ADVANCES IN MARINE BIOGEOCHEMISTRY,
ECOTOXICOLOGY AND CONTAMINATION
OF COASTAL SYSTEMS
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SCIENTIA MARINA 77S1
January 2013, 69-78, Barcelona (Spain)
ISSN: 0214-8358
doi: 10.3989/scimar.03731.27F

Biomarkers in *Nereis diversicolor* (Polychaeta: Nereididae) as management tools for environmental assessment on the southwest Iberian coast

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SUMMARY: The environmental quality of the southwest Iberian coast was assessed in different areas (Ria Formosa Lagoon, Guadiana Estuary and Cádiz Bay) focusing on metal contamination (Cd, Cu, Ni, Pb and Zn) in *Nereis diversicolor* whole tissues. In addition, associated toxicological effects were assessed using a multibiomarker approach combining several conventional biomarkers. The set of biomarkers selected included antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase), metallothionein and lipid peroxidation. *N. diversicolor* from the Ria Formosa Lagoon, Guadiana River and Cádiz Bay responded differently to metal contamination with different biomarker responses showing a clear site trend, suggesting different sources and/or magnitudes of contamination. Cadmium was a source of oxidative stress in polychaetes, mainly in Cádiz Bay, with a significant influence on antioxidant enzymes and enhancement of lipid peroxidation. The highest MT concentrations were in the Ria Formosa Lagoon and in the Guadiana River, where there was a direct relationship with high nickel concentrations. Biomarker responses of *N. diversicolor* are useful tools for environmental quality assessment on the southwest Iberian coast, and more specific metal biomarkers should be included in future assessments.

Keywords: biomarkers, metals, *Nereis diversicolor*, antioxidant enzymes, metallothionein, lipid peroxidation, southwest Iberian coast.

RESUMEN: BIOMARCADORES EN *NEREIS DIVERSICOLOR* (POLYCHAETA: NEREIDIDAE) COMO HERRAMIENTAS DE GESTIÓN DE LA EVALUACIÓN DEL MEDIO AMBIENTE EN EL SUROESTE DE LA PENÍNSULA IBÉRICA. – El estudio de la calidad ambiental de los ecosistemas costeros del SO de la Península Ibérica (Ría Formosa, estuario del Guadiana y bahía de Cádiz) en relación a la contaminación metálica (Cd, Cu, Ni, Pb y Zn) se ha llevado a cabo mediante el análisis del contenido de metales en *Nereis diversicolor*. Además, los efectos ecotoxicológicos fueron evaluados mediante una aproximación “multibiomarcadores”, en donde se combinan varios biomarcadores convencionales. La batería de biomarcadores seleccionadas han sido: las enzimas antioxidantes (catalasa (CAT), superóxido dismutasa (SOD) y glutatión peroxidasa (GPX), metalotioneína (MT) y peroxidación lipídica (LPO). Los ejemplares de *N. diversicolor* recolectados en las distintas zonas, mostraron una respuesta diferente a la contaminación metálica, lo que sugiere diferentes fuentes y/o magnitud de la contaminación. Los niveles de cadmio estan directamente relacionados con las actividades SOD y CAT y los niveles de LPO. En el caso del níquel, sus niveles se hallaban correlacionados positivamente con los de MT en la Ría Formosa y en el río Guadiana. La integración de las respuesta de los biomarcadores en *N. diversicolor* ha demostrado ser una herramienta útil para la evaluación de la calidad ambiental de los ecosistemas costeros del SO de la Península Ibérica.

Palabras clave: biomarcadores, metales, *Nereis diversicolor*, enzimas antioxidantes, metalotioneínas, peroxidación lipídica, Suroeste Península Ibérica.

INTRODUCTION

Estuaries are among the aquatic ecosystems most susceptible to the continuous influx of heterogeneous contaminant mixtures largely associated with nearby agricultural and/or industrial activities (Kennish 2002, Berthet *et al*. 2003). This leads irrevocably to a high inherent environmental risk because estuaries and related saltmarshes are key habitats for many ecologically relevant species, such as the endobenthic polychaete *Nereis/Hediste diversicolor* (Zhou *et al.* 2003, Durou *et al.* 2007a, Gillet *et al.* 2008). These quite abundant omnivorous organisms dwell in the sediment (often regarded as a long-term reservoir of contaminants), being crucial species not only due to the important role they play in the food web but also due to the remobilization of contaminant and nutrient loads linked to their burrowing and dietary behaviour (Amiard *et al.* 2007, Durou *et al.* 2007a,b, Gillet *et al*. 2008). These features confer to *N. diversicolor* its resistance and consequent ability to inhabit metal-rich sediments, and thus its importance as a bioindicator of estuarine environmental quality (Moreira *et al*. 2006, Poirier *et al*. 2006, Durou *et al.* 2008, Gillet *et al.* 2008, Solé *et al*. 2009).

Metals present at high concentrations induce deleterious effects in aquatic organisms, such as oxidative stress, because they enhance reactive oxygen species (ROS) production (Winston and Di Giulio 1991, Livingstone 2001). These effects are often assessed by analysing certain metal detoxification mechanisms where metallothioneins (MTs) assume an important role because they show both the ability to bind or form an inactive complex with certain metals favouring their storage or excretion as well as recognized oxyradical scavenger properties (Langston *et al.* 1998, Viarengo *et al.* 1999). Moreover, toxic metal-induced ROS are also counteracted by the action of an antioxidant enzyme cascade initiated by superoxide dismutase (SOD) activity that converts the highly reactive superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2), which in turn is transformed by both catalase (CAT) and glutathione peroxidase (GPX) in water and oxygen. Lipid peroxidation (LPO) is associated with an over presence of ROS due to antioxidant defences system failure. The formation of lipid peroxides is characterized by the presence of a by-product: malondialdehyde (MDA) and hydroxyalkenals that is considered a useful damage biomarker (Winston and Di Giulio 1991, Livingstone 2001).

The aim of this study is to assess metal contamination at several sites of the southwest Iberian coast through metal analysis of *N. diversicolor* whole tissues, namely Cd, Cu, Ni, Pb and Zn. In addition, associated ecotoxicological effects and seasonal and spatial trends were determined using a multibiomarker approach including antioxidant enzymatic activities alterations, metallothionein and lipid peroxidation levels. The overview given by this study provides useful complementary data for environmental quality assessment.

MATERIAL AND METHODS

Study Area

The Ria Formosa Lagoon is a shallow, mesotidal lagoon system that is highly productive and dynamic, with high nutrient concentrations and constant water renewal (Mudge and Bebianno 1997, Dionísio *et al.* 1999). In recent years the water quality in the lagoon has deteriorated due to unsustainable economic development with a consequent decrease in bivalve production (Bebianno 1995, Coelho *et al*. 2002).

The Guadiana River is the fourth most important river of the Iberian Peninsula, located along the southern border between Portugal and Spain. Several point and diffuse pollution sources affect the quality of the Guadiana waters, originating from urban centres, industry, landfills, cattle breeding, and olive and vineyard crops. The intensive agricultural activities and pesticide treatments carried out in the Spanish areas close to the hydrological basin of the river also influence the discharges into the river (Cravo *et al*. 2006, Almeida *et al*. 2007).

