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A weight of evidence approach for quality assessment of sediments impacted by an oil spill: The role of a set of biomarkers as a line of evidence

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ABSTRACT

In an attempt to incorporate both line of evidence (LOE) and classical weight of evidence (WOE) approaches for the assessment of sediment quality, a set of biomarkers were analyzed in target tissues of two invertebrate species after 28 days of exposure to sediments impacted by oil (derived from the tanker *Prestige* (2002)). The integration of biomarkers with sediment contamination, acute toxicity and benthic alteration parameters provides an "early warning" tool which not only indicates the environmental quality of an area, but also constitutes an advisory tool for potential ecological risks. The selected biomarkers provide information about the first biological responses due to the presence of contaminants in the environment providing predictable reports about further effects to the ecosystem. The present study demonstrates that the use of a set of biomarkers as part of a WOE approach designed to assess contaminated sediments contributes added value to the classical LOE and allows characterization of the environmental status of the studied area in a more precise and accurate way.

1. Introduction

Chemical analyses normally provide the main tool in sediment quality assessment, even though chemical concentrations alone are inadequate for the prediction of biological consequences. Biological effects can be established using laboratory tests that determine toxic responses and field surveys of the communities living within impacted sediments, i.e., observing pollution-induced degradation of the biota (Chapman et al., 1991). Weight of evidence (WOE) investigations determines the possible ecological impacts of chemicals or other stressors based on multiple lines of evidence (LOE) (Chapman, 2007). They have been widely used in recent years to assess sediment quality around the world (Chapman, 2000; Borgmann et al., 2001), including different areas within the Iberian Peninsula (DelValls and Chapman, 1998; Caeiro, 2004; Riba et al., 2004; Martín-Díaz, 2004; Morales-Caselles et al., 2008a).

The sinking of the tanker *Prestige* (2002) spilt about 63,000 tonnes of heavy fuel oil (a mixture of saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes, with most of the PAHs being of medium to high molecular weight) mainly affecting the Galician Coast, NW Spain. Several investigations have focused on determining the biological and environmental effects of this dramatic episode by following single lines of evidence, such as chemical analyses (CSIC, 2003a; Franco et al., 2006; González et al., 2006), toxicity (Mariño-Balsa et al., 2003; Martínez-Gómez et al., 2006; Marigómez et al., 2006; Morales-Caselles et al., 2006) or benthic alteration (Junoy et al., 2005; Serrano et al., 2006). Recently, authors presented a report (Morales-Caselles et al., 2008a) where a classical WOE approach, the sediment quality triad (SQT), based on three lines of evidence (physicochemical characterization of the sediments, determination of acute toxicity and benthic alteration) was carried out in the Galician Coast. This study suggested a general recovery of the environmental health within the area. However, there were signs (Morales-Caselles et al., 2008a) that other sources of contaminants apart from the *Prestige* oil spill could be producing some environmental stress. The aim of the present study is to use a biomarker line of evidence (LOE) with the weight of evidence (WOE) approach to further improve the sediment quality assessment of the Galician Coast. Chapman and Hollert (2006) suggested that future applications of the SQT should consider specific LOE in terms of risk assessment, ensuring that both exposure and assessment of biological effects are adequately addressed, as both are of causation and ecological relevance. Thus, the LOE selected in this study constitutes an improvement of the classical WOE laboratory

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and field studies based on biomarker determinations. Biomarkers can act as an important early warning system by indicating whether environmental pollutants are present at sufficiently high concentrations to cause an effect (Walker et al., 2006). In addition, biomarker responses are specific to certain groups of contaminants, providing information related to possible causation (Galloway et al., 2004). Chemicals such as the polycyclic aromatic hydrocarbons (PAHs) have a very short biological half-life in most species, yet nevertheless, may have long-term effects (Walker et al., 2006). In this sense, some compounds may not produce acute toxic effects, however sublethal effects are likely to be observed; carefully selected biomarkers can highlight these sublethal effects, thus providing increased sensitivity and robustness to WOE assessments. Thus, the aim of this research was to examine the feasibility and viability of incorporating a biomarker LOE within the classical WOE approach as applied to the assessment of oil contaminated sediments. This new approach is demonstrated by examining the extent of biological impact and identity of contaminants within sediments influenced by the accidental oil spill of the *Prestige* (2002) on the Galician Coast.

