UNIVERSIDADE NOVA DE LISBOA

Faculdade de Ciências e Tecnologia

Departamento de Ciências e Engenharia do Ambiente

EVALUATION OF THE POTENTIAL OF TRANSLOCATED COMMON COCKLE FOR ECOLOGICAL RISK ASSESSMENT STUDIES: BIOACCUMULATION AND BIOMARKERS TEST

Thesis submitted to the Faculdade de Ciências e Tecnologia to obtain the Master's degree in Environmental Engineering, profile in Ecological Engineering

By: Jorge Lobo Arteaga

Under the guidance: Professor Doctor Maria Helena Ferrão Ribeiro da Costa

Lisbon
October 2009

Acknowledgements

First of all, thanks to Professor Maria Helena Costa for support and time spent in guidance, and special attention for Pedro Costa for teaching and giving me the support and comprehension needed during all this time. Special thanks to Sandra Caeiro for the cooperation and availability. Thanks to Mr. Manuel Ribeiro for the provision of cockles. Special thanks have to be given to the Instituto Nacional dos Recursos Biológicos (IPIMAR_INRB) for the collaboration in this work, particularly to Marta Martins. And most of all, I want to thank my friends and family for their support, encourage, advice, and for always being there.

The present research was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB 57281/104) and financed by FEDER (European Fund for regional Development).

Sumário

A contaminação sedimentar é um factor de grande preocupação em estuários e outras massas de águas costeiras confinadas, muitas vezes submetidas a fontes antropogénicas de poluição. Com o objectivo de investigar os efeitos e respostas do berbigão comum (*Cerastoderma edule*) aos contaminantes no sedimento e avaliar o potencial da espécie como organismo indicador, o bivalve foi submetido a um ensaio de translocação com sedimentos colectados de diferentes locais do estuário do Sado (Portugal). Os berbigões foram recolhidos num local de maricultura do estuário do Sado (Portugal), identificado como local D, e foram expostos em ensaios de laboratório semi-estáticos, a sedimentos colectados noutros três locais do estuário (A, B e C) que revelaram diferentes níveis de metais, contaminantes orgânicos e distintas propriedades fisico-químicas, correspondendo a condições que vão desde ausência de impacte a impacte moderado quando comparados com os valores-guia de qualidade sedimentar disponíveis. Os bivalves foram analisados para bioacumulação de metais (Ni, Cu, Zn, As, Cd e Pb) e contaminantes orgânicos (PAHs, PCBs e DDTs). Empregaram-se dois conjuntos de biomarcadores para avaliar a toxicidade potencial: indução de metalotioninas (MT) e histopathologia da glândula digestiva. Estimaram-se o factor de bioacumulação (BAF) e o factor de acumulação biota-sedimento (BSAF) como índices ecológicos de exposição a metais e compostos orgânicos. Encontraram-se correlações significativas e positivas entre BSAF e MT para PHAs, e entre cada factor (BAF e BSAF) e MT para o Cd. Encontraram-se alterações histopatológicas nos berbigões expostos a todos os sedimentos para onde foram translocados, houve uma degradação da integridade da glândula digestiva principalmente nos organismos do sedimento B e C e no dia 28 do sedimento A. Os resultados permitiriam concluir que *C. edule* responde a sedimentos contaminados e é capaz de regular e eliminar contaminantes, sendo adequado para a biomonitorização. Ainda assim, os níveis de contaminação sedimentar não explicam a variação na bioacumulação e níveis de MT, que podem resultar de concentrações moderadas de contaminantes nos sedimentos e, mais importante, ainda não se conhecem os efeitos das interacções de xenobióticos.

Abstract

Sediment–bound contamination is a major concern factor in estuaries and other confined coastal water bodies, frequently subjected to anthropogenic sources of pollution. In order to investigate the effects and responses of the common cockle (*Cerastoderma edule,* L. 1558, Bivalvia: Cardiidae) to sediment contaminants and to assess the species' potential as an indicator organism, the bivalve was subjected to a laboratorial translocation assay with sediments collected from distinct sites of the Sado Estuary (Portugal). Cockles were collected from a mariculture site of the Sado estuary (Portugal), herewith identified as site A, and exposed through 28–day, semi–static laboratorial essays, to sediments collected from three other sites (B, C and D) of the estuary that revealed different levels of metals, organic contaminants and physico–chemical properties and that ranged from globally unimpacted to moderately impacted levels when compared to available sediment quality guidelines. The animals were surveyed for bioaccumulation of metals (Ni, Cu, Zn, As, Cd and Pb) and organic contaminants (PAHs, PCBs and DDTs). Two sets of potential biomarkers were employed to assess toxicity: whole–body metallothionein (MT) induction and digestive gland histopathology. The bioaccumulation factor (BAF) and the biota-to-soil accumulation factor (BSAF) were estimated as ecological indices of exposure to metals and organic compounds. Significant positive correlations between BSAF and MT were found for PHAs, and between each factor (BSAF and BAF) and MT were found for Cd. Histopathological alterations were found in cockles exposed to all sediments where they were translocated. The digestive gland integrity was found to be especially compromised in cockles from sediment B and C and at day 28 from sediment A. Results allowed concluding that *C. edule* responds to sediment–bound contamination and is capable to regulate and eliminate both types of contaminants and might, therefore, be suitable for biomonitoring. Still, the sediment contamination levels do not explain the variation in bioaccumulation and MT levels, which may result from the moderate contaminant concentrations found in sediments and, more importantly, from yet unexplained xenobiotic interaction effects.

Symbology

T0	sampling day 0
T14	sampling day 14
T28	sampling day 28
ww	wet weight
dw	sediment dry weight
Eh	sediment redox potential
TOM	total organic matter
MT	metallothionein
Ni	nickel
Cu	copper
Zn	zinc
Cd	cadmium
Pb	lead
As	metalloid arsenic
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
DDT	dichloro-diphenyl-trichloroethane
tPAH	total PAH (sum of all individual PAHs)
tPCB	total PCB (sum of all congeners)
tDDT	total DDT (pp'DDD+pp'DDD+pp'DDT)
DDD	1,1-dichloro-2,2-bis(*ρ*-chlorophenyl)ethane
DDE	1,1-dichloro-2,2-bis (*ρ*-chlorophenyl)ethylene
DDT	1,1,1-trichloro2,2-bis (*ρ*-chlorophenyl)ethane
GC–MS	gas chromatography–mass spectrometry
ICP-MS	inductive coupled plasma atomic emission spectrometry
TEL	threshold effects level
PEL	probable effects level
PEL-Q	PEL quotient
SQG-Q	sediment quality guideline quotient indice
BAF	bioaccumulation factor
BSAF	biota-sediment accumulation factor

Index

Index Figures

Index Tables

Index Annexes

1 Introduction

Marine bivalve molluscs are mainly filter-feeding organisms which due to their sedentary lifestyle, are characterised by their very high capability to bioaccumulate chemical substances dissolved in the water or bound to suspended particles (Machreki-Ajmi et al. 2008; Solé et al. 2009). These substances can be organic compounds and trace metals (essential or not), both with potential to cause toxic effects. Bivalve filter-feeders are therefore considered good bioindicators for the assessment of environmental quality (Cajaraville et al. 2000; Hédouin et al. 2007). The assessment of polluted environments based only in chemical analyses is difficult, particularly the assessment of polluted sediments due to the complex nature of the sediment matrix and the potential for exposure of aquatic organisms to in-place contaminants via several routes (Del Valls et al. 1998). The use of biomarkers has been considered to be viable measures of impact of toxicity (Huggett et al. 1992; Peakall and Shugart 1993). In recent years, biomarkers that may provide information on the effects of xenobiotics in organisms have received considerable interest and many of them have been validated in bivalves (Geret et al. 2003; Bergayou et al. 2009). Mussels and oysters are the marine bivalves most used in pollution monitoring. However, other species have been intensively studied because of their importance for human consumption or their close contact with sediment (Amiard et al. 2006). Some of these species have been widely employed in toxicity test and biomarker techniques have already been validated (Table 1).

Table 1 - Bivalve species in which Biomarkers have been shown to be induced by contaminant exposure.

Biomarker	Specie	Tissue/Organ	Exposure condition	Studied contaminant	Change	Reference
Lipid peroxidation [a]	*R. decussatus*	Digestive gland	Lab	Cu	Increase	Roméo and Gnassia-Barelli 1997
		Gill	Lab	Cu	No change	Roméo and Gnassia-Barelli 1997
	U. tumidus	Digestive gland	Field	PAHs, PCBs, others	Increase	Cossu et al. 1997, 2000
		Gill	Field	PAHs, PCBs, others	No change	Cossu et al. 1997, 2000
Metallothionein [b]	*Adamussium colbecki*	Gills	Lab	Cu	Increase	Viarengo et al. 1997