The Bay of Cádiz is a littoral ecosystem that covers four different regions: the outer bay, which has oceanic characteristics (wave, wind and tide action); the inner bay, which is strongly influenced by tides; the amphibious bay, which has a rich and varied ecosystem, including tidal marshes; and the terrestrial bay, which is permanently emerged. These areas have intense maritime traffic and have supported decades of industrial activities related to naval construction (Blasco *et al*. 2000, 2010, Carrasco *et al*. 2003).

Sampling

N. diversicolor were collected during two seasonal campaigns, at the end of autumn (2006) and spring (2007). Sampling sites with different contamination levels were chosen in the Ria Formosa Lagoon and Cádiz Bay (Fig. 1). Due to constraints on polychaete abundances, in autumn individuals were collected at only one site in the Ria Formosa Lagoon, Faro (F-RF), while in spring two other sites were chosen, Ramalhete (R-RF) and Ribeira de Almargem (A-RF). In the Guadiana River (GD), sampling was only possible in spring. In Cádiz Bay, three sites were chosen for the autumn and spring campaigns: Puente Zuazo (PZ), Trocadero (TR) and Rio San Pedro (RSP). After collection, the polychaetes were transported alive to the laboratory in cool boxes filled with sediment and covered with seawater from the site of origin. At the laboratory, polychaetes were cleaned to eliminate sediment residues, frozen in liquid nitrogen and stored at –80ºC until analysis.

Metal analysis

Cadmium, copper, nickel, lead and zinc concentrations were determined in dried (80°C) subsamples

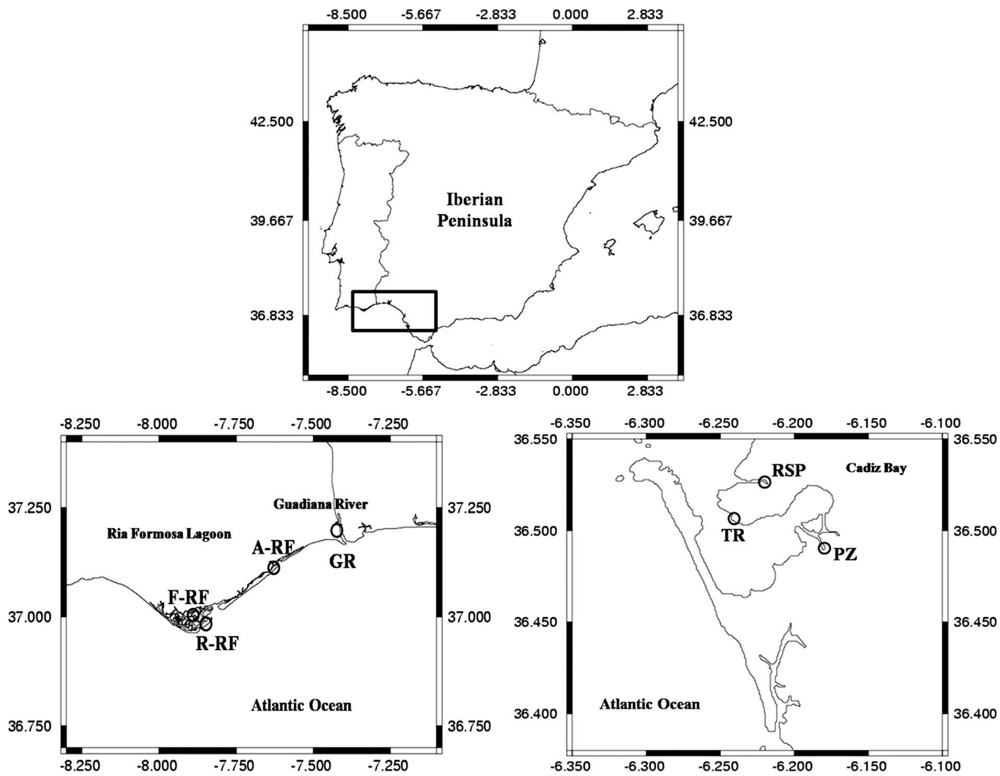

FIG. 1. – Location of sampling stations on the southwest Iberian coast.

of the homogenate of the total tissue of the polychaetes. Samples were submitted to a wet digestion with concentrated nitric acid (HNO_3). Analyses were performed by atomic absorption spectrophotometry, using standard addition methods. The analytical procedure was checked using a standard reference material (Lobster Hepatopancreas) provided by the National Research Council, Canada – TORT II. The values (mean±standard deviation) obtained were 27.21±0.88 μg g^{-1} for Cd, 106.54±1.81 μg g^{-1} for Cu, 2.31±0.08 μg g^{-1} for Ni, 0.30±0.02 μg g^{-1} for Pb and 193.66±7.13 μg g^{-1} for Zn compared with the certificated values of 26.7±0.6 μg g^{-1}, 106.0±10.0 μg g^{-1}, 2.50±0.19 μg g^{-1}, 0.35±0.13 μg g^{-1} and 180.0±6.0 μg g^{-1} respectively. All metal concentrations are expressed on a dry tissue weight basis.

Antioxidant enzymes

To determine the levels of enzymatic activities, total tissues of *Nereis diversicolor* were homogenized in 20 mM Tris-HCl buffer, pH 7.6, containing 1 mM of EDTA, 0.5 M of saccharose, 0.15 M of KCl and 1 mM of DTT. The homogenates were centrifuged at 500 *g* for 15 minutes at 4°C to precipitate large particles and re-centrifuged at 12000 g for 45 minutes at 4°C to precipitate the mitochondrial fraction. The cytosolic fraction was purified on a Sephadex G-25 gel column (PD10, Pharmacia) to remove low molecular weight proteins. All enzymatic activities were determined in the cytosolic fraction.

Superoxide dismutase activity (SOD, EC 1.15.1.1) was determined by measuring the reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at 550 nm. One unit of SOD is defined by the amount of the enzyme that inhibits 50% of the reduction of cytochrome c (McCord and Fridovich 1969). The SOD activity obtained is expressed as U mg^{-1} of total protein concentration.

Catalase activity (CAT, EC 1.11.1.6) was measured following the method described by Greenwald (1985) as a result of the decrease in absorbance at 240 nm due to hydrogen peroxide consumption. The results are expressed as μmoles min^{-1} mg^{-1} of total protein concentration.

Total glutathione peroxidase (GPX) was measured following NADPH oxidation at 340 nm in the presence of excess glutathione reductase, reduced glutathione and cumene hydroperoxide as substrate (Lawrence and Burk 1976). GPX activities are expressed in nmoles min^{-1} mg^{-1} of total protein concentration.

Metallothionein analysis

Metallothionein (MT) was quantified in the total tissues of polychaetes. Samples were homogenized in three volumes of Tris-HCl buffer (0.02 M pH 8.6) in an ice bath. An aliquot of the homogenate was used for metal analysis. A further aliquot of the homogenate was centrifuged at 30000 g for 45 minutes at 4°C. The supernatant was separated from the pellet and two aliquots were used for lipid peroxidation and total protein determination. The remaining supernatant was heat-treated at 80°C for 10 min to precipitate the high molecular weight proteins and re-centrifuged at 30000 g for 45 minutes at 4°C. Aliquots of the heat-treated cytosol were used to quantify the MT concentration using differential pulse polarography according to the method developed by Bebianno and Langston (1989). In the absence of a polychaete MT standard, quantification of the MT in the cytosol was based on rabbit liver metallothionein, MT-I, using the method of standard additions. MT concentrations were determined as a milligram per gram of total protein concentration.

Lipid peroxidation analysis

Lipid peroxidation (LPO) was assessed by determining malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations upon decomposition by polyunsaturated fatty acid peroxides, following the method described by Erdelmeier *et al*. (1998). This procedure is based on the reaction of two moles of *N*-methyl-2-phenylindole, a chromogenic reagent, with one mole of either MDA or 4-HNE at 45°C for 60 min to yield a stable chromophore that has maximum absorbance at 586 nm, using malondialdehyde bis-(tetrametoxypropan) as a standard. Lipid peroxidation is expressed as μmols of MDA and 4-HNE per gram of total protein concentration.

Total protein analysis

The total protein content of the cytosolic fractions of *N. diversicolor* was measured with the Lowry method (Lowry *et al*. 1951) using Folin's Reagent and Bovine Serum Albumin (BSA) as reference standard materials.

Statistical analysis

The data obtained was tested for normality and homogeneity of variances to determine whether they satisfy the assumptions associated with parametric tests, and one-way analysis of variance (ANOVA) or the Kruskal-Wallis were applied. The Tukey (parametric data) or the Dunn's tests (non-parametric data) were used to discriminate significant differences. Pearson's correlation analysis was applied between biomarkers and metals to verify existing relationships. Canonical Correspondence Analysis (CCA) was used to assess the

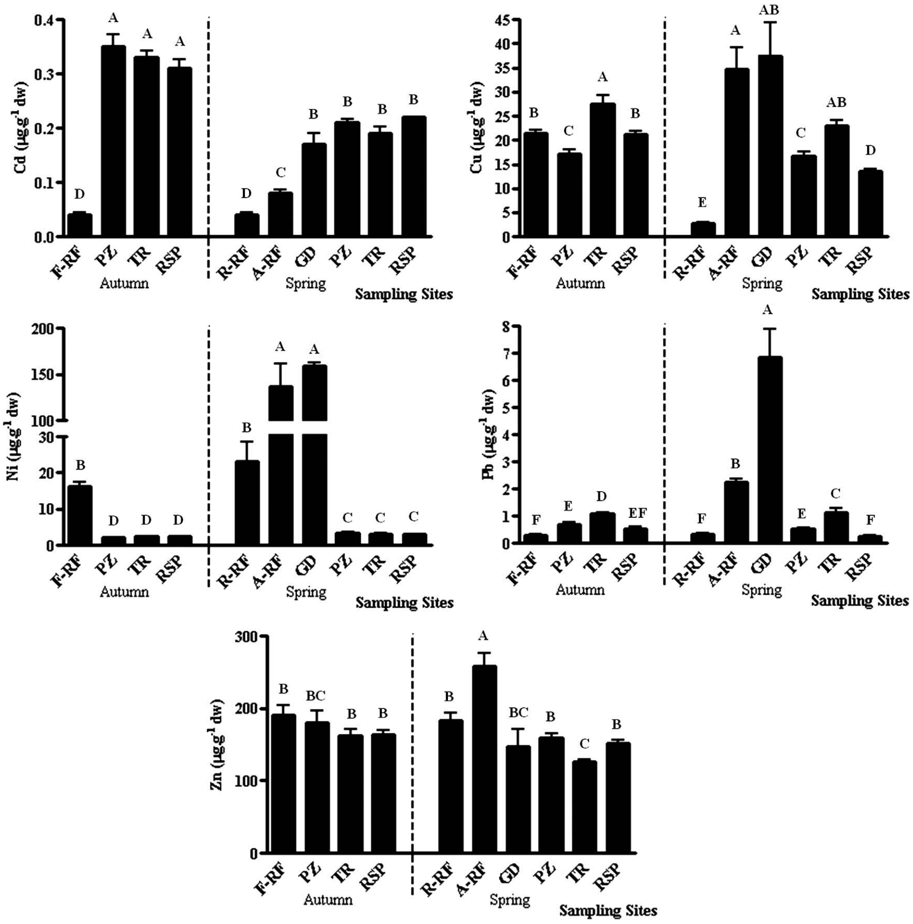

FIG. 2. – Concentrations of cadmium, copper, nickel, lead and zinc (μg g^{-1} dry weight) in polychaete tissues collected at the sampling sites along the southwest Iberian coast in autumn and spring (mean±std). Different letters represent significant differences among sampling locations in autumn and spring, at $p<0.05$ using Tukey or Dunn's post hoc tests.

Table 1. – *Nereis diversicolor* biomarker levels (mean±sd) in the southwest Iberian Peninsula in the two seasonal periods. Different letters represent significant differences among sampling locations in autumn and spring, at $p<0.05$ using Tukey or Dunn's post hoc tests. TP – total protein levels. F-RF: Faro, Ria Formosa Lagoon; PZ: Puente Zuazo, Cádiz Bay; TR: Trocadero, Cádiz Bay; RSP: Rio San Pedro, Cádiz Bay; R-RF: Ramalhete, Ria Formosa Lagoon; A-RF: Ribeira de Almargem, Ria Formosa Lagoon; GD: Guadiana River.

Season	Location	SOD (U mg^{-1} TP)	CAT (µmol min mg^{-1} TP)	GPX-T (nmol min mg^{-1} TP)	MT (mg g^{-1} TP)	LPO (µmol.g^{-1} TP)
Autumn	F-RF	9.4±2.4^{e}	45.8±13.9^{d}	12.0±1.0^{bc}	3.1±0.3^{bc}	0.3±0.0^{e}
	PZ	34.2±4.3^{b}	83.5±19.6^{ab}	16.2±4.1^{b}	1.0±0.3^{d}	1.6±0.4^{b}
	TR	54.6±14.2^{a}	95.3±33.2^{ab}	19.1±5.0^{a}	3.4±0.9^{b}	1.3±0.2^{bc}
	RSP	48.9±9.9^{ab}	106.2±18.6^{ac}	17.5±4.8^{ab}	2.0±0.3^{c}	1.0±0.2^{b}
Spring	R-RF	13.3±2.0^{d}	67.8±11.3^{bc}	25.7±5.1^{a}	4.9±1.0^{a}	0.2±0.0^{e}
	A-RF	12.9±2.6^{de}	43.7±7.5^{d}	13.2±2.5^{b}	6.0±1.5^{a}	0.1±0.0^{f}
	GD	25.7±1.9^{c}	32.7±2.5^{d}	10.0±2.3^{cd}	4.1±1.4^{ab}	0.4±0.1^{d}
	PZ	45.4±11.5^{ab}	70.8±6.5^{bc}	7.6±1.7^{de}	3.0±0.7^{bc}	2.5±0.2^{a}
	TR	37.7±9.9^{abc}	74.7±24.8^{ac}	6.7±0.7^{e}	2.7±0.8^{bc}	0.9±0.0^{c}
	RSP	34.9±11.0^{bc}	56.6±17.0^{cd}	5.3±0.5^{e}	2.5±0.7^{bc}	1.0±0.2^{c}

influence of biomarkers associated with metal levels on the different sites. Statistical significance was defined at the $p<0.05$ level. Statistical analyses were performed using XLSTAT® 2010.

RESULTS

Metal concentrations

Metal (Cd, Cu, Ni, Pb and Zn) concentrations were determined in the whole tissues of *N. diversicolor* and are shown in Figure 2. Polychaetes from all the sites in Cádiz Bay had higher cadmium levels in autumn (1.8, 1.7 and 0.71-fold, $p<0.05$) compared to spring. The highest concentrations were found in polychaetes from PZ, TR and RSP for both seasons, followed by the ones from Guadiana River (0.17±0.04 µg g^{-1} d.w.). The lowest concentrations were detected in the Ria Formosa Lagoon, with the minimum levels in F-RF in autumn (0.04±0.01 µg g^{-1} d.w.) and R-RF in spring (0.04±0.01 µg g^{-1} d.w.).

N. diversicolor from the Rio San Pedro (RSP) were the only ones with significant differences in Cu concentrations between seasons (21.17±1.20 µg g^{-1} d.w. in autumn and 13.64±0.47 µg g^{-1} d.w. in spring, p<0.05). The highest levels of Cu were detected in polychaetes from GD and A-RF in spring and TR in autumn and spring, ranging from 37.47±11.88 µg g^{-1} d.w. in GD to 23.08±1.93 µg g^{-1} d.w. in TR in spring. The lowest concentrations were detected in polychaetes from RSP (13.64±0.47 µg g^{-1} d.w.) and R-RF (2.66±0.65 µg g^{-1} d.w.) in spring.

Polychaetes from all the sites in Cádiz Bay had higher nickel concentrations in spring than in autumn (1.7, 1.3 and 1.2-fold, $p<0.05$). The highest Ni concentrations were detected in polychaetes from the Guadiana River (159.30±6.13 µg g^{-1} d.w.) and A-RF (136.75±41.65 µg g^{-1} d.w.), followed by the individuals from the F-RF and R-RF (16.13±2.89 µg g^{-1} d.w. and 23.26±7.33 µg g^{-1} d.w. respectively), all in the Ria Formosa Lagoon. The lowest levels were found in individuals from Cádiz Bay, ranging from 3.47±0.39 µg g^{-1} d.w. in PZ in spring to 2.05±0.06 µg g^{-1} d.w. in PZ in autumn.

No differences were found for Pb levels in *N. diversicolor* between seasons except for those from TR, with a 1.1-fold increase in spring. The highest Pb concentration was found in the Guadiana River (6.84±1.50 µg g^{-1} d.w.), followed by the A-RF in the Ria Formosa Lagoon (2.23±0.24 µg g^{-1} d.w.) in spring. The lowest concentrations were also detected in the Ria Formosa Lagoon, in F-RF in autumn (0.30±0.05 µg g^{-1} d.w.) and R-RF in spring (0.34±0.03 µg g^{-1} d.w.), and in Cádiz Bay in RSP in autumn (0.54±0.14 µg g^{-1} d.w.) and spring (0.23±0.08 µg g^{-1} d.w.).

Zinc concentrations in *N. diversicolor* from all sites were similar between seasons except for TR, where a higher Zn level was detected in spring (1.3-fold, $p<0.05$). Zinc levels ranged between 257.50±37.41 µg g^{-1} d.w. and 126.13±4.65 µg g^{-1} d.w. in A-RF and TR in spring respectively, with similar concentrations between the remaining sites (p>0.05).

There were several significant relationships between metal levels in *N. diversicolor* tissues, namely between Cu and Ni ($r=0.70$, $p<0.05$), Cu and Pb ($r=0.72$, $p<0.05$) and Ni and Pb ($r=0.86$, $p<0.01$).

Biomarkers

The biomarkers analysed in *N. diversicolor* per site and sampling period are presented in Table 1. There was no seasonal influence on the SOD activity of *N. diversicolor*. The highest SOD activities were detected in polychaetes from TR (54.6±14.6 U mg^{-1} TP) and RSP (48.9±9.9 U mg^{-1} TP) in autumn, and PZ (45.4±11.5 U mg^{-1} TP) and TR (37.7±9.9 U mg^{-1} TP) in spring. The lowest levels were found in polychaetes from the Ria Formosa Lagoon, namely F-RF (9.4±2.4 U mg^{-1} TP), A-RF (12.9±2.6 U mg^{-1} TP) and R-RF (13.3±2.0 U mg^{-1} TP), with no significant differences between them ($p>0.05$).

A similar trend was found for CAT activity. The activities of this enzyme in *N. diversicolor* were similar between seasons for the Ria Formosa Lagoon and Cádiz Bay ($p>0.05$). Levels ranged from 106.2±18.6 µmol min mg^{-1} TP to 32.7±2.5 µmol min mg^{-1} TP, with the highest values in polychaetes from Cádiz Bay in both seasons and the lowest values in F-RF in autumn and GD and A-RF in spring.

For GPX, a seasonal variation was observed in polychaetes from PZ, TR and RSP, with a 0.53, 0.65 and 0.70 fold decrease, respectively, in autumn. The maximum levels were found both in spring in R-RF (25.7±5.1 nmol min mg^{-1} TP) and autumn in TR and RSP (19.1±5.0 nmol min mg^{-1} TP and 17.5±4.8 nmol min mg^{-1} TP respectively). The minimum activities were detected in polychaetes from Cádiz Bay in spring (7.6±1.7 nmol min mg^{-1} TP for PZ, 6.7±0.7 nmol min mg^{-1} TP for TR and 5.3±0.5 nmol min mg^{-1} TP for RSP).

For MT, significant differences between autumn and spring were only found in polychaetes from PZ, with a 3-fold increase in spring. *N. diversicolor* from GD, A-RF and R-RF had the highest MT concentrations (4.1±1.4 mg g^{-1} TP, 6.0±1.5 mg g^{-1} TP and 4.9±1.0 mg g^{-1} TP, respectively) and the lowest concentrations were found in those from PZ in autumn (1.0±0.3 mg g^{-1} TP).

Lipid peroxidation expressed as MDA and 4-HNE concentrations followed the same seasonal pattern as MT, that is, polychaetes from PZ had a higher concentration in spring (1.56-fold, $p<0.05$). Lipid peroxidation was higher in *N. diversicolor* from PZ in spring (2.5±0.2 μmol g^{-1} TP), followed by those from PZ and TR in autumn (1.6±0.4 μmol g^{-1} TP and 1.3±0.2 μmol g^{-1} TP). The lowest levels were detected in polychaetes from the Ria Formosa Lagoon, ranging from 0.1±0.0 μmol g^{-1} TP in A-RF in spring to 0.3±0.0 μmol g^{-1} TP in F-RF in autumn.

There were two significant relationships between biomarkers: SOD activity is directly related to CAT activity ($r=0.75$, $p<0.05$) and negatively to LPO ($r=-0.74$, $p<0.05$).

Relationship between metals and biomarkers

Multiple correlations were conducted to assess the relationships between metals in *N. diversicolor* whole tissues and biochemical responses. Cadmium and nickel were the only metals that showed a significant relationship with biomarker levels. Cd is directly related to SOD activity ($r=0.88$, $p<0.01$), CAT activity ($r=0.73$, $p<0.05$) and LPO levels ($r=0.66$, $p<0.05$). Nickel is directly related to MT ($r=0.68$, $p<0.05$) and negatively to CAT ($r=-0.71$, $p<0.05$).

Metal concentrations in the sediments from the same sites and collected at the same time (Blasco *et al.* 2010) were also included in the analysis due to the importance of metal transfer from ingested sediments to these species and to higher trophic levels (Coelho *et al.* 2008). Cadmium was the only metal with a significant relationship between its concentration in polychaete tissues and in sediments ($r=0.69$, $p<0.05$).

Canonical Correspondence Analysis (CCA) was applied to biomarkers and metal levels in polychaetes from the different sites to determine the factors that influence the variance of biomarkers (Fig. 3). The two main axes explain 94.67% of the total variance, where only PC1 represents 78.59%. Polychaetes from the Ria Formosa Lagoon, Guadiana River and Cádiz Bay responded differently to metal contamination with different biomarker responses, showing a clear site trend. The overall CCA shows a site related trend but no seasonal differentiation in biomarker responses. The sampling sites are clearly separated, with a close association between the three sites within Cádiz Bay (TR, PZ and RSP) as well as the three sites within the Ria Formosa Lagoon (F-RF, R-RF and A-RF). SOD and CAT activities are higher in Cádiz Bay in association with higher cadmium concentrations that are directly related to lipid peroxidation. In the Ria Formosa Lagoon, GPX responds primarily to the Zn concentration, most closely associated with R-RF. The highest MT concentrations are in the Ria Formosa Lagoon and in the Guadiana River, and are closely related to higher nickel concentrations. Pb is the main metal influencing polychaetes in the Guadiana River, while Ni is more associated with the Guadiana River and A-RF in spring.

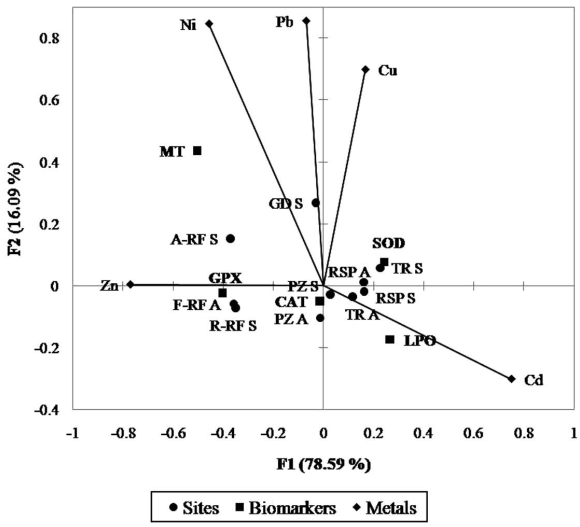

Fig. 3. – Canonical correspondence analysis (CCA) of the set of biomarkers and metal concentrations in the polychaete *Nereis diversicolor* collected at the sampling sites along the southwest Iberian coast in autumn and spring.

DISCUSSION

The coastal ecosystems from the southwest Iberian Peninsula are strongly impacted by human activities. This anthropogenic pressure influences the structure and dynamics of the estuaries and saltmarshes of this area, mainly through mining, urban, agricultural and harbour effluents and discharges (Carrasco *et al.* 2003, Pérez *et al.* 2004, Almeida *et al.* 2007, Solé *et al.* 2009).

Considering the importance and potential complexity of aquatic pollution in estuaries, it is recommended to apply a multibiomarker approach in order to obtain a more comprehensive and integrated view of the biological responses, and complement the information given by chemical measures of contaminant contents in the selected species (Cajaraville *et al.* 2000, Sun and Zhou 2008, Cravo *et al.* 2009).

Numerous environmental variables have the capacity to trigger the antioxidant defence system in polychaete species, such as solar radiance, hydrogen peroxide, salinity, water temperature, oxygen, and also exposure to several classes of contaminants (Bocchetti *et al*. 2004, Ait Alla *et al*. 2006, Moreira *et al*. 2006, Ferreira-Cravo *et al*. 2007, Sun and Zhou 2008, Douhri and Sayah 2009).

The overall metal concentrations determined in *N. diversicolor* tissues showed different spatial responses, reflecting different origins and intensities of contamination, but with no clear seasonal patterns. Guadiana River and Ria Formosa Lagoon are strongly influenced by metallic contamination with higher Cu, Ni and Pb levels in *N. diversicolor* tissues compared to those from Cádiz Bay. Only cadmium was higher in polychaetes from Cádiz Bay. Both Cu and Cd are known to accumulate in *H. diversicolor* and other polychaete species proportionally to their concentration in the surrounding medium (Díez *et al*. 2000, Berthet *et al*. 2003, Poirier *et al*. 2006). The higher concentration of these two metals in these particular ecosystems is of anthropogenic origin associated with sewage and industrial effluents and agriculture runoffs (Bebianno 1995, Blasco *et al*. 2000, Carrasco *et al*. 2003, Caetano *et al*. 2006). Nickel is one of the trace-element constituents of the sediments of the south coast of Portugal and one of the major input sources of this metal is land runoff due to rainfall (Boski *et al*. 1999, Machado *et al*. 1999). The polychaetes in Guadiana River exhibited the highest lead levels. The presence of Pb in this river is related to its location within the Iberian Pyrite Belt, where the untreated mine wastes and discharges of acid mine waters represent the two major sources of this metal. Concurrently, industrial activities, sewage waste and remains of leaded petrol are considered additional Pb inputs (Caetano *et al*. 2006, Company *et al*. 2008, 2011, Kalman *et al*. 2008). The strong influence of Pb in polychaetes from the Guadiana River was also supported by the CCA; however, this was not particularly associated with any of the biomarkers selected. As an essential metal used as a co-factor in numerous enzymes, Zn concentrations in marine organisms can be regulated, as in *N. diversicolor* (Bocchetti *et al*. 2004, Wang and Rainbow 2005, Durou *et al*. 2007a,b). In the present study no significant enhancement or differences between sites were detected in Zn concentration in *N. diversicolor* tissues despite the high anthropogenic-derived metal concentrations in the sediments, which is in agreement with previous studies (Berthet *et al*. 2003, Carrasco *et al.* 2003, Blasco *et al*. 2010).

The metal concentrations detected are characteristic of low or moderate levels of contamination (see levels in sediments from the same sites) (Blasco *et al*. 2010). Overall, metal concentrations in *N. diversicolor* tissues are in the same range of those reported in Cádiz Bay in previous years (Solé *et al*. 2009). Several industries that use metals have developed in recent years in the areas within the bay, namely manufacturers of car and aircraft components, which have resulted in a continuous input of metals into the bay (Blasco *et al*. 2000, 2010, Carrasco *et al*. 2003). For the other sites, the only metal concentrations in the literature are from bivalve species that are not directly comparable to polychaetes. Nevertheless, Blasco *et al*. (2010) reported that metals in sediments from the Ria Formosa Lagoon and Guadiana River are also in agreement with previous studies, indicating similar levels of contamination (Bebianno 1995, Caetano *et al*. 2006).

The possible effects that sediment-based metals can have on aquatic organisms living in sediments depend on the bioavailability of the metals (Díez *et al*. 2000, Berthet *et al*. 2003). As for the metal contents in sediments, Cd concentrations are directly reflected in polychaete tissues, as corroborated by the correlation analysis. Cadmium in sediments from all sites appears to have high availability to polychaetes. *N. diversicolor* is a good indicator of Ag, Cd, Co, Cr, Cu and Pb concentrations, reflecting metal bioavailabilities in this compartment (Díez *et al*. 2000, Berthet *et al*. 2003, Poirier et *al*. 2006).

Metals are able to produce ROS through redox cycling by depleting glutathione and protein-bound sulphydryl groups (Cd, Cu, Hg, Ni and Pb) and inhibiting some antioxidant enzymes, among others (Wang and Rainbow 2005, Moreira *et al*. 2006, Valavanidis *et al*. 2006). In this study, polychaetes antioxidant efficiency was assessed by analysing antioxidant enzymes (SOD, CAT and GPX). These enzymes responded efficiently to metal contamination, allowing site differentiation but no seasonal discrimination. SOD, CAT and GPX had similar patterns with higher activities in organisms from Cádiz Bay compared to those from the Ria Formosa Lagoon and Guadiana River. These antioxidant enzymes were activated due to the presence of high metal concentrations, principally cadmium. A metal pollution gradient exist at these sites, that includes discharges from domestic sources, offshore industries and shipbuilding, in which the presence of cadmium is a recent input into the bay with a high bioavailability from sediments (Pérez *et al*. 2004, Solé *et al*. 2009, Blasco *et al*. 2010). Several studies have reported the enhancement of antioxidant enzyme activities in polychaetes to cope with metal stress both in laboratory exposures and in the field (e.g. Cd, Cu) (Geracitano *et al*. 2004, Bocchetti *et al.* 2004, Ferreira-Cravo *et al.* 2007, Sun and Zhou 2008, Douhri and Sayah 2009). This result is confirmed by the statistical analyses, which showed a significant relationship between the Cd concentrations and SOD and CAT activities in polychaete tissues associated with the sites within Cádiz Bay. CAT and SOD are therefore linked to counteract ROS production originated by the high Cd concentrations found within the bay. Catalase is also associated with Ni, as high concentrations of this metal inhibit CAT enzymatic activity. As for GPX, the CCA analysis showed an association between Zn concentration and GPX activities in polychaetes from the Ria Formosa Lagoon; however, the relationship was not significant.

Table 2. – Biomarker levels in *Nereis diversicolor*, *Mytilus galloprovincialis* and *Scrobicularia plana* collected at different sites.

Species	Location	SOD (U mg^{-1} TP)	CAT (µmol min mg^{-1} TP)	GPX-T (nmol min mg^{-1} TP)	MT (mg g^{-1} TP)	LPO (µmol g^{-1} TP)	References
Nereis diversicolor	Seine estuary, France		15-98				Durou *et al.* 2007a
	Cádiz Bay, Spain			36.9-42.1	8.7-15.5		Pérez *et al.* 2004
			25-225		7.6-24.7		Solé *et al.* 2009
	west coast of Portugal	15-45	19-25	7-15		0.5-1.2	Moreira *et al.* 2006
	Oued Souss, Morrocco		16.9-87.9				Ait Alla *et al.* 2006
M. galloprovincialis	south coast of Portugal	6-19	35-60	4-8	5-17	1-14	Cravo e*t al.* 2009
Scrobicularia plana	south coast of Portugal	17.9-132.3	14.3-249.4	3.3-20.3	0.2-4.3	0.1-0.97	Data not published
	Cádiz Bay, Spain	8.9-28.1	136.7-269.7	19.4-45.3	2.0-7.7	0.2-4.1	Data not published

The enzymatic results from this study are in the same range as those reported for the same species in Cádiz Bay (Pérez *et al*. 2004, Solé *et al*. 2009), on the west coast of Portugal (Moreira *et al*. 2006), in the Seine estuary in France (Durou *et al*. 2007a) and in the Oued Souss in Morocco (Ait Alla *et al*. 2006) (Table 2). In the Guadiana River and Ria Formosa Lagoon, CAT, SOD and GPX activities were similar to the ones reported for bivalves *Mytilus galloprovincialis* (Cravo *et al*. 2009) and *Scrobicularia plana* (data not published) (Table 2).

Besides their role in metal detoxification and homeostasis, MTs are also known as ROS scavengers (Langston *et al*. 1998, Viarengo *et al*. 1999). The MT concentrations in polychaetes from the Ria Formosa Lagoon and the Guadiana River were higher than in polychaetes from Cádiz Bay. Although polychaetes from the Guadiana River and the River Formosa Lagoon also showed high concentrations of Cu, Ni and Pb, the general response of MTs did not reflect the overall metal concentrations present in polychaetes from all the sites sampled. The lack of MT induction after metal exposure in polychaete species has also been reported by other authors (Poirier *et al*. 2006, Solé *et al*. 2009), and could be related to the presence of other physiological detoxification mechanisms in polychaetes to cope with high metal concentrations, such as storage of insoluble metals in detoxification granules, increases in mucus secretion, accumulation in specific body parts and tissues and exocytosis (Gibbs *et al*. 2000, Berthet *et al*. 2003, Mouneyrac *et al*., 2003). There was a significant relationship between MT and Ni (correlation analysis and CCA), reflecting the MT action in detoxifying the higher Ni concentrations found in the Guadiana River and the Ria Formosa Lagoon (A-RF). Nickel can generate ROS, leading to alterations in the cellular antioxidant system (Wang and Rainbow 2005, Moreira *et al*. 2006, Valavanidis *et al*. 2006) and MT may act as an ROS scavenger, detoxifying the excess of ROS originated by this metal and preventing further oxidative damage in polychaete tissues. The presence of this metal at these two sites is associated with freshwater inputs that lead to a higher Ni availability in sediments and consequently to a higher accumulation by polychaetes. Other study showed high degrees of Ni accumulation in polychaete tissues associated with high Ni concentrations in interstitial water due to the oxidation of metal sulphides present in the sediment (Pesch *et al*. 1995, Otero *et al*. 2000, Ruus *et al*. 2005).

The MT levels in *N. diversicolor* from this study were lower than those reported by Pérez *et al*. (2004) and Solé *et al*. (2009) in Cádiz Bay (Table 2), where no relationship between metals and MT levels was found. MTs were also in the range of those obtained in bivalves *S. plana* (data not published) and *M. galloprovincialis* (Cravo *et al*. 2009) collected at the same sites in both the Guadiana River and Ria Formosa Lagoon (Table 2).

When ROS production exceeds antioxidant defences, oxidative stress occurs, which causes membrane lipid peroxidation, among other effects (Matés 2000, Livingstone 2001, Viarengo *et al*. 2007). In the present study, LPO levels were, as for the antioxidant enzymes, site specific and higher in polychaetes from Cádiz Bay. The higher enzymatic activities and lipid peroxidation detected suggest that these antioxidant enzymes were unable to eliminate ROS and prevent deleterious effects in lipids of cellular membranes, originated by the higher cadmium concentration at these sites (Winston and Di Giulio *et al*. 1991, Matés 2000). CCA and correlation analysis showed an association between SOD and CAT activities, LPO levels and cadmium concentrations.

Few field studies report the presence of lipid peroxidation in polychaete species. The LPO levels in polychaetes from the southwest Iberian coast are similar to those reported by Moreira *et al*. (2006) for the west coast of Portugal from both clean and impacted sites (Table 2). In comparison to bivalve species, the levels are similar to the ones in *S. plana* collected from the same sites in the Guadiana River, Ria Formosa Lagoon and Cádiz Bay (data not published) and in the same range as the ones in *M. galloprovincialis* from the Ria Formosa Lagoon and Guadiana River (Table 2).

A specific biomarker for lead exposure should have been included in the multibiomarker approach used in this study, specifically the enzyme δ-aminolevulinic acid dehydratase (ALAD), since this metal does not induce MT. This enzyme has been shown to be a potential biomarker for Pb contamination with several bivalve species from the Gulf of Cádiz to the Ria Formosa Lagoon (Company *et al.* 2011).

The fluctuation in biomarker levels in polychaetes from each site reflects not only the differences between metal loads, but also the interactions between mixtures of contaminants. The presence of complex mixtures of metals and other contaminants in these ecosystems is well recognized, with interactions that are reflected in the biomarker response of polychaetes (Kennish 2002, Sun and Zhou 2008, Cravo *et al*. 2009, Solé *et al*. 2009).

In conclusion, *N. diversicolor* showed different responses at the different sites along the southwest coast of the Iberian Peninsula, which suggests different sources and/or magnitudes of contamination in association with diverse anthropogenic activities. Although the metal concentrations found in *N. diversicolor* tissues are indicative of a low or moderate level of contamination, the applied biomarkers responded efficiently to specific site differences, except for MT, which did not reflect the overall metal concentrations present. The selected biomarkers can be integrated into future environmental quality assessments of the southwest Iberian coast, along with more specific biomarkers of metal exposure, such as ALAD (Pb), or other physiological detoxification mechanisms specific to polychaetes. For these species, MT can also be included as a biomarker for oxidative stress but not a biomarker for metal exposure. The particular features of *N. diversicolor* evidence its role as a sentinel species, validating its capacity to bioaccumulate several metals from sediments.

ACKNOWLEDGEMENTS

We would like to thank the Interreg III-A Program for funding the projects RED CONTAMAR and PROTEOBIOMAR.

REFERENCES

Ait Alla A., Mouneyrac C., Durou C., Moukrim A., Pellerin, J. 2006. Tolerance and biomarkers as useful tools for assessing environmental quality in the Oued Souss estuary (Bay of Agadir, Morocco). *Comp. Biochem. Physiol. C.* 143: 23-29.

Almeida C., Serôdio P., Florêncio M.H., Nogueira J.M.F. 2007. New strategies to screen for endocrine-disrupting chemicals in the Portuguese marine environment utilizing large volume injection-capillary gas chromatography-mass spectrometry combined with retention time locking libraries (LVI-GC-MS-RTL). *Anal. Bioanal. Chem*. 387: 2569-2583.

Amiard J., Geffard A., Amiard-Triquet C., Crouzet C. 2007. Relationship between the lability of sediment-bound metals (Cd, Cu, Zn) and their bioaccumulation in benthic invertebrates. *Estuar. Coast. Shelf Sci.* 72: 511-521.

Bebianno M.J., Langston W.J. 1989. Quantification of metallothioneins in marine invertebrates using differential pulse polarography. *Port. Electrochim. Acta* 7: 59-64.

Bebianno M.J. 1995.Effects of pollutants in the Ria Formosa Lagoon, Portugal. *Sci. Total Environ.* 171: 107-115.

Berthet B., Mouneyrac C., Amiard J.C., Amiard-Triquet C., Berthelot Y., Le Hen A., Mastain O., Rainbow P.S., Smith B.D. 2003. Accumulation and soluble binding of cadmium, copper, and zinc in the polychaete *Hediste diversicolor* from coastal sites with different trace metal bioavailabilities. *Arch. Environ. Contam. Toxicol.* 45: 468-478.

Blasco J., Sáenz V., Gómez-Parra A. 2000. Heavy metal fluxes at the sediment – water interface of three coastal ecosystems from south-west of the Iberian Peninsula. *Sci. Total Environ*. 247: 189-199.

Blasco J., Gomes T., Garcia-Barrera T., Rodriguez-Romero A., Gonzalez-Rey M., Moran-Roldan F., Tromibini C., Miotk M., Gomez-Ariza J.L., Bebianno M.J. 2010. Metal occurrence in recent sediments from the southwest of the Iberian Peninsula. *Sci. Mar*. 74S1(1): 99-106.

Bocchetti R., Fattorini D., Gambi M.C., Regoli F. 2004. Trace metal concentrations and susceptibility to oxidative stress in the polychaete *Sabella spallanzanii* (Gmelin) (Sabellidae): potential role of antioxidants in revealing stressful environmental conditions in the Mediterranean. *Arch. Environ. Contam. Toxicol.* 46: 353-361.

Boski T., Moura D.M., Machado L.M., Bebianno M.J. 1999. Trace metals on the Algarve coast, I: Associations, origings and remobilization of natural components. *Bul. Inst. Esp. Oceanogr.* 15(1-4): 457-463.

Caetano M., Vale C., Falcão, M. 2006. Particulate trace metal distribution in Guadiana estuary punctuated by flood episodes. *Estuar. Coast. Shelf Sci*. 70: 109-116.

Cajaraville M.P., Bebianno M.J., Blasco J., Porte C., Sarasquete C., Viarengo A. 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *Sci. Total Environ.* 247: 295-311.

Carrasco M., López-Ramírez J.A., Benavente J., López-Aguayo F., Sales D. 2003. Assessment of urban and industrial contamination levels in the bay of Cádiz, SW Spain. *Mar. Pollut. Bull*. 46: 335-345.

Coelho M.R., Bebianno M.J., Langston M.J. 2002. Organotin levels in the Ria Formosa lagoon, Portugal. *Appl. Organometal. Chem.* 16: 384-390.

Company R., Serafim A., Lopes B., Cravo A., Shepherd T.J., Pearson G., Bebianno M.J. 2008. Using biochemical and isotope geochemistry to understand the environmental and public health implications of lead pollution in the lower Guadiana River, Iberia: A freshwater bivalve study. *Sci. Total Environ*. 405: 109-119.

Company R., Serafim A., Lopes B., Cravo A., Kalman J., Riba I., DelValls T.A., Blasco J., Delgado J., Sarmiento A.M., Nieto J.M., Shepherd T.J., Nowell G., Bebianno M.J. 2011. Source and impact of lead contamination on ●aminolevulinic acid dehydratase activity in several marine bivalve species along the Gulf of Cádiz. *Aquat. Toxicol*. 101: 146-154.

Cravo A., Madureira M., Felícia H., Rita F., Bebianno M.J. 2006. Impact of outflow from the Guadiana River on the distribution of suspended particulate matter and nutrients in the adjacent coastal zone. *Estuar. Coast. Shelf Sci.* 70: 63-75.

Cravo A., Lopes B., Serafim A., Company R., Barreira L., Gomes T., Bebianno M.J. 2009. A multibiomarker approach in *Mytilus galloprovincialis* to assess environmental quality. *J. Environ. Monit.* 11: 1673-1686.

Díez G., Soto M., Canton L., Vaquero C., Marigomez I. 2000. *Hediste* (*Nereis*) *diversicolor* as bioindicator of metal and organic chemical bioavailability: A field study. *Ecotoxicol. Environ. Restor.* 3: 7-15.

Dionísio L.P.C., Rheinheimer G., Borregos J.J. 1999. Microbial pollution in the Ria Formosa (South of Portugal). *Mar. Pollut. Bull*. 40(2): 186-193.

Douhri H., Sayah F. 2009. The use of enzymatic biomarkers in two marine invertebrates *Nereis diversicolor* and *Patella vulgate* for the biomonitoring of Tangier's bay (Morocco). *Ecotoxicol. Environ. Saf*. 72: 394-399.

Durou C., Poirier L., Amiard J.-C., Budzinski H., Gnassia-Barelli M., Lemenach K., Peluhet L., Mouneyrac C., Rómeo M., Amiard-Triquet C. 2007a. Biomonitoring in a clean and a multi-contaminated estuary based on biomarkers and chemical analyses in the endobenthic worm *Nereis diversicolor*. *Environ. Pollut.* 148: 445-458.

Durou C., Smith B., Roméo M., Rainbow P., Mouneyrac C., Mouloud M., Gnassia-Barelli M., Gillet P., Deutsch B., Amiard-Triquet C. 2007b. From biomarkers to population responses in *Nereis diversicolor*: Assessment of stress in estuarine ecosystems. *Ecotoxicol. Environ. Saf.* 66: 402-411.

Durou C., Mouneyrac C., Amiard-Triquet C. 2008. Environmental quality assessment in estuarine ecosystems: Use of biometric measurements and fecundity of the ragworm *Nereis diversicolor* (Polychaeta, Nereididae). *Water Res.* 42: 2157-2165.
Erdelmeier I., Gerard-Monnier D., Yadan J.C., Acudiere J. 1998. Reactions of N-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Mechanistic aspects of the colorimetric assay of lipid peroxidation. *Chem. Res. Toxicol*. 11: 1184-94.
Ferreira-Cravo M., Piedras F.R., Moraes T.B., Ferreira J.L.R., de Freitas D.P.S., Machado M.D., Geracitano L.A., Monserrat J.M. 2007. Antioxidant responses and reactive oxygen species generation in different body regions of the estuarine polychaete *Laeonereis acuta* (Nereididae). *Chemosphere*. 66: 1367-1374.
Geracitano L.A., Bocchetti R., Monserrat J.M., Regoli F., Bianchini A. 2004. Oxidative stress responses in two populations of *Laeonereis acuta* (Polychaeta, Nereididae) after acute and chronic exposure to copper. *Mar. Environ. Res*. 58: 1-17.
Gibbs P.E., Burt G.R., Pascoe P.L., Llewellyn C.A., Ryan K.P. 2000. Zinc, copper and chlorophyll-derivatives in the polychaete *Owenia fusiformis*. *J. Mar. Biol. Assoc. U.K*. 80: 235-248.
Gillet P., Mouloud M., Durou C., Deutsch B. 2008. Response of *Nereis diversicolor* population (Polychaeta, Nereididae) to the pollution impact - Authie and Seine estuaries (France). *Estuar. Coast. Shelf Sci.* 76: 201-210.
Greenwald R.A. 1985. *Handbook of methods for oxygen radical research*. CRC Press, Boca Raton, FL, 464 pp.
Kalman J., Riba I., Blasco J., DelValls T.A. 2008. Is δ-aminolevulinic acid dehydratase activity in bivalves from south-west Iberian Peninsula a good biomarker of lead exposure? *Mar. Environ. Res*. 66(1): 38-40.
Kennish M.J. 2002. Environmental threats and environmental future of estuaries. *Environ. Conserv.* 29(1): 78-107.
Langston W.J, Bebianno M.J., Burt G.R. 1998. Metal handling strategies in molluscs. In: Langston W.J. and Bebianno M.J. (eds.), *Metal Metabolism in Aquatic Environments*. Chapman and Hall, London, pp. 219-283.
Lawrence R.A., Burk R.F. 1976. Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem. Biophy. Res. Commun.* 71: 952-958.
Livingstone D.R. 2001. Contaminated-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Mar. Pollut. Bull.* 42: 656-666.
Lowry O.H., Rosenbrough N.J., Farr A.L., Randall R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. 193: 265-275.
Machado L.M., Bebianno M.J., Boski T., D.M. Moura. 1999. Trace metals on the Algarve coast, II: Bioaccumulation in mussels *Mytilus galloprovincialis* (Lamarck, 1819). *Bol. Inst. Esp. Oceanogr.* 15(1-4): 465-471.
Matés J.M. 2000. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicol.* 153: 83-104.
McCord J.M., Fridovich I. 1969. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). *J. Biol. Chem*. 244(22): 6049-6955.
Moreira S.M., Lima I., Ribeiro R., Guilhermino L. 2006. Effects of estuarine sediment contamination on feeding and on key physiological functions on the polychaete *Hediste diversicolor*: Laboratory and in situ assays. *Aquat. Toxicol.* 78: 186-201.
Mouneyrac C., Mastain O., Amiard J.C., Amiard-Triquet C., Beaunier P., Jeantet A.-Y., Smith B.D., Rainbow P.S. 2003. Trace metal detoxification and tolerance of the estuarine worm *Hediste diversicolor* chronically exposed in their environment. *Mar. Biol*. 143: 731-744.
Mudge S.M., Bebianno M.J. 1997. Sewage contamination following an accidental spillage in the Ria Formosa, Portugal. *Mar. Pollut. Bull.* 34(3): 163-170.
Otero X.L., Sanchez J.M., Macias F. 2000. Bioaccumulation of heavy metals in thionic fluvisols by a marine polychaete: the role of metal sulfides. *J. Environ. Qual*. 29: 1133-1141.
Pérez E., Blasco J., Solé M. 2004. Biomarker responses to pollution in two invertebrate species: *Scrobicularia plana* and *Nereis diversicolor* from the Cádiz bay (SW Spain). *Mar. Environ. Res.* 58: 275-279.
Pesch C.E., Hansen D.J., Boothman W.S., Berry W.J., Mahony J.D. 1995. The role of acid-volatile sulfide and interstitial water metal concentrations in determining bioavailability of cadmium and nickel from contaminated sediments to the marine polychaete *Neanthes arenaceodentata*. *Environ. Toxicol. Chem*. 14: 129-141.
Poirier L., Berthet B., Amiard, J.C., Jeantet A.Y., Amiard-Triquet C. 2006. A suitable model for the biomonitoring of trace metal bioavailabilities in estuarine sediments: the annelid polychaete *Nereis diversicolor*. *J. Mar. Biol. Ass. U.K.* 86: 71-82.
Ruus A., Schaaning M., Øxnevad S., Hylland K. 2005. Experimental results on bioaccumulation of metals and organic contaminants from marine sediments. *Aquat. Toxicol.* 72: 273-292.
Solé M., Kopecka-Pilarczyk J., Blasco J. 2009. Pollution biomarkers in two estuarine invertebrates, *Nereis diversicolor* and *Scrobicularia plana*, from a Marsh ecosystem in SW Spain. *Environ. Int.* 35(3): 523-531.
Sun F., Zhou Q. 2008. Oxidative stress biomarkers of the polychaete *Nereis diversicolor* exposed to cadmium and petroleum hydrocarbons. *Ecotoxicol. Environ. Saf.* 70: 106-114.
Valavanidis A., Vlahogianni T., Dassenakis M., Scoullos M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotox. Environ. Saf.* 64: 178-189.
Viarengo A., Burlando B., Dondero F., Marro A., Fabbri R. 1999. Metallothionein as a tool in biomonitoring programmes. *Biomarkers* 4: 455-466.
Viarengo A., Lowe D., Bolognesi C., Fabbri E., Koehler A. 2007. The use of biomarkers in biomonitoring: A 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comp. Biochem. Physiol. C* 146: 281-300.
Wang W.-X., Rainbow P.S. 2005. Influence of metal exposure history on trace metal uptake and accumulation by marine invertebrates. *Ecotoxicol. Environ. Saf.* 61: 145-159.
Winston G.W., Di Giulio R.T. 1991. Prooxidant and anti- oxidant mechanisms in aquatic organisms. *Aquat. Toxicol.* 19: 137-161.
Zhou Q.X., Rainbow P.S., Smith B.D. 2003. Tolerance and accumulation of the trace metals zinc, copper and cadmium in three populations of the polychaete *Nereis diversicolor*. *J. Mar. Biolog. Assoc. U.K.* 83: 65-72.

Received April 18, 2011. Accepted September 30, 2011.
Published online January 7, 2013.