2. Methodology

2.1. Approach

The study was performed in two areas of the Galician Coast (NW Spain) affected by the *Prestige* oil spill in 2002 (Fig. 1): Cies Island (A, B and C) within the Atlantic Island National Park (AINP) and the Bay of Corme–Laxe (D, E and F). Cies Island acted as a natural barrier protecting the rias from entry by the fuel. The Bay of Corme–Laxe is also considered a place with high ecological relevance, with a low anthropogenic and industrial influence (fishing and farming being the main economic activities).

The four LOE employed within the WOE approach were:

(a) Sediment contamination; physicochemical characterization of sediments by analyzing PAHs (acenaphtalene, acenaphtylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i) perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h) anthracene, fenanthrene, fluoranthene, fluorene, indene(1,2,3,cd)pyrene, naphthalene, and pyrene) using GC–MS with selected ion monitoring and trace metals (Zn, Pb, Cu, Ni and Hg) with anodic stripping voltammetry (Morales-Caselles et al., 2006).

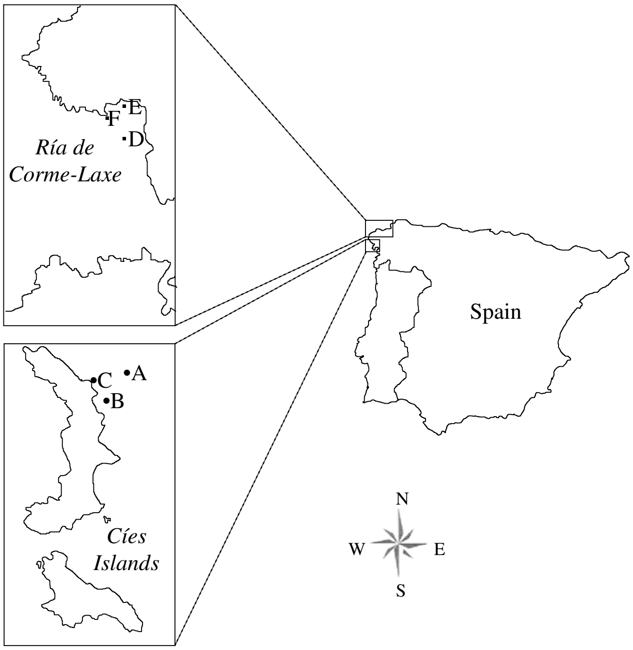

Fig. 1. Map of the coastal area of Galicia (NW Spain) showing the sampling sites in the Atlantic Island National Park (A, B and C) and the area of Corme–Laxe (D, E and F).

(b) Acute toxicity and bioaccumulation; by performing sediment bioassays such as the commercial assay Microtox® (Morales-Caselles et al., 2007), the amphipod mortality test with *Corophium volutator* (Morales-Caselles et al., 2007), the polychaetes mortality assay (Casado-Martínez et al., in press) and bioaccumulation experiments with *Arenicola marina* (Morales-Caselles et al., 2008b).

(c) 'In situ alteration'; benthic alteration was selected and determined by measuring parameters in situ based on taxonomic identification and community descriptive statistics (abundance–biomass analysis, species richness, diversity, dominance and proportions of the major taxonomic groups) (DelValls and Chapman, 1998).

(d) Laboratory and field studies based on biomarkers; using two invertebrate species the crab *Carcinus maenas* and the clam *Ruditappes philippinarum*, and a set of biomarkers (Morales-Caselles et al., 2008c): mixed function oxygenase activity, which is the first mode of detoxification in many organic pollutants, was measured using the adapted ethoxyresorufin-O-deethylase (EROD) assay; the ferric reducing ability of plasma (FRAP) assay allows a measure of the antioxidant capacity; antioxidant glutathione-S-transferase (GST) activity was determined by monitoring the rate of conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm; oxidation of 1 mM NADPH by glutathione reductase activity (GR) in the presence of 10 mM oxidized glutathione was also monitored at 340 nm; phase II metabolizing enzyme glutathione peroxidase activity (GPX); all the biomarker responses were normalized with the total protein content ($n = 5$).

2.2. Data integration

The data obtained from the different LOE were integrated through a multivariate analysis approach based on linking all the variables obtained (Riba et al., 2004) and a pie chart representation of comparisons between sites of multivariate factors (Riba et al., 2004; Morales-Caselles et al., 2008a). The multivariate analysis was performed using principal component analysis (PCA) as the extraction procedure, which is a multivariate statistical technique to explore variable distributions (Riba et al., 2003). The original data set used in the analysis included the variables obtained from the 4-LOE and its objective was to derive a reduced number of new variables as linear combinations of the original variables. This provides a description of correlations between the set of the original variables and their meaning in each study site with a minimum loss of information. For the representation of the pie charts the new factors obtained from the PCA were submitted to ANOVA and Tukey tests which identified significant differences in sensitivity among stations and controls for each factor (Morales-Caselles et al., 2008a). Every study site has a pie chart divided into the obtained factors which use different colours depending on the level of significant differences in relation to the reference.

3. Results and discussion

Table 1 shows the summarized results of the different parameters analyzed. Sediment Hg and Pb content was significantly higher in all Corme–Laxe sites; The highest metal contents were observed at stations A and F. There was no observed general pattern in toxicity and benthic parameters between the sampling sites.

Factor analysis revealed that the original variables could be grouped into three principle components (factors) that explained 79% of the total variance (Table 2). The multivariate analysis is a tool that allows us to interpret large groups of different variables by grouping them using correlations; in addition it indicates the importance of each factor in every single study site.

3.1. Factor #1

Factor 1 explained 35.2% of the variance and was influenced by the relationship between different variables related with chemicals, sublethal responses, bioaccumulation and benthic alteration. Sediment PAHs, Pb and Hg contents were positively correlated to the bioaccumulation of PAHs in *A. marina* (under laboratory conditions), but negatively correlated to their mortality in the acute assays. A set of antioxidant and detoxification biomarkers analyzed in crabs (activity of GPX, GR, GST, EROD and FRAP) and the digestive gland of clams (GR and GST activity) under laboratory conditions were also correlated with Factor #1. EROD and FRAP of organisms exposed to sediments in situ were not significantly correlated with other variables (Table 2). The aforementioned contaminants and the toxicity variables are slightly connected to the benthic alteration explained by alteration of the specific richness and an increase of the polychaete population, while a positive development of crustaceans was detected. Thus, Factor #1 may be interpreted as representing contamination by PAHs, Hg and Pb, which does not produce lethal effects; although PAH bioaccumulation and sublethal responses in organisms (under laboratory and field conditions) are associated with a slight alteration of the in situ benthic community. Environmental alterations due to these contaminants have been reported by other authors (DelValls et al., 1998). Factor 1 scores for stations E (1.0) and F (1.4) located in Corme–Laxe (Fig. 2) were rather high, suggesting an accumulation of contaminants including fuel oil from the *Prestige* (Labandeira et al., 2001; Prego et al., 2006). Although initial studies did not detect the presence of Pb and Hg in emulsified samples of the *Prestige* fuel (with 54–59% water) (CSIC, 2003b), the presence of Pb was later corroborated by other authors (Andrade et al., 2004). Thus, the contamination by PAHs, Hg and Pb observed at Corme–Laxe

Table 1

Summarized results and SD of chemical analysis (mg kg^{-1} for metals, µg kg^{-1} for PAHs) the acute toxicity tests (*Corophium volutator* and *Arenicola marina*: % mortality; Microtox: IC50; bioaccumulation of PAHs: µg kg^{-1}), biomarker responses under field and laboratory conditions (glutathione peroxidase activity GPX: nmol min^{-1} mg $prot^{-1}$, glutathione transferase GST activity nmol min^{-1} mg $prot^{-1}$, glutathione reductase GR activity nmol min^{-1} mg $prot^{-1}$, ferric reducing ability of plasma FRAP activity µM mg^{-1} min^{-1} and EROD activity pmol mg^{-1} min^{-1}) and the alteration parameters for sediments from the Atlantic Islands National Park (AINP) (A, B and C) and Corme–Laxe (D, E and F). ND: not detected; NA: not available.

	A	B	C	D	E	F
Chemical analyses						
Zn	377 ± 36.06	91 ± 1.34	164 ± 11.31	25 ± 1.41	19.9 ± 1.34	271 ± 16.26
Pb	1.5 ± 0.14	0.9 ± 0.00	0.85 ± 0.01	3.7 ± 0.21	7.3 ± 0.07	5.9 ± 0.14
Cu	5.2 ± 0.35	1.4 ± 0.14	1.4 ± 0.21	0.7 ± 0.21	0.43 ± 0.05	4.2 ± 0.21
Ni	13.3 ± 1.91	2.4 ± 0.21	4.5 ± 0.14	1.7 ± 0.14	1.5 ± 0.00	5.7 ± 0.57
Hg	0.7 ± 0.21	0.8 ± 0.07	0.6 ± 0.07	2 ± 0.14	2.1 ± 0.28	3.4 ± 0.28
PAH	108 ± 7.78	67 ± 16.33	ND	38 ± 2.47	52 ± 0.71	323 ± 13.44
Toxicity tests						
Corophium	23 ± 1.18	20 ± 1.53	17 ± 0.02	10 ± 1.77	17 ± 1.18	20 ± 3.54
Arenicola	28 ± 1.96	28 ± 1.97	22 ± 1.96	39 ± 0.79	17 ± 0.02	17 ± 0.24
Microtox	5631 ± 134	9422 ± 301	1801 ± 101	3977 ± 214	21041 ± 407	4398 ± 282
Bioaccumulation PAH	2927 ± 1.48	2573 ± 1.48	2666 ± 1.24	2616 ± 2.40	3911 ± 0.71	3285 ± 0.71
Biomarkers (laboratory)						
GPX-crab-lab	11.6 ± 0.59	9.7 ± 0.55	8.2 ± 1.72	19.3 ± 0.00	19.5 ± 2.88	15.9 ± 1.18
GPX-clam-lab	2.1 ± 0.01	2.9 ± 0.59	4.5 ± 0.04	6.1 ± 2.36	3.1 ± 1.47	4.2 ± 2.42
GR-crab-lab	1.1 ± 0.06	0.7 ± 0.08	0.9 ± 0.12	0.9 ± 0.18	0.6 ± 0.01	1.5 ± 0.04
GR-clam-lab	2.1 ± 0.65	1.6 ± 0.00	2.3 ± 0.36	3.4 ± 0.43	11.7 ± 3.17	3.9 ± 0.03
GST-crab-lab	139.6 ± 52.3	218.1 ± 44.2	407.3 ± 8.97	429.6 ± 36.1	684.1 ± 422	1070.9 ± 198
GST-clam-lab	1292.8 ± 339	838.6 ± 0.00	1624.5 ± 209	1199.1 ± 174	910.2 ± 566	847.7 ± 293
EROD-crab-lab	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	0.10 ± 0.00	0.09 ± 0.00
EROD-clam-lab	0.26 ± 0.00	0.27 ± 0.00	0.42 ± 0.00	0.41 ± 0.00	0.44 ± 0.00	0.22 ± 0.00
FRAP-crab-lab	3.85 ± 0.92	2.08 ± 1.01	2.56 ± 0.47	2.86 ± 0.43	2.93 ± 0.56	1.56 ± 0.30
FRAP-clam-lab	10.58 ± 3.8	7.77 ± 0.5	4.04 ± 0.70	13.72 ± 1.9	12.09 ± 2.6	6.43 ± 0.30
Biomarkers (field)						
GPX-crab-field	17.8 ± 0.07	23.1 ± 2.12	15.9 ± 4.10	41.4 ± 2.83	193.1 ± 0.1	125.7 ± 11
GPX-clam-field	10.5 ± 0.49	3.6 ± 0.14	4.0 ± 0.21	25.5 ± 0.35	3.2 ± 0.49	7.0 ± 2.40
GR-crab-field	0.7 ± 0.21	1.4 ± 0.92	1.4 ± 0.99	9.9 ± 0.71	9.5 ± 2.83	23.4 ± 1.06
GR-clam-field	2.9 ± 0.00	1.3 ± 0.00	3.8 ± 0.00	9.7 ± 0.85	14.7 ± 0.07	8.1 ± 1.77
GST-crab-field	1097.8 ± 571	1563.8 ± 224	690.3 ± 10.6	1489.0 ± 219	7523.4 ± 1129	6072.9 ± 0.0
GST-clam-field	2060.8 ± 926	371.6 ± 130	1198.7 ± 45	3365.6 ± 250	130.5 ± 2.5	1557.9 ± 192
EROD-crab-field	0.13 ± 0.00	2.96 ± 0.00	0.02 ± 0.00	8.51 ± 0.00	0.39 ± 0.00	0.48 ± 0.00
EROD-clam-field	0.15 ± 0.00	0.07 ± 0.00	0.09 ± 0.00	0.63 ± 0.00	0.07 ± 0.00	0.13 ± 0.00
FRAP-crab-field	2.72 ± 0.35	NA	NA	2.42 ± 0.32	NA	NA
FRAP-clam-field	10.39 ± 1.4	3.09 ± 0.53	2.63 ± 0.91	23.58 ± 2.5	2.01 ± 0.65	6.58 ± 1.01
Benthic alterations						
Number species	28.5	33.9	42.4	28.6	32.1	48.2
specific richness	5.1	5	4.3	3	3	2.9
Diversity	15.3	28.4	39.1	30	40.1	15.4
Dominance	0.50	0.10	0.06	0.15	0.19	0.20
% Mollusca	15.3	28.4	39.1	30.0	40.1	15.4
% Polychaete	20.0	21.5	21.7	20.0	22.2	23.1
% Crustacea	37.0	41.0	39.1	50.0	51.4	61.5

appears to be caused by the remnants of the *Prestige* (Morales-Caselles et al., 2008b).

Table 2
Sorted rotated factor loadings of 36 variables for the three principal factors resulting from the multivariate analysis of the single results obtained from the chemical analysis, the acute toxicity tests, the suite of biomarkers and the alteration parameters for the study of the sediments quality in the Galician Coast. *Chemicals:* loadings are related to the concentration of contaminants in sediments. *Acute effects:* loadings explain the toxicity detected by the acute assays and the bioaccumulation of PAHs in *Arenicola marina*. *Sublethal effects:* loadings are related to the induction of biomarkers. *Benthic alteration:* loadings are related to alteration of the biota (decrease of number of species, specific richness, diversity, diminution in the percentage of molluscs and crustacea and increase in the polychaete population). The group of variables selected for the interpretation represented a loading of 0.40 or higher for a good association between an original variable and a factor.

	Factor 1 35.2	Factor 2 24.5	Factor 3 19.2
Chemicals			
Zn	–	–	0.90
Pb	0.95	–	–
Cu	–	–	0.93
Ni	–	–	0.76
Hg	0.95	–	–
PAH	0.59	–	0.76
Acute effects			
Corophium	–	−0.52	0.75
Arenicola	−0.41	0.87	–
Microtox	–	–	–
Bioaccumulation	0.73	–	–
Sublethal effects (laboratory and field)			
GPX-crab-lab	0.48	−0.77	–
GPX-clam-lab	–	0.53	–
GR-crab-lab	0.41	–	0.78
GR-clam-lab	0.65	–	0.49
GST-crab-lab	0.85	–	–
GST-clam-lab	−0.53	–	–
EROD-crab-lab	0.62	−0.78	–
EROD-clam-lab	–	–	−0.89
FRAP-crab-lab	−0.46	–	–
FRAP-clam-lab	–	0.64	–
GPX-crab-field	0.88	–	–
GPX-clam-field	–	0.99	–
GR-crab-field	0.91	–	–
GR-clam-field	0.77	–	−0.46
GST-crab-field	0.89	–	–
GST-clam-field	–	0.75	–
EROD-crab-field	–	0.88	–
EROD-clam-field	–	0.98	–
FRAP-crab-field	–	0.76	–
FRAP-clam-field	–	0.99	–
Benthic alteration			
Number of species	–	0.56	–
Specific richness	0.91	–	–
Diversity	–	–	0.97
% Molusca	–	–	0.96
% Polychaeta	0.62	−0.68	–
% Crustacea	−0.96	–	–

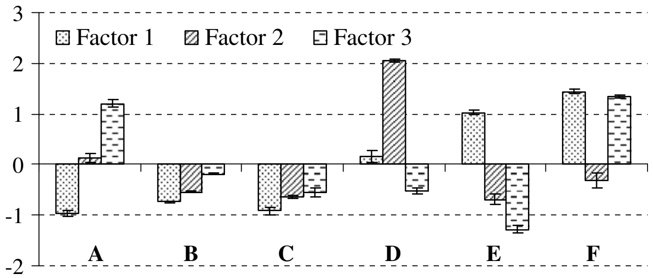

Fig. 2. Estimated factor scores for the three factors in each of the six cases. The factor scores quantify the prevalence of each factor for every station and are used to establish the definition of each factor.

Although no lethal effects were observed, the bioaccumulation of PAHs by *A. marina* demonstrates the high bioavailability of these compounds. The induction of different biomarkers in the hepatopancreas of crabs and in the digestive glands of clams was also correlated with the presence of these contaminants (PAHs, Hg and Pb), suggesting that the deployed organisms suffered stress due to the presence of these substances in the sediments (Morales-Caselles et al., 2008c). The correlation observed among the biomarkers and the different variables defined by Factor #1 was stronger under laboratory conditions, implying that field deployments result in less sensitivity (Astley et al., 1999). The flushing action of the open water environment may act to reduce the impacts of sediment contaminants when compared with laboratory incubations.

Although, a good correlation was observed between the biomarkers induced in both invertebrate species, the crab *C. maenas* was more sensitive, i.e., showed stronger responses, than the clam *R. philippinarum* to this kind of pollution. On the other hand some of the variables related to the benthic alteration present in Factor #1 corroborate the effects observed in the sublethal experiments.

3.2. Factor #2

The second factor, Factor #2 (24.5% of the variance) connects the set of biomarkers measured under field conditions (EROD and FRAP activity in crabs and clams, and GPX and GST in clams), the mortality of *Arenicola* in the acute experiment, no toxicity for amphipods, the alteration in the number of species and the decrease of the polychaete population. Positive and negative correlations for a few biomarkers were also detected under laboratory conditions and no concordance with the amphipod toxicity test was observed. The relationships between the biological responses identified in Factor #2 are not correlated with any of the chemicals analyzed suggesting that a contaminant or group of compounds that was not analyzed, is the cause of the biomarker responses. Taking into account that the acute toxicity observed in *Arenicola* and the rest of the bioassays was relatively low (less than 30% mortality) (Morales-Caselles et al., 2008b), a source of contaminant not related to the sediment is the most likely cause of these effects. Station A (0.1) located on Cies Island and site D (2.0) in Bay of Corme–Laxe had the highest positive correlation with Factor #2 (Fig. 2). The good correlation observed between the biomarkers in both crabs and clams incubated under field conditions suggests that these locations suffer the stress of non-measured variables which, in the case of site D, could be related to the proximity of aquaculture infrastructures (mussel culture). Other authors (Otero et al., 2006), have described the negative impacts of these mussel aquaculture, including: the discharge of a large volume of biodeposits containing high concentrations of nutrients; the release of drugs and pesticides into the environment; an increase in sedimentation and accumulation of organic matter and an increase in the concentration of nutrients in sediments and waters (mainly N and P). Negative effects on wild populations of animals have also been observed, ranging from genetic interaction and disease transmission, to changes in the composition of the structure of benthic fauna due to a change from oxic to anoxic conditions (Otero et al., 2006).

3.3. Factor #3

The third factor, Factor #3 explained 19.2% of the variance and was correlated with sediments contaminated by the metals Zn, Cu and Ni, and PAHs; these contaminants were correlated with the antioxidant responses under laboratory assays with crabs and clams (GR) and the acute toxicity as determined by the amphipods assay, which was not significant (the toxicity detected was not enough to consider sediment samples as toxic according to this acute assay). Samples where the mortality rate of the amphipods was 20% higher than the mortality recorded in the reference and showed significantly different (*$p < 0.05$) results compared to those obtained in the reference are considered as toxic (Morales-Caselles et al., 2008b). The effects on the benthic community were manifested as an alteration in the diversity and percentage of molluscs. This suggests the presence of a stress related to the source of metals (Zn, Cu and Ni) and organic compounds (PAHs), which is different than the source explained by Factor #1. Sites A (1.2) in the AINP and F (1.3) in the Bay of Corme–Laxe had positive Factor #3 values (Fig. 2). Previous studies in Bay of Corme–Laxe detected severe contamination of the sediments by Cu (Prego et al., 2006), however a fuel oil origin was unlikely, with the major source of Cu being antifouling paints from the hulls of fishing vessels (Cobelo-García and Prego, 2003). Although contamination by Cu and Zn was observed in the uppermost sediment layers of the *Prestige* shipwreck area (Prego and Cobelo-García, 2004; Cobelo-García et al., 2005), this contamination is unlikely not be related to the shipwreck, because levels of Cu in the fuel oil carried by the *Prestige* were relatively low (3.39 mg kg^{-1}) and previous studies have shown that inputs from terrestrial sources are probably higher than from the spilled fuel oil (Carballeira et al., 1997; Villares et al., 2007). Some of the studied variables demonstrate the stress of these contaminants and the effects on the benthic community; however, the results mostly point to chronic contamination with low bioavailability, and potential, but unconfirmed biological risk. Thus, despite the high trace metal content of sediments from the Galician Coast, their reactivity and bioavailability were very low (Otero et al., 2005), which maybe related to the high degree of pyritization found for some of the most toxic trace metals, favouring their release by oxidation of the sulphides that they form, thus making them bioavailable to benthic fauna.

3.4. Significant differences among stations

Station C, with an absence of PAH contamination and the lowest biological effects, was considered as the most suitable site to use as a reference station. Factor loadings obtained in the MAA were submitted to ANOVA and post-hoc examination using the Tukey test (Fig. 3).

Factor #1 scores of sediments from Cies Island in the AINP were not significantly different, suggesting that the effects of the *Prestige*

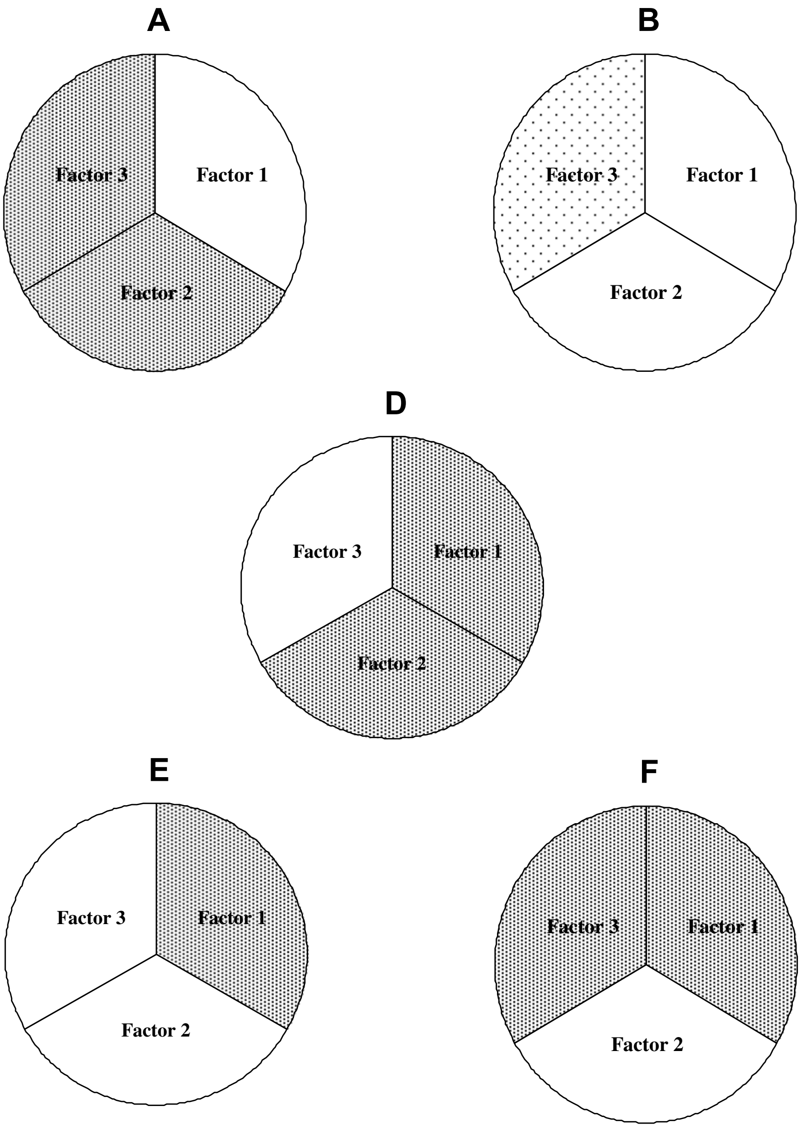

Fig. 3. Pie charts which represent the significant differences of the factors score in every study site related to the reference site C (heavy shading: $p < 0.01$; light shading: $p < 0.05$; no shading: no significant differences, $p > 0.05$).

oil spill were no longer detectable within the area. However, Factors #2 and #3 scores for station A were significantly different ($p < 0.01$) from the reference station (C), suggesting the influence of non-measured stressors from sources other than the *Prestige*. The Factor #3 score of station B was also significantly different ($p < 0.05$) from the reference station, suggesting the presence of some contaminants considered a potential risk within the area, although in general, the environmental quality of the sediments was relatively good. On the other hand, Factor #1 scores of sediments from Corme–Laxe D, E and F were significantly different ($p < 0.01$) from the reference station, suggesting the remaining contaminants from the *Prestige* oil spill are still producing sublethal effects to the biota of the bay. In addition, the significant difference ($p < 0.01$) in Factor #2 score observed at station D suggests other unknown sources of contaminants responsible for biological stress in the study site. In the case of station F a mixture of metals and PAHs from different sources could be considered a potential risk in the area as shown by Factor #3 ($p < 0.01$).

Biomarkers are useful "early warning" indicators of the health status of animals from impacted areas (Galloway et al., 2004; Montserrat et al., in press), including systems impacted by oil (Anderson and Lee, 2006), where complex mixtures of pollutants are usually present. The use of a set of biomarkers, as part of a WOE approach designed to assess contaminated sediments, contributed added value to the classical LOE concept and allows for the characterization of the environmental status of the studied area in a more precise and accurate way. In addition, the inclusion of chronic bioassays with two invertebrate species, not only under laboratory conditions but also in field deployments, elucidate different sources of contaminants apart from the sediments permitting a more realistic approach to the original situation of the ecosystem, and the potential ecological risks.

4. Conclusions

Multiple lines of evidence suggest that four years after the impact of the *Prestige* oil spill, the fuel is not producing acute toxic effects within the environment (Morales-Caselles et al., 2008a); however, the use of a biomarker LOE within the WOE approach demonstrated that sublethal responses may occur in the area of Corme–Laxe, related to PAHs, Pb and Hg. No effects of oil spill were observed in the study sites located in AINP although contamination by metals, especially Zn, Cu and Ni was observed in some sites on Cies Island. The inclusion of biomarkers in the SQT indicates that coastal anthropogenic influences (a mixture of pollutants that act as environmental stressors) are evident in both areas and should be considered a potential risk. In the case of Corme–Laxe, a possible impact related to the aquaculture of mussels was detected. Biomarkers demonstrated higher sensitivity when compared to acute toxicity approaches providing a more comprehensive and integrated weight of evidence approach (Morales-Caselles et al., 2008a), that as demonstrated, can be easily incorporated as a fourth line of evidence into the classical SQT. Furthermore, this study confirms the suitability of biomarkers as a tool to assess the sources and potential ecological risk of metallic and petrogenic contaminants within sediments.

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