Biomarker	Specie	Tissue/Organ	Exposure condition	Studied contaminant	Change	Reference
	Anadara granosa	Blood	Lab	Cd	Increase	Chan et al. 2002
	Anodonta anatina	Kidney	Lab	Cd	Increase	Streit and Winter 1993
	Anodonta cygnea	Whole soft tissue	Lab	Cu	No change	Tallandini et al. 1986
		Whole soft tissue	Lab	Zn	No change	Tallandini et al. 1986
	Anodonta grandis	Gills	Field	Cd	Increase	Couillard et al. 1993, Giguère et al. 2003
		Digestive gland	Field	Cd	Increase	Couillard et al. 1993
		Remaining soft tissue	Field	Cd	Increase	Couillard et al. 1993
	(*Anodonta*) *Pyganodon grandis*	Whole soft tissue	Field	Cd	Increase	Couillard et al. 1995a
		Gills	Field	Cd	Increase	Wang et al. 1999
	Corbicula fluminea	Gill, mantle and adductor muscle	Lab	Cd	Increase	Doherty et al. 1988
		Visceral mass	Lab	Cd	Increase	Doherty et al. 1988
		Whole soft tissue	Field	Cd	Increase	Baudrimont et al. 1999
		Whole soft tissue	Field	Zn	Increase	Baudrimont et al. 1999
	Macoma balthica	Whole soft tissue	Lab	Cu	Increase	Johansson et al. 1986
		Whole soft tissue	Field	Cu	Increase	Ag Johansson et al. 1986
		Whole soft tissue	Field	Zn	Increase	Ag Johansson et al. 1986
		Whole soft tissue	Field	Ag	Increase	Ag Johansson et al. 1986
		Whole soft tissue	Lab	Cd	Increase	Bordin et al. 1994, 1997
		Whole soft tissue	Lab	Cu	Increase	Bordin et al. 1994, 1997
		Whole soft tissue	Lab	Zn	Increase	Bordin et al. 1994, 1997
		Whole soft tissue	Field	Cd	Increase	Bordin et al. 1997
		Whole soft tissue	Field	Cu	Increase	Bordin et al. 1997
		Whole soft tissue	Field	Zn	Increase	Bordin et al. 1997
		Whole soft tissue	Lab	Cd	Increase	Mouneyrac et al. 2000
		Whole soft tissue	Lab	Ag	Increase	Mouneyrac et al. 2000
		Whole soft tissue	Lab	Hg	Increase	Mouneyrac et al. 2000
		Whole soft tissue	Field	Cu	Increase	Bray et al. 1983
		Whole soft tissue	Field	Ag	Increase	Bray et al. 1983
	Mercenaria mercenaria	Kidneys	Lab	Cd	Increase	Robinson et al. 1985
	Mizuhopecten yessoensis	Gills	Lab	Cd	Increase	Evtushenko et al. 1986
		Hepatopancreas	Lab	Cd	Increase	Evtushenko et al. 1986
	Protothaca straminea	Viscera and Kidney	Lab	Cd	Increase	Roesijadi 1980
		Viscera and Kidney	Lab	Cu	Increase	Roesijadi 1980
		Viscera and Kidney	Lab	Zn	Increase	Roesijadi 1980
	Rangia cuneata	Whole soft tissue	Field	Cu	Increase	Bray et al. 1983
		Whole soft tissue	Field	Ag	Increase	Bray et al. 1983
	Ruditapes decussatus	Whole soft tissue	Lab	Cd	Increase	Bebianno et al. 1993
		Digestive gland	Lab	Cd	Increase	Bebianno et al. 1993
		Gills	Lab	Cd	Increase	Bebianno et al. 1993
		Digestive gland	Lab	Cu	Increase	Hamza-Chaffai et al. 1998
		Gills	Lab	Cu	Increase	Roméo and Gnassia-Barelli 1995
		Gills	Lab	Cd	Increase	Bebianno and Serafim 1998
	(=*Ruditapes*) *Tapes philippinarum*	Gills	Field	Cu	Increase	Irato et al. 2003

Biomarker	Specie	Tissue/Organ	Exposure condition	Studied contaminant	Change	Reference
		Digestive gland	Lab	Cd	Increase	Ng and Wang 2004
		Digestive gland	Lab	Zn	No change	Ng and Wang 2004
		Digestive gland	Lab	Ag	Increase	Ng and Wang 2004
		Whole soft tissue	Lab	Cd	Increase	Ishiguro et al. 1982
	Scapharca inoequivalis		Lab	Cd	Increase	Serra et al. 1995
AchE	*Scrobicularia plana*	Digestive gland	Field	metals/organics (PCB, PAH)	Change	Solé et al. 2009

a Adapted from Livingstone 2001
b Adapted from Amiard et al. 2006

The common cockle (*Cerastoderma edule*, Bivalvia: Cardiidae) is a filter-feeding bivalve common in the North Sea and north-east Atlantic, being widely distributed from north-east Norway to West Africa. It lives buried in the upper few centimetres of the sediment, frequently forming high populational densities, in marine and estuarine environments. High inter-individual variability of reproduction stage, parasite load, metallothionein (MT) concentration, etc. is generally observed in *C. edule* populations (Baudrimont et al. 2006). It is highly tolerant to environmental variations of physico-chemical parameters such as sediment grain size and salinity, and may thus be employed as an indicator organism along an estuarine gradient. In Sado estuary, i.e. this cockle colonizes all intertidal sediments, from the sand beach of Tróia Peninsula close to the estuarine mouth to the mudflats in the channel of Águas de Moura. *C. edule* has been tested in recent studies (Jung et al. 2006) but despite its characteristics, there are very few ecotoxicological studies with this bivalve.

The response to sediment–bound contamination and the capacity to regulate and eliminate both organic and metallic contaminants are reflected in biomarkers. Biomarkers are defined as indicators of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001) Biomarkers can be indicators of either exposure or effects. Biomarkers of exposure indicate that exposure to a chemical or class of chemical has occurred, but do not provide knowledge of toxic effects at the level of the organisms, and the biomarkers of effect reflect a deteriorating condition (Koeman et al. 1993). MT induction and histopathology are two biomarkers usually

employed. MT is a protein involved in metal (essential or not) accumulation and elimination strategies and, in many bivalves MT response has been found revealing higher sensitivity with increase of pollution (Marie et al. 2006; Serafim and Bebianno 2009). However, metals are not the only factor in the MT induction, also other factors can interfere: in *C. edule* the possible impact of the period of reproduction, the infection of digenean parasites as well as other factors related with the metabolic state of each specimen supposed to be important contributors to fluctuations in MT concentration (Baudrimont et al. 2006). Most of the histopathological lesions could be related to environmental stressors, as soft-tissue concentrations of contaminants (Gold-Bouchot et al. 1995). Gills and digestive gland in molluscs appear to be good survey organs for pollution studies (Gold-Bouchot et al. 1995; Syasina et al. 1997; Zaldibar et al. 2007, 2008), but the histopathology in *C. edule* has received little or no focus at all.

The Sado estuary, located on the west coast, is the second largest in Portugal with an area of approximately 24,000 ha. The estuary comprises the Northern and the Southern Channels, partially separated by intertidal sandbanks. Water exchange is conducted mainly through the Southern Channel, which reaches a depth of 25 meters, whereas the maximal depth of the Northern Channel is generally 10 meters. Most of the estuary is classified as a natural reserve, with a weighty ecological and landscape value. The region equally plays an important role for the leisure and recreation, and therefore, is important in the local and national economy. The city of Setúbal located in the North edge of Sado estuary, has a large resident population and an important heavy-industry in the adjacent area. The estuary is an important fishing area and many aquaculture facilities have been settled here along the past few years. The southernmost section of the estuary is mainly characterized by an important tourism-based economy. The major sources of anthropogenic contaminant input are mainly the pyrite mines in the river basin, the industries that involving pulp and paper, pesticides, fertilizers, yeast, food and shipyards (Catarino et al. 1987) along the North shore of the lower estuary and the runoffs

from extensive agriculture grounds located upstream. The results of previous studies indicate that anthropogenic sources play a major role on the elemental composition of the Sado estuarine sediments (Cortesão and Vale 1995) and, this estuary has a low contamination level with some local hotspots and a moderate potential for observing adverse biological effects (Caeiro et al. 2005).

Translocation of bivalves between areas with different levels of water and sediment contamination has long been employed for standard biomonitoring of aquatic ecosystems. These procedures have been proved to provide valuable information on the molluscs' responses and defences to contamination, with especial respect to the kinetics of xenobiotic uptake and elimination (see De Kock and Kramer 1994 for a thorough review).

The present work intends to recreate a translocation assay with *C. edule*, under controlled laboratorial conditions in order to determine the species' potential as an indicator organism for contaminated estuarine sediments and to assess the effects and responses of exposure to metallic and organic toxicants.

2 Materials and methods

2.1 Experimental assay

The sediments were collected from four different sites (designated as sites A, B, C and D) of the Sado estuary (Fig. 1) on November 2006, selected by their different levels of metallic and organic contamination. Site A is located near an environmentally protected area, the Sado estuary Natural Reserve, and is the most distant from sources of direct contamination. Due to its location in the south channel of estuary, this site has a greater influence of oceanic hydrodynamics and a lower residence time than the others. Site B is located near the port of Setúbal and site C in the industrial zone near factories for the production of fertilizers, pesticides and others (such as paper mills, thermoelectric, shipyards, etc), having been identified as potentially contaminated. They are both located in the North Channel, an area of low hydrodynamics which facilitate the retention of contaminants and fine particles of sediment coming from the upper estuary. Site D, located near aquaculture and small-scale fishery grounds consist of a lower hydrodynamics, relatively confined, area. Site D is the only one located in the intertidal zone, and the other sites (A, B and C) are located in the subtidal zone.

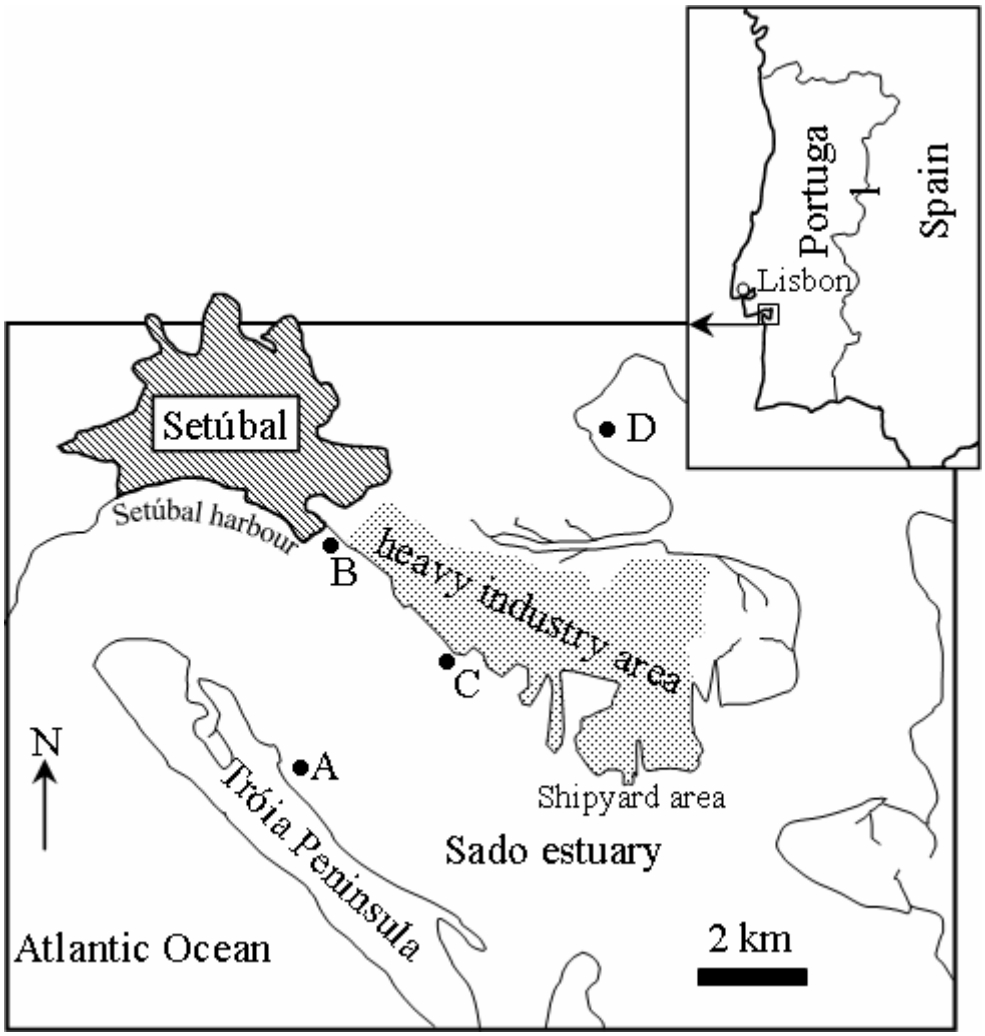

Fig. 1 - Map of the study area.

Cockles [28 ± 1.6 mm shell length, 8.0 ± 1.4 g wet weight (ww)] were collected on November 2006 from site D of the Sado estuary and acclimatized to test conditions (temperature of 18ºC and salinity of 34) in clean sand and seawater for 48h. The bivalves were exposed to the sediments (A, B and C) for 28 days through a static arrangement of bioassays (performed in duplicate). Each replicate consisted of a tank (24x11x39 cm) in which were allocated 2 L of sediment and 5 L of clean seawater. Forty animals were distributed per tank, exposed to continuous aeration and fed with commercial fish food (Avipar Lda., Portugal) during assay. Each week, 50% of the seawater was changed and the following parameters monitored: salinity, dissolved oxygen, ammonia, pH and temperature. The animals were collected and sacrificed for analysis on day 0 (T0), 14 (T14) and 28 (T28) in order to determine bioaccumulation of metallic and organic contaminants, metallothionein induction and

histopathological alterations of the digestive gland. For each test and sampling time, 20 individuals were used to determine the organic contaminants, 10 individuals to determine the metals and metallothioneins and 10 to examine the histopathology. Animals collected at T0 consisted of 15 individuals collected directly from the acclimatization tanks.

2.2 Sediments analyses

2.2.1 Physico chemical characterization

Sediment redox potential (Eh) was measured immediately after collection, using an Orion model 20A meter with a H3131 Ag/AgCl reference electrode (Orion Research Inc., USA). For the analysis of organic matter, the sediment was previously dried in stove at 60-80 °C in a and then combusted in oven at 500 ± 25 °C for 4 hours. The content of organic matter (extrapolated from total combustible carbon, TOM) is given in percent (%) sediment dry weight (dw). Fine fraction (particle size < 63 µm) was determined by sieving after treating the samples with hydrogen peroxide and disaggregation with pyrophosphate.

2.2.2 Contaminant determination

The sediments were analysed for the metals nickel (Ni), copper (Cu), zinc (Zn), cadmium (Cd) and lead (Pb), and the metalloid arsenic (As). Sediment samples (≈100 mg dw) were mineralized completely with 6 cm^3 of HF (40%) and 1 cm^3 of Aqua Regia (HCl-36%: HNO_3-60%; 3:1) in closed Teflon vials in a water bath at 100 °C during 1 h. Contents were evaporated to near dryness redissolved in HNO_3 and Milli-Q water, heated for 20 min at 75 °C and diluted to 50 mL with Milli-Q grade ultrapure water (Caetano et al. 2007). The metal

concentrations were determined in the same samples but in separate runs using a quadropole ICP-MS (Inductive coupled plasma atomic emission spectrometry) (Thermo Elemental, Xseries, USA) equipped with a Peltier Impact bead spray chamber and a concentric Meinhard nebulizer. MESS-2 (NRC, Canada), PACS-2 (NRC, Canada) and MAG-1 (USGS, USA) were the references materials used to validate the procedure and were found within the certified range. Results are given in mg kg^{-1} sediment dw.

The determination of PAHs (polycyclic aromatic hydrocarbons) was performed on a GCQ Trace Finnigan gas chromatography–mass spectrometry (GC-MS) system with a 30 m · 0.25 mm · 0.25 lm film thickness DB-5 MS column (Argilent, USA) in selected ion mode (SIM) (Martins et al. 2008). Seventeeen three- to six-ring PAHs were quantified. For PCB (polychlorinated biphenyls) and DDT (dichloro-diphenyl-trichloroethane) analysis, dry sediment samples and glass-fibre filters with suspended particulate matter were Soxhlet extracted with n-hexane for 16 h. The extracts were cleaned up with Florisil and sulfuric acid (Ferreira et al. 2003). Eighteen PCB congeners and pp'DDD, pp'DDE and pp'DDT as total DDT were analysed. The SMR 1941b reference sediment (NIST, USA) was used to validate of the analysis and were found within the certified range. The detection limit was 0.01 ng.g-1. All concentrations are expressed in ng g^{-1}.sediment dw.

The PEL quotient (PEL-Q) was calculated to evaluate the impact potential for observing adverse biological effects of the tested sediments. This quotient is based on the published guideline values for coastal waters, namely the threshold effects level (TEL) and the probable effects level (PEL) (MacDonald et al. 1996). These guidelines have been largely used in estuarine sediment ecological risk assessment studies. This index was calculated for all contaminants of each sediment as given by the formula (Long and MacDonald 1998):

Note: the quantification of organic conaminants in sediments (PAHs, PCBs and DDTs) by GC-MS was performed by the Instituto Nacional dos Recursos Biológicos (IPIMAR_INRB)

$$PEL - Q_i = \frac{C_i}{PEL} \quad [1]$$

where PEL is the guideline value for the contaminant *i* and C_i the measured concentration of the contaminant in the surveyed sediment. The sediment quality guideline quotient indice (SQG-Q) was calculated to compare the four sites impacted by mixtures as described by Long and MacDonald (1998):

$$SQG - Q = \frac{\sum_{i=1}^{n} PEL - Q_i}{n} \quad [2]$$

where PEL-Q_i is the indice deriving from [1] for the contaminant i and n the number of contaminants under analysis. Stations were scored according to their overall potential of observing adverse biological effects, as proposed by MacDonald et al. (2004): SQG-Q < 0.1 - unimpacted; 0.1 ≤ SQG-Q < 1 - moderately impacted; SQG ≥ 1 - highly impacted.

2.3 Organism analysis

2.3.1 Bioaccumulation

For the analysis of metals, whole-body individual samples were dried (0.025 ± 0.003 g dw) in borosilicate, lead free, glass vials at 60 ºC during 5 days and then transferred to Teflon vessels adding 5 ml nitric acid (65%) to digest for 24 hours at room temperature. They were placed in a water bath at 95 ºC during 4 hours, then 1 ml hydrogen peroxide (30% v/v) was added and Teflons were placed in the water bath for another hour (Clesceri et al. 1999). Finally, the samples are stored in HDPE plastic bottles (25 ml) after elution with Mili-Q water and were kept at 4 ºC until reading. The quantification of the concentrations of metals (Ni, Cu, Zn, Cd

and Pb) and metalloid (As) was determined using ICP-MS. The organic contaminants were determined in the same sample by GC-MS after soxhlet extraction (3- to 6 ring PAH, 18 PCB congeners plus pp'DDD, pp'DDE and pp'DDT as total DDT). Quantification was carried out similarly to the procedure described in the sediments, adapted to biological tissue (Martins et al. 2008).

2.3.2 Metallothionein induction

Metallothionein induction was determined by quantification of thiols in whole soft tissue samples according to Costa et al. (2008). In brief: samples were homogenized in Tris-HCl 0.02 M buffer (pH 8.6). Homogenates were centrifuged at 17,000 rpm at 4 ° C for one hour. The supernatant was heated in a water bath at 80 °C for 10 minutes to destroy the proteins with less thermal stability, and were centrifuged as previously described. Finally, the metallothioneins were quantified from heat-treated cytosols by differential-pulse polarography with a static mercury-drop electrode (DPP-SMDE) using a 693 VA processor and a 694 VA stand (Metrohm, Herisau, Switzerland). In absence of a commercial form of bivalve MT, Rabbit MT isoforms I & II (Sigma, St Louis, MO, USA) was used for the standard addition method.

2.3.3 Histopathology

Cockles were fixed in Bouin-Holland's solution (27% formaldehyde, 7% acetic acid, and picric acid until saturation) for approximately 48 hours at room temperature. Afterwards, the samples were washed with water for 24 hours to remove the excess picric acid, dehydrated in

---

Note: the quantification of organic conaminants in organisms (PAHs, PCBs and DDTs) by GC-MS was performed by the Instituto Nacional dos Recursos Biológicos (IPIMAR_INRB)

a progressive series of ethanol, an intermediate embedding with xylene (≈ 100%) was carried out. Samples were then embedded in paraffin for about 12 hours. The blocks were cut in sections of 5 µm and then stained with haematoxylin and eosin (H & E) (Martoja and Martoja 1967) and mounted with DPX resin (BDH).

2.4 Bioaccumulation and biota-sediment accumulation factors

The bioaccumulation factor (BAF) and the biota-to-sediment accumulation factor (BSAF) were measured regarding the metals (Ni, Cu, Zn, Cd and Pb), metalloid (As) and organic contaminants (PAHs, PCBs and DDTs). The BAF was calculated after 14 and 28 days of exposure according to the formula (Lee 1992):

$$BAF = \frac{C_o}{C_s} \quad [3]$$

The BSAF after 14 and 28 of exposure = BAF normalized to the organic carbon content in the sediment (adapted from formula of USEPA 1995):

$$BSAF = \frac{C_o}{\left(\frac{C_s}{TOC}\right)} \quad [4]$$

where C_o was contaminant concentration in organism expressed in $mg.kg^{-1}$ dry weight of tissue, C_s was contaminant concentration in sediment expressed in $mg.kg^{-1}$ dry weight of sediment, and TOM was expressed in % Total Organic Matter of sediment on a dry weight basis.

2.5 Statistical analysis

The non-parametric tests Kruskall-Wallis H and Mann-Whitney U were employed to assess global and pairwise statistical differences, respectively. The Chi-square predicted x observed test was applied to assess significant differences between the concentrations of organic contaminants (in sediment and bioaccumulation) of all tests and sampling points. The non-parametric Spearman's Rank Order Correlation ρ statistic was used to assess the correlation between BAFs/BSAFs and metallothioneins. A significance level of 5% was set for all analyses. All the statistical results were obtained using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA), version 15.0.1.

3 Results

The values obtained of the parameters monitored each week were under standard conditions. Values were the following: salinity = 34 ± 1, dissolved oxygen = 42 ± 2 %, ammonia < 0,5 mg L-1, pH = 7.8 ± 0.1, and temperature = 18 ± 1 ºC.

3.1 Physical characterization of sediments

There is a linear relation between FF and TOM content in sediments from the 4 sites (Fig. 2).

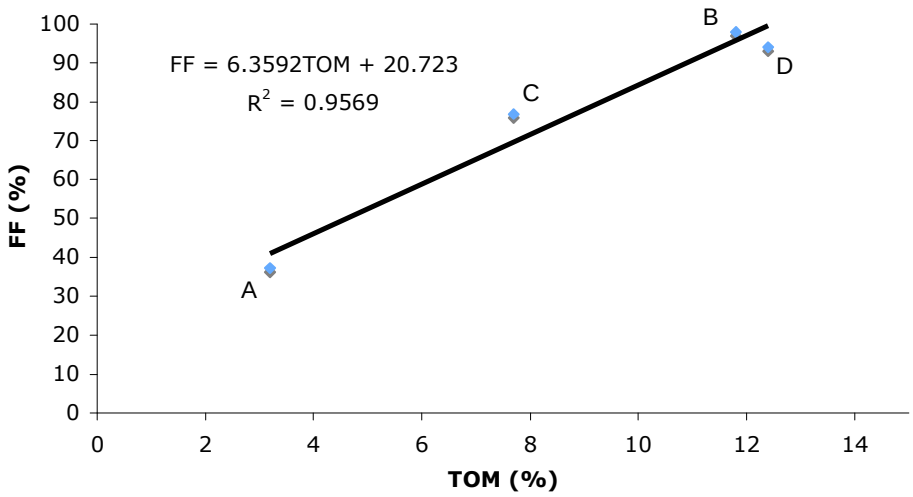

Fig. 2 - Linear relation between FF and TOM for all sediments tested.

The sediment A has less FF and TOM. The other sediments have high FF and TOM, the grain size of B and D is almost entirely FF (97,9 and 94,1 % respectively) and have a high organic matter content (11,8 and 12,4 % respectively).

Redox potential is negative for all sites, being C the most reduced sediment. D is the only intertidal sediment and it is the less reduced (Table 2).

Table 2 - Characterization of sediments from sites D, A, B, and C.

Site	FF[a] (%)	TOM[b] (%)	Eh[c] (mV)
D	94.1	12.4	-187
A	37.3	3.2	-233
B	97.9	11.8	-290
C	76.8	7.7	-316

a Particle size < 63 µm
b Total organic matter
c Redox Potential

3.2 Contaminants in sediments

The results of the metal and organic concentrations on sediments from the 4 sites are presented in Annex I. The sediments B and D present higher concentrations of metals and metalloid (As), with values above TEL for all metals and metalloid except Cd, highlighting concentrations of Zn and Cu above PEL in sediment B. As, Cu and Zn present values above TEL on sediment A and As, Cu and Pb present values above TEL on sediment C (Fig. 3). Values of tPAHs obtained decreased in the following order on sediments: C>B>D>>>A. Concentrations of 4- and 5-ring PAHs were higher on all sediments. Values above TEL were not found in the sediment A, and only a few compounds of 3-, 4- and 5-ring PAHs had concentrations above TEL on sediments B, C and D (Fig. 4 and 5). Values of tPCBs obtained, decreased in the following order on sediments: C>>B>D>A, with any value above TEL (Fig. 6). The levels of the organic contaminants analysed were irrelevant in the sediment A compared with other sediments; PCB-26 (tri-chlorinated) was the congener with higher concentration on sediment D; penta-, hexa- and hepta-chlorinated were the higher concentrations on sediment B; and penta- and hexa-chlorinated on sediment C. Congeners

with the highest concentration on the sediment C were PCB-101 and PCB- 118 (penta-chlorinated), and PCB-138, PCB-151 and PCB-153. The values of tDDTs obtained decreased in the following order on sediments: B>C>A>D. Sediment D presents very low concentrations of tDDTs, pp'DDE was the only above detection limit. pp'DDT were the forms with the highest concentrations on sediments A, B and C, particularly on sediment B (Fig. 6), being the major metabolite of tDDTs. Value obtained of SQG-Q for the 4 sediments follow the sequence (from worst to best sediment quality): B>D>C>A. Due to the weight of SQG-Q is mainly metallic for all sediments, SQG-Q discriminated calculated for metals follow the same sequence as for the total, but those calculated for organics: B>C>D>A.

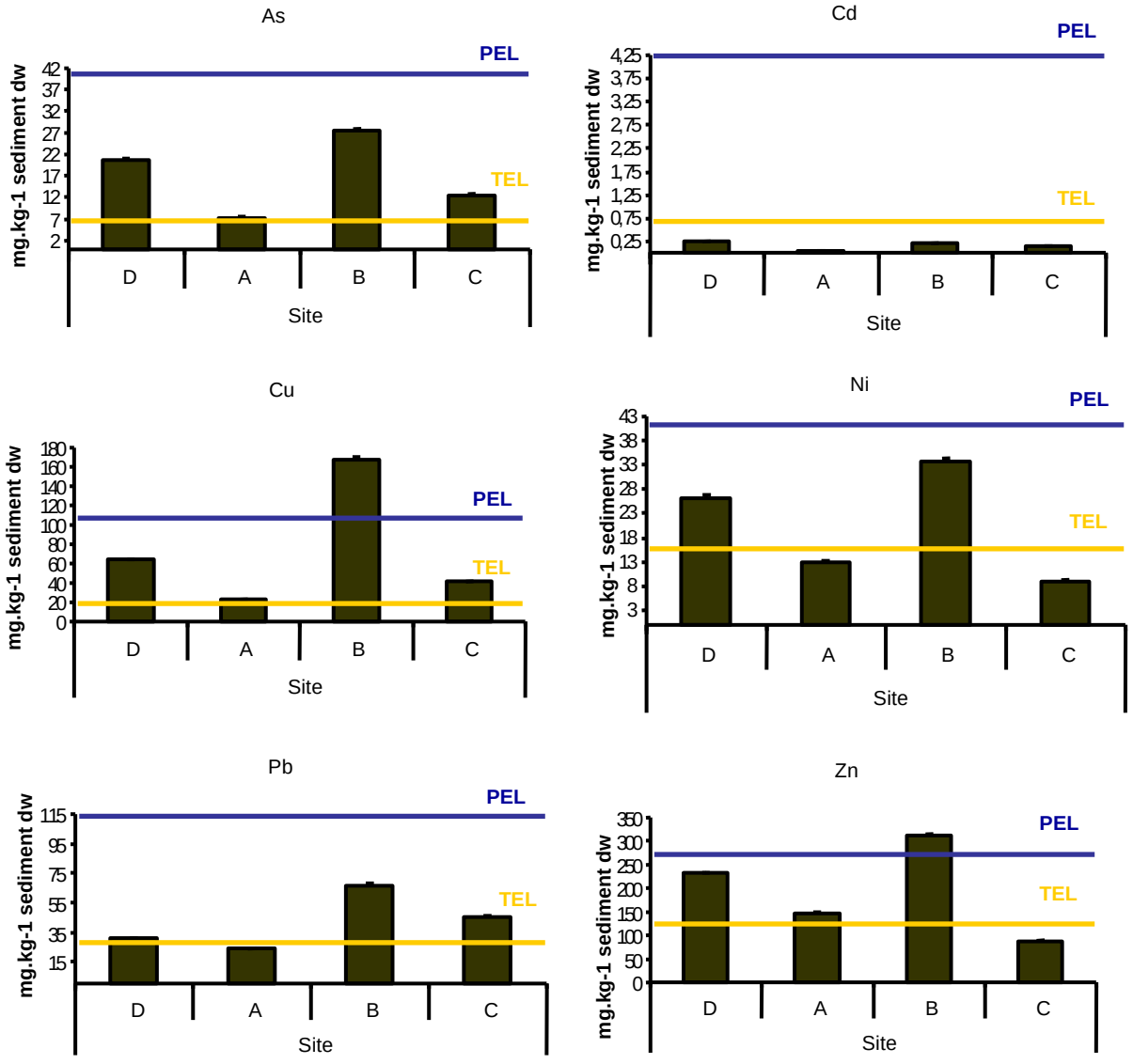

Fig. 3 - Metal concentrations of sediments from sites D, A, B and C and TEL and PEL for each metal and metalloid.

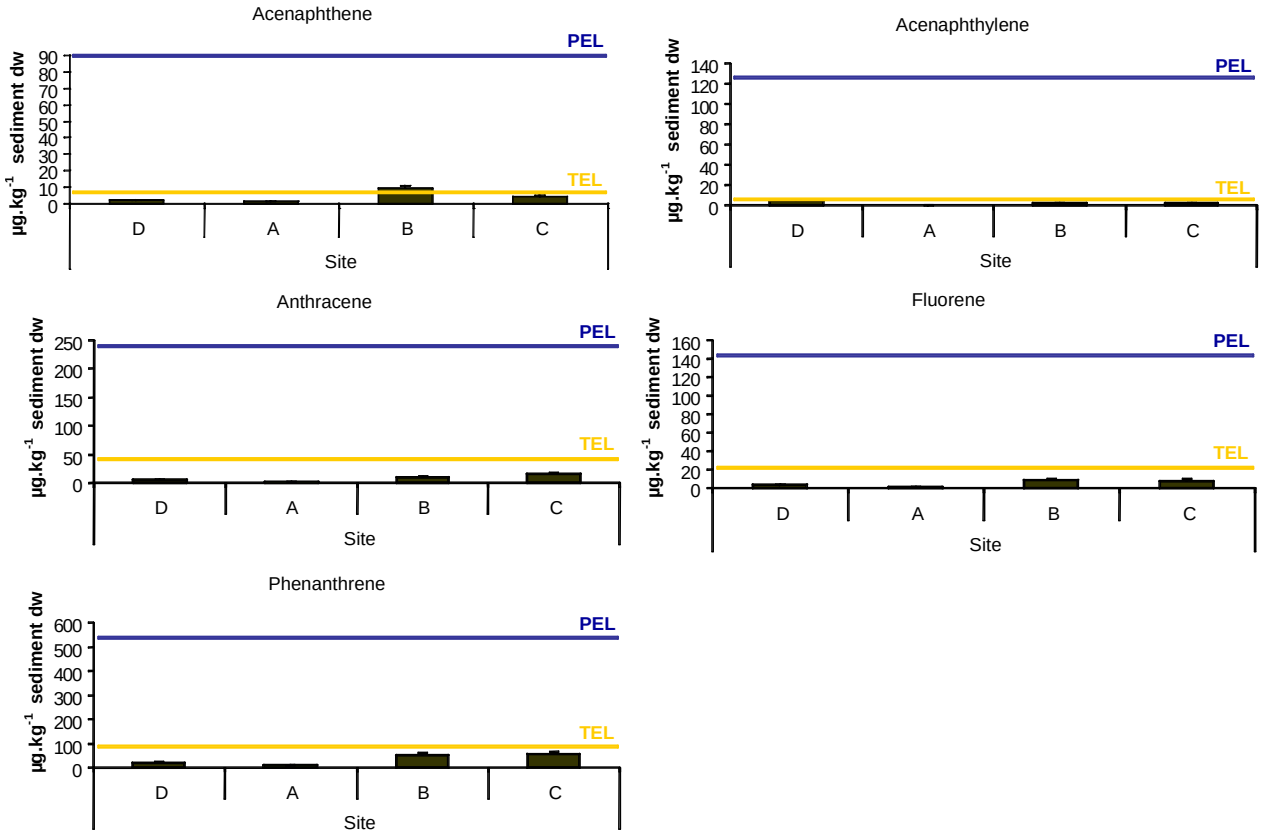

Fig. 4 - Concentrations of 3- ring PAHs of sediments from sites D, A, B and C and TEL and PEL for each compound.

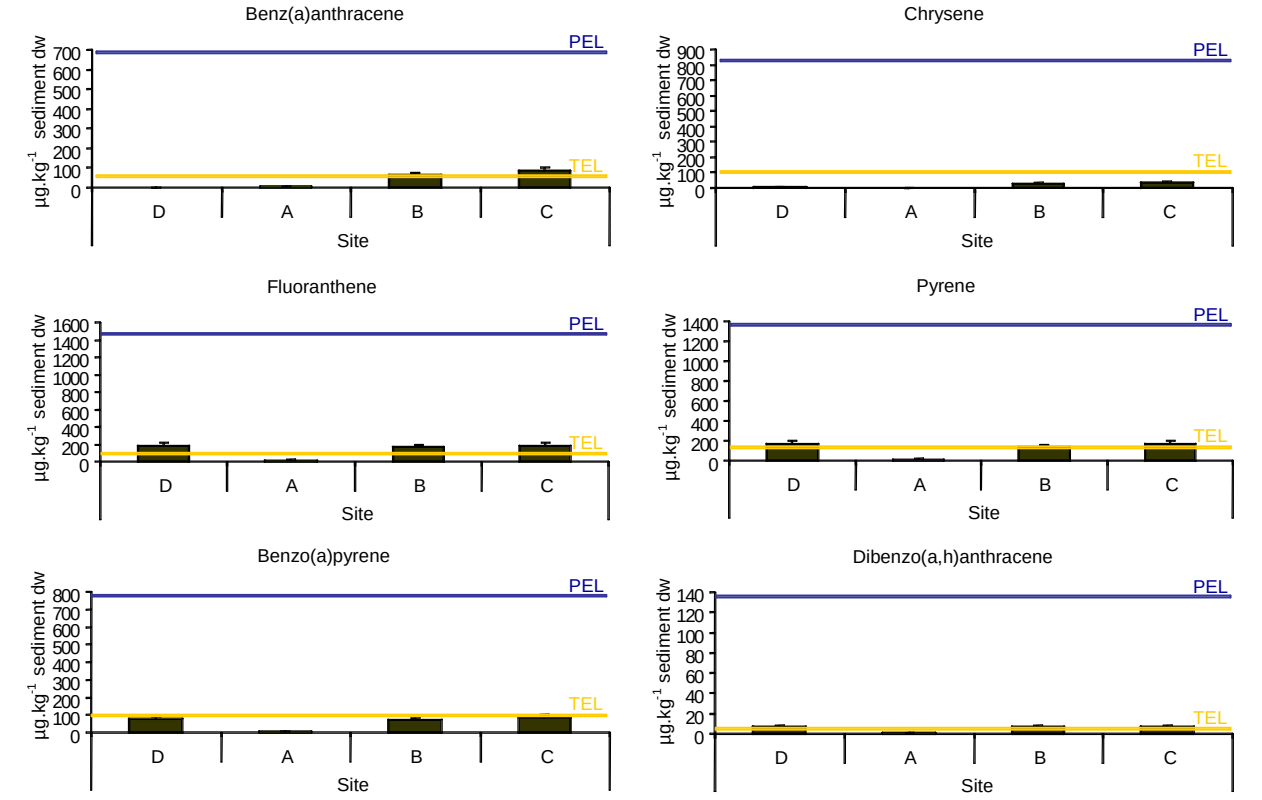

Fig. 5 - Concentrations of 4- and 5- ring PAHs of sediments from sites D, A, B and C and TEL and PEL for each compound.

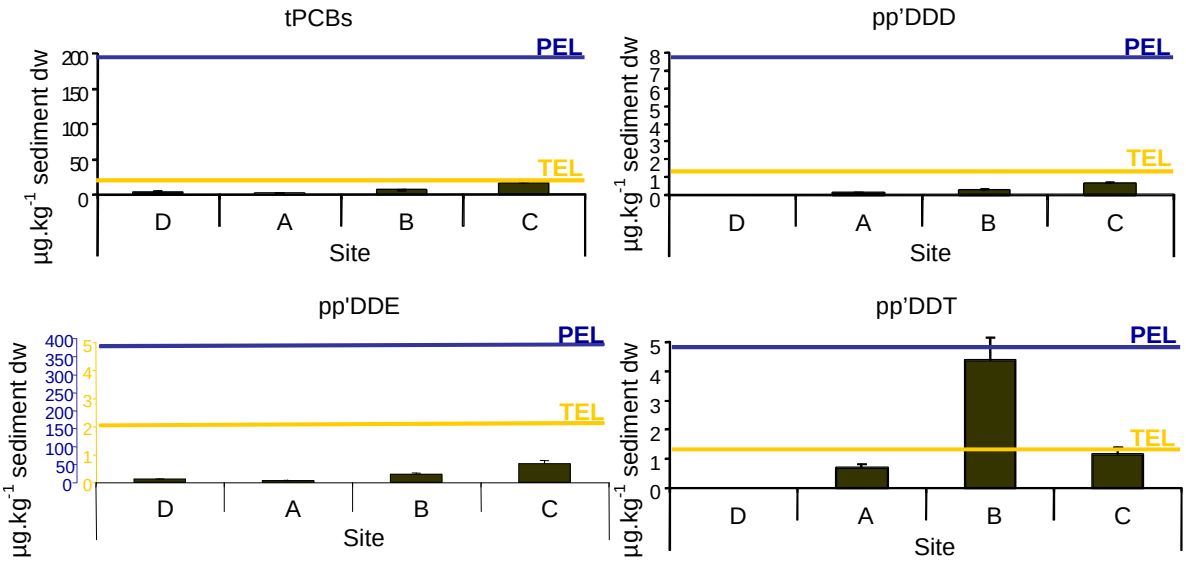

Fig. 6 - tPCB and PAH (pp'DDD, pp'DDE and pp'DDT) concentrations of sediments from sites D, A, B and C and TEL and PEL for each compound.

3.3 Bioaccumulation and metallothioneins in *C. edule*

The results of metallothioneins and bioaccumulation in *C. edule* are present in Annex II. Significant decrease of metallothioneins over time stands out in organisms of the sediment C (Fig. 7).

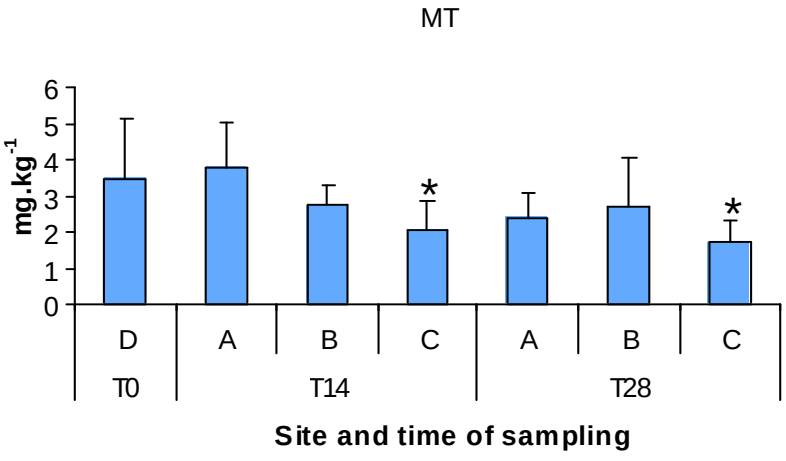

Fig. 7 - Concentration of metallothioneins in *C. edule* after 14 and 28 days of exposure to all sediments.

* indicates significant differences (ρ<0,05) between tests and D (Mann–Whitney U test)

There is a great variability of the bioaccumulation of metals in each individual and over time. Main rates of bioaccumulation are found in organisms of sediment B, with statistically significant differences in As ($p < 0,01$), Pb ($p < 0,05$) and Zn ($p < 0,01$) (Fig. 8 and 9).

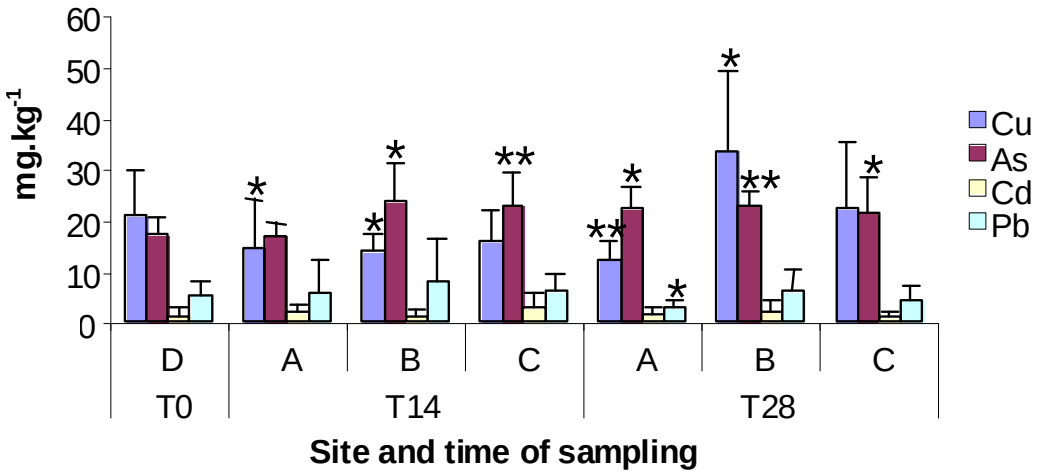

Fig. 8 - Bioaccumulation of Cu, As, Cd and Pb after 14 and 28 days of exposure to all sediments.

* and ** indicate significant differences ($p < 0,05$ and $p < 0,01$, respectively) between tests and D (Mann–Whitney U test)

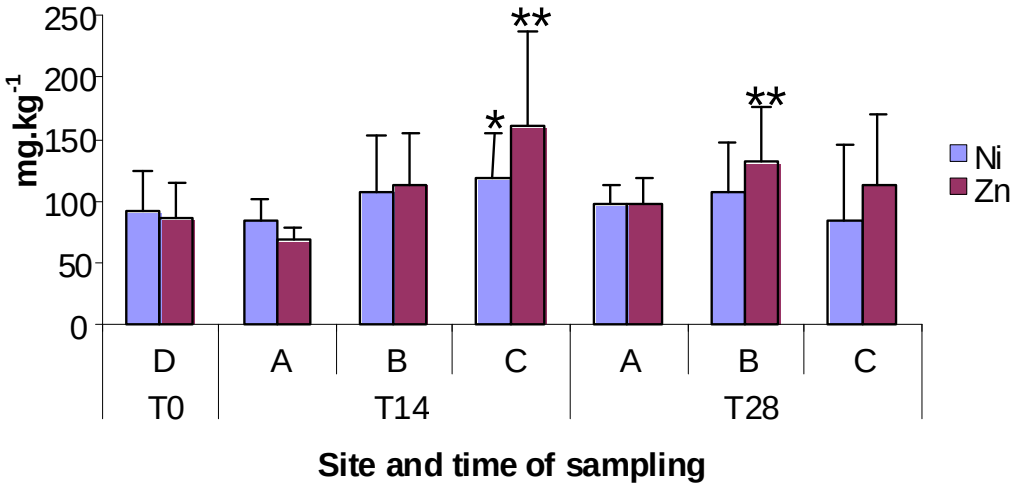

Fig. 9 - Bioaccumulation of Ni and Zn after 14 and 28 days of exposure to all sediments.
* and ** indicate significant differences ($p < 0,05$ and $p < 0,01$, respectively) between tests and D (Mann–Whitney U test)

3- and 4-ring were the major compounds identified in PAHs, and they were bioaccumulated in sediment C with statistically significant differences ($p < 0,01$). PCBs were bioaccumulated in all sediments. Tri-chlorinated had the main weight in tPCBs in the sediment B and T28 in the

sediment C, and penta-chlorinated in T14 in the sediment C. DDTs were only bioaccumulated in T28 in the sediment B with statistically significant differences ($p < 0,05$). pp'DDT had the main weigh in tDDTs (Fig. 10).

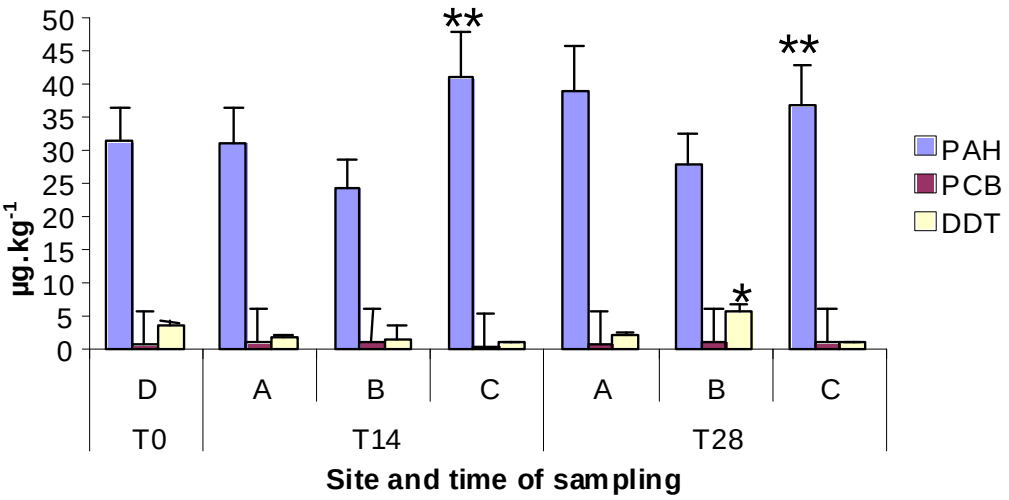

Fig. 10 - Bioaccumulation of organic contaminants after 14 and 28 days of exposure to all sediments.

* and ** indicate significant differences ($\rho<0,05$ and $\rho<0,01$, respectively) between tests and D (chi-square test)

3.4 BAFs and BSAFs

The bioaccumulation factor and the biota-sediment accumulation factor are presented in the Annex III. On sediment A, BAFs for all contaminants (except DDTs) were higher than in the sediment D. On sediment C, BAFs for all metals (except Pb) were higher than in sediment D and BAFs for PCBs were extremely lower than in the other sediments.

BSAFs were lower for organic contaminants on sediments A, B and C than in D. In general, they were lower also for metals, except Cd, and PAHs for sediment A, Cd and PCBs for sediment B, and As, Cd, Ni and Zn for sediment C. Both factors for Cd and BSAF for PAHs

were highly correlated to MT induction ($\rho = 0.943$, $p < 0.01$ and $\rho = 0.886$, $p < 0.05$ respectively) (Fig. 11 and 12), revealing a positive interaction between the contaminants.

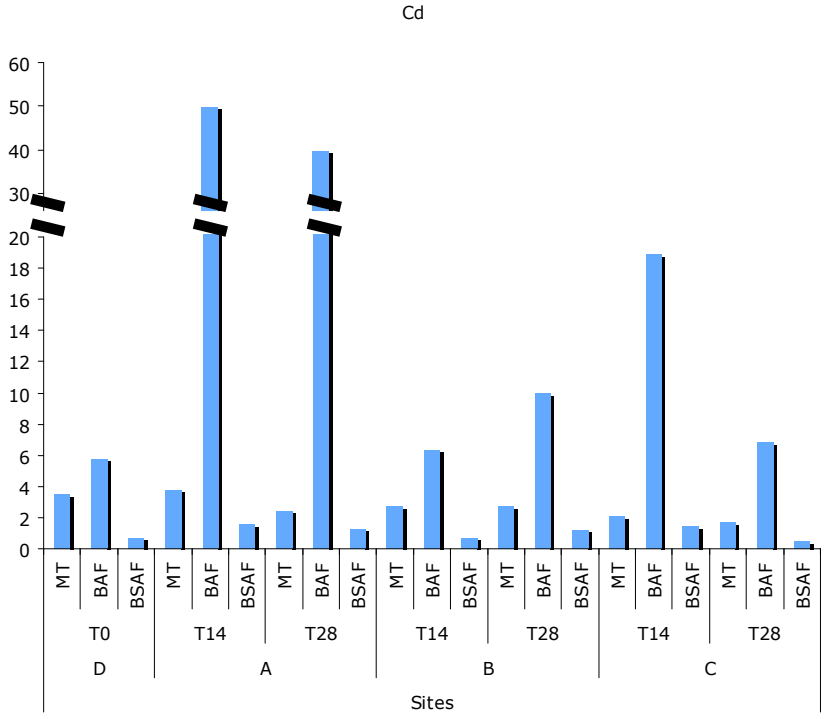

Fig. 11 – MT (mg.g^{-1} whole soft tissue dry weight) and BAFs and BSAFs for Cd after 14 and 28 days of exposure to all sediments.

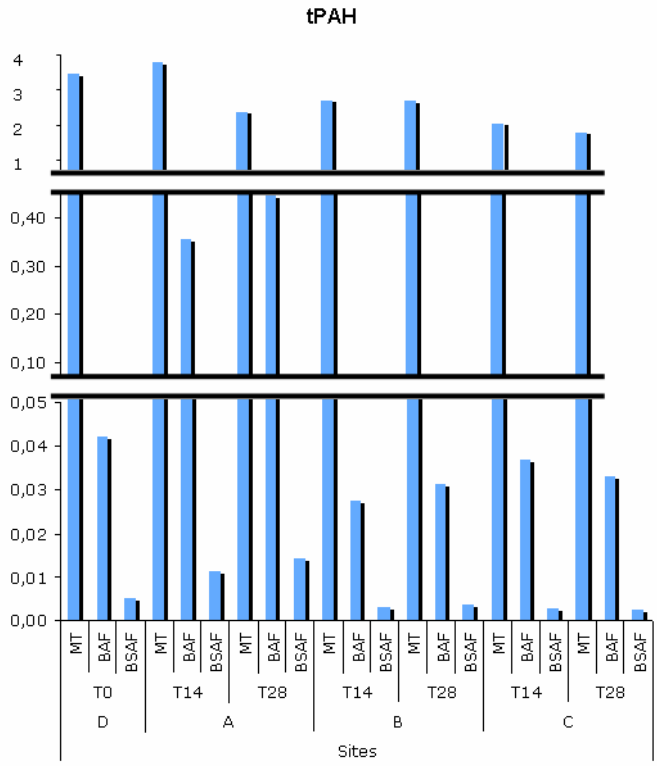

Fig. 12 - MT (mg.g^{-1} whole soft tissue dry weight) and BAFs and BSAFs for tPAHs after 14 and 28 days of exposure to all sediments.

3.5 Histopathology

The Fig. 13 shows the digestive gland in cockles from sediment D in T0. The digestive gland apparently seems normal, without alterations. In comparison with the sediment D, the digestive gland of cockles from other sediments showed significant differences. Degradation in the digestive gland tubule integrity is shown in the organisms from all sediments, except the sediment D. The histological damages are present in most organisms and varied depending on sediment and time of exposure. In general, there is a decrease of connective tissue (Fig. 14C, D, Fig. 15A-D, Fig. 16A-D). The excretory cells increased slightly in sediment A (Fig. 14A, B) and highly in sediment C (Fig. 16A). The cells of tubules detached from epithelium are identified in the cockles from sediment B, that is the most contaminated (Fig. 15A-D) and at day 28 from sediment A (Fig. 14C, D) and C (Fig. 16C, D), where the level of contaminants are lower. Increase of lumen size of the tubules of digestive gland is observed in cockles from sediment C (Fig. 16B). Occasionally, hyperplasia of epithelial cells was found in sediment B (Fig. 14A).

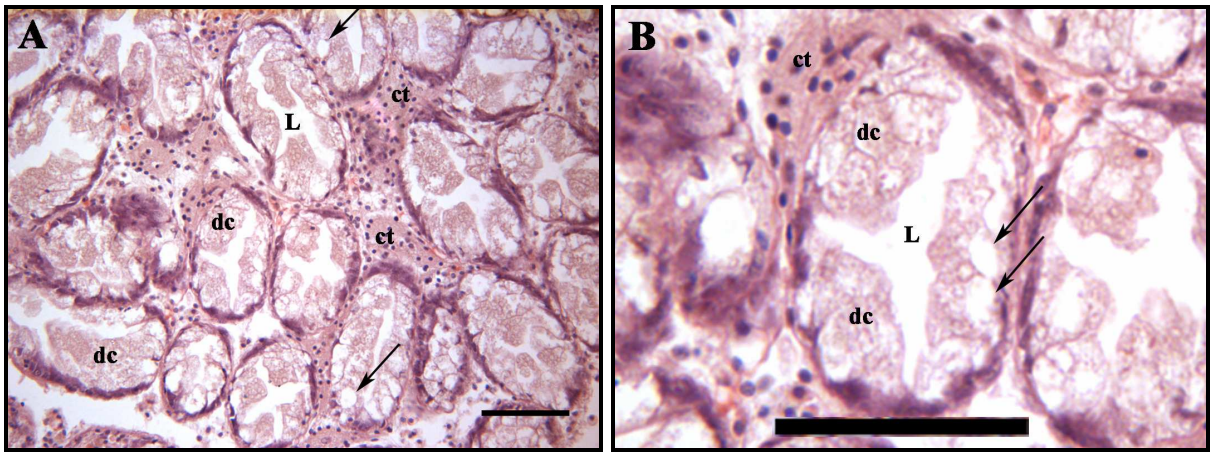

Fig. 13 - Histological sections stained with haematoxylin and eosin of digestive gland of *C. edule*. Scale bar: 50 μm.

Image A: cockles collected from site D. they show the digestive gland without obvious alterations, high connective tissue content (ct), and lumen (L), digestive cells (dc), excretory cells (arrows), epithelium and cells of digestive gland tubules intact. Image B: is an enlargement of image A. It shows a digestive gland tubule, where the lumen (L) and connective tissue (ct) are intact, and the digestive cells (dc) and excretory cells (arrows) are easily distinguished.

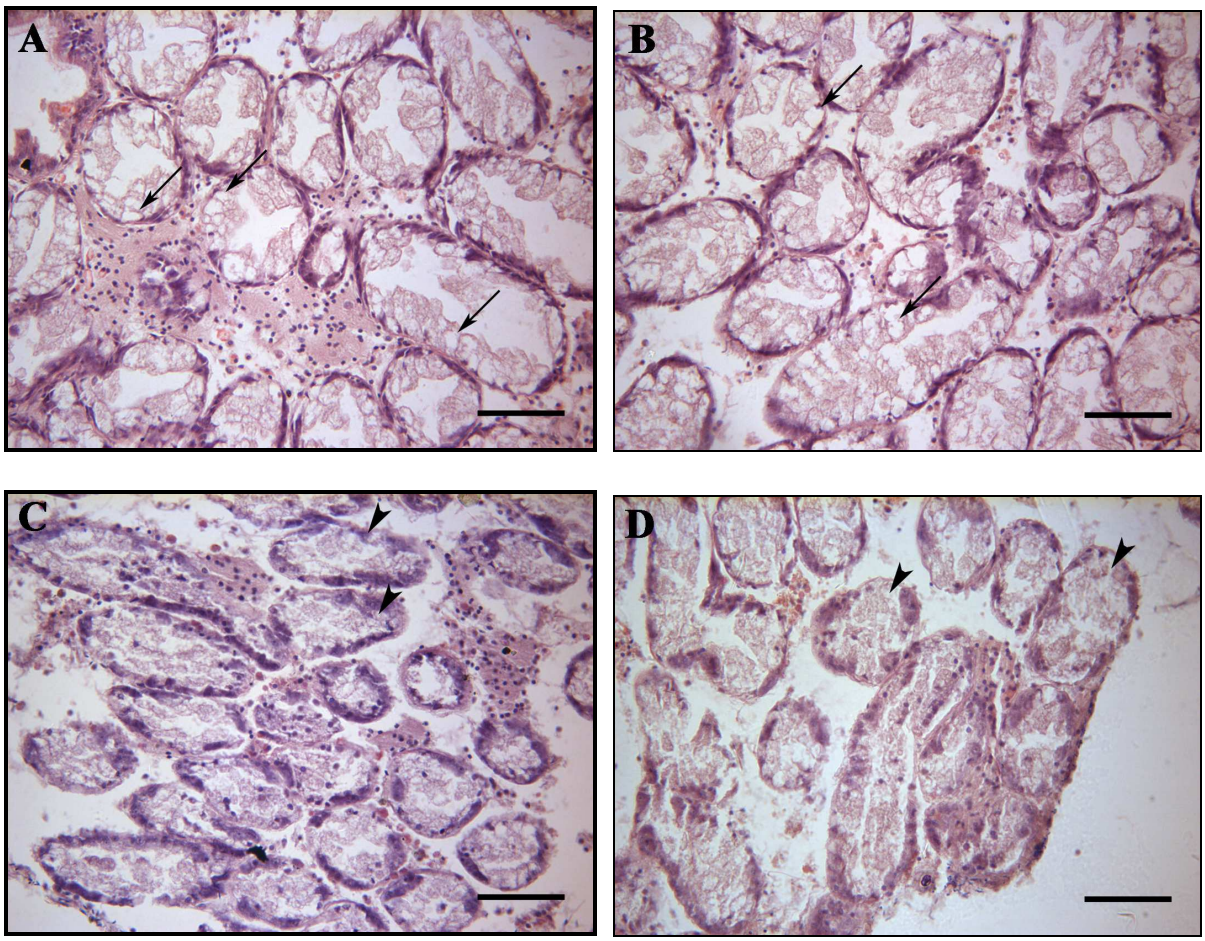

Fig. 14 - Histological sections stained with haematoxylin and eosin of digestive gland of *C. edule*.

Images A and B: from site A on day14. The number of excretory cells (arrows) increased slightly but the digestive glands seem still in good condition. Images C and D: from site A on day 28. It shows intact epithelium and significant damages: disaggregation and undifferentiated cells (arrowheads), decreased in connective tissue. Scale bar: 50 μm.

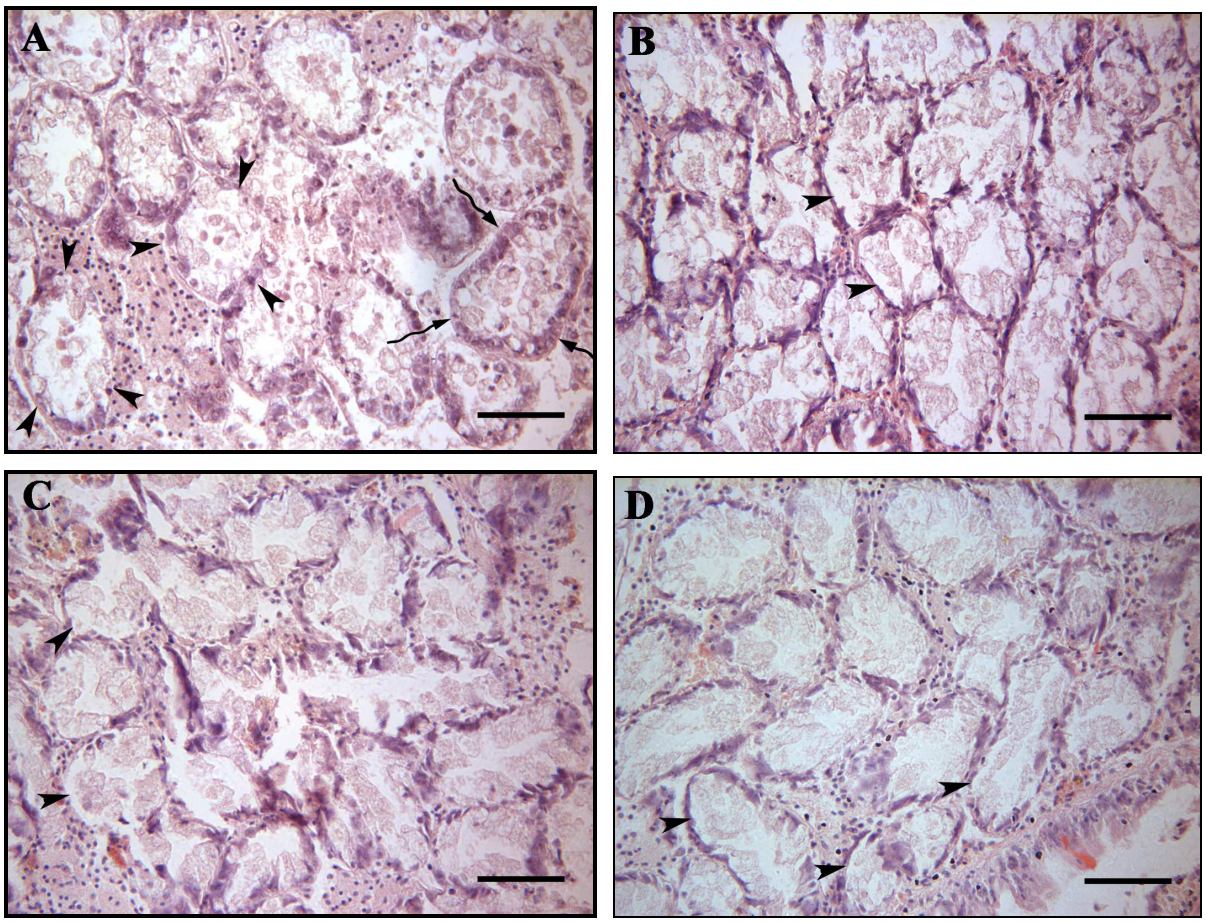

Fig. 15 - Histological sections stained with haematoxylin and eosin of digestive gland of *C. edule*.

Images A and B: from site B on day 14. There is an obvious degradation in the digestive gland integrity, cells of tubules detached from epithelium (arrowheads), slight decrease of connective tissue and occasionally hyperplasia of epithelial cells (arrowcurves). Images C and D: from site B on day 28. Slight decrease of connective tissue and cells of tubules detached from epithelium (arrowheads). Scale bar: 50 μm.

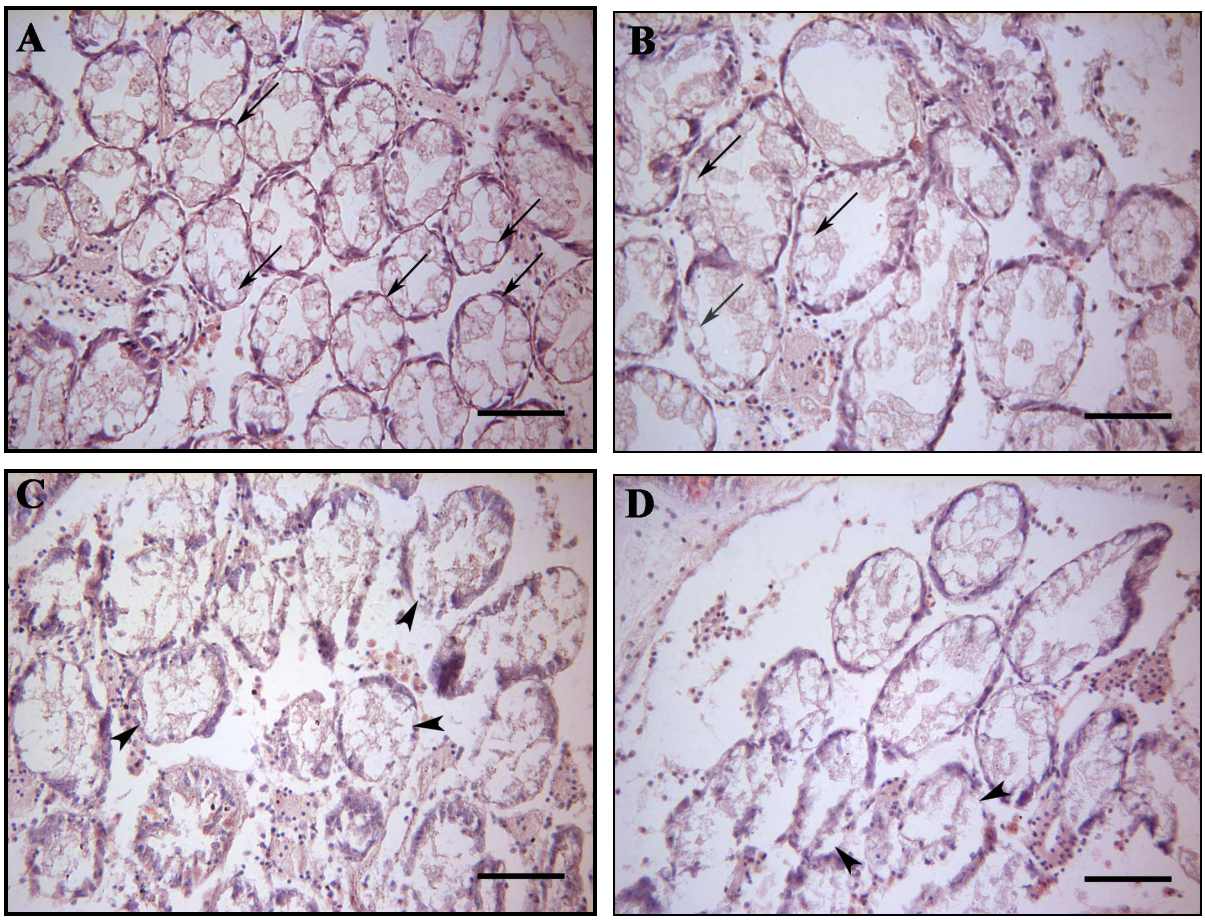

Fig. 16 - Histological sections stained with haematoxylin and eosin of digestive gland of *C. edule*.

Images A and B: from site C on day 14. Image A shows epithelium of tubules intact but almost no connective tissue and high increase of excretory cells (arrows). Image B shows the number of excretory cells (arrows) increased slightly and the lumen size of the tubules increased significantly. Images C and D: from site C on day 28. The histological damages are very obvious: degradation in the digestive gland tubule integrity, in which may be observed loss of the connective tissue and epithelial tissue structure and epithelial lifting from tubule basal laminae (arrowheads). Scale bar: 50 μm.

4 Discussion

In our study, BAF values generally presented a similar evolution, decreasing when sediment TOM increased. A similar results was observed in a study of bioaccumulation of atrazine and chlorpyrifos in the land worm *Lumbriculus variegatus* (Jantunen et al. 2008). An exception, however, was observed regarding the organic contaminants. BAF of PCBs in the sediment C is much lower than in other sediments, this may be due to the existence of a higher concentration of PCBs in the sediment and cockles maintain the same capacity for assimilation of PCBs, regardless of the initial concentration in the sediment. On the other hand, the opposite was observed with PAHs and DDTs. The concentration of PAHs in the sediment A was lower than in the other sediments and therefore, BAF of PAHs was much higher. BAF of DDTs in sediment D was observed to be higher although the concentration of DDTs in this sediment was very low. BAFs were more elevated in Cd and Ni (>> 1) and DDTs (almost always > 1). For metals, BSAF value was always lower in sediment A (except for Cd) and for organics it was always lower in sediment C. Theoretically, if the bioavailability of contaminants depends only on the existence of a perfect correlation between contaminant concentration and TOM, BSAF should be constant, but for the other sediments, the BSAF values obtained were very variable. The large variability found in BSAF values may be explained by the different quality of organic matter contents, sorption behavior and other physico-chemical parameters affecting to the bioavailability of contaminants. Variable values of BSAF have also been found in the bioaccumulation to *Lumbriculus variegatus* (Jantunen et al. 2008) and on the bioaccumulation of cadmium and BDE-99 by Baltic Sea benthic invertebrates (Thorsson et al. 2008). Significant positive correlations were found between BAF and BSAF of Cd and MT. This indicates that cockles respond not only to the concentration of Cd bioaccumulated but also to the relationship between the concentration of Cd in the organism and in the sediment, i.e. the concentration at which they are exposed. This was also verified in a study with the metalloid arsenic in the bivalve *Corbicula fluminea*

(Costa et al. 2009). Significant positive correlations were found between BSAF of PAHs and MT, being this response probably related with oxidative stress in cockles.

The time-of-exposure factor is known crucial for the bioaccumulation of contaminants (see Luoma and Rainbow 2005). Cockles may need an adaptation period to reach the limit of accumulation in relation to the concentration in the sediment. This is reflected in the total values of BAFs for all sediments. The sediment A is the least polluted (SQG-Q = 0.082) and has a total BAFs higher, and the sediment B is the most contaminated (SQG-Q = 0.313) and has a total BAFs lower than the others. The sediment D is the second more contaminated (SQG-Q = 0.181) but has BAF slightly below to sediment A. The cockles came from sediment D, where they were exposed to local contamination throughout their lifes and it is possible that a steady state could be attained between the levels of contaminants in the districts compartments of the ecosystem.

Translocation from one site to another caused significant damages to the cockles' digestive gland. The histological analysis identified different responses of the organisms from different stations. In a study of histopathological effects of petroleum hydrocarbons and heavy metals on the American oyster, they related the histopathological lesions to certain contaminants but also to salinity (Gold-Bouchot et al. 1995), this can be verified in our study because the histopathological damages were found in the cockles from all sediments where they were translocated, as the sediment A and C have less contamination that the sediment D according to the SQG-Q calculated. Early lesions (day 14) and higher intensity are presented in the cockles from sediment B and C; these sediment have higher contamination that the sediment A, where the lesions are present only at day 28. This could indicate a greater effect caused by contaminants than by other factors. The sediment A has a SQG-Q below 0.1, so that contamination should not cause impact (MacDonald et al. 2004). However in several studies it is verified that stress in molluscs provokes enhanced excretory activity in digestive cells and

due to this, the cell-type composition of the digestive gland epithelium may result severely altered (Zaldibar et al. 2008).

The slight increase in the number of excretory cells in cockles from sediment A at day 14 (Fig. 14A, B) could be due to the low level of contaminants (SQG-Q < 0.1) but at day 28 the excretory cells are rarely identified due to the presence of severe lesions. Decrease of connective tissue and dissagregation and unidentified cells were presented (Fig. 14C, D). These damages do not appear to be caused by the contaminants, since they are in relatively small quantities, unless non analysed chemicals were present in this sediment or due to the local low TOM and FF, the bioavailability was higher. The highly increased of the number of excretory cells in cockles from sediment C at day 14 (Fig. 16A, B) seems to be caused by contaminants. This sediment has SQG-Q > 0.1, and according to MacDonald et al.(2004) this could have a moderate impact in organisms. At day 28, the damages presented were loss of epithelial tissue structure and epithelial lifting from tubule basal laminae (Fig. 16C, D), there is an evident degradation in the digestive gland tubule integrity, so the excretory cells are hardly identified. In cockles from sediment B, histological damages were very pronounced at day 14 and 28. There is an early degradation in the digestive gland integrity, cells of tubules detached from epithelium and slight decrease of connective tissue (Fig. 15A-D) and occasionally hyperplasia of epithelial cells (Fig. 15A). This could be due to the sediment which has SQG-Q higher than the other sediments but below 1, so that contaminants would have a moderate impact.

Induction of MT is usually related with the metal concentrations. However, metals are not the only factor in the MT induction, also other factors can interfere. In a study with *Corbicula fluminea*, MT transcripts were higher in the digestive gland and the gills of bivalves collected in July, where the result could be linked to the increasing metabolic activity related to the seasonal temperature elevations (Bigot et al. 2009). Additionally, in a study with *C. edule*, it

is suggested that parasite infection in cockles can modulate MT synthesis that could consequently interfere with the response of these protective proteins in case of metal contamination. They monitored MT concentrations in cockles and they demonstrated that MT levels were higher in parasitized individuals (Baudrimont et al. 2006). Cockles were dying after a few months in systems of acclimatization, probably due to the development of parasites found in these animals (Fig. 17). