T.M., 1996. Relatório do subprojeto Química Ambiental do Programa de Monitoramento dos Ecossistemas ao Norte da Baía de Todos os Santos. Convênio PETROBRAS/UFBA.

UNEP/IOC/IAEA. 1992. Determination of petroleum hydrocarbons in sediments. Reference methods for marine pollution studies no. 20 UNEP, Monaco, p. 75.

Verardo, D.J., Froelich, P.N., McIntyre, A., 1990. Determination of organic carbon and nitrogen in marine sediments using the Carlo Erba NA-1500 Analyser. Deep Sea Res. 37 (1), 157–165.

Volkman, J.K., Holdsworth, D.G., Neill, G.P., Bavor Jr., H.J., 1992. Identification of natural, anthropogenic and petroleum hydrocarbons in aquatic sediments. Sci. Total Environ. 112, 203–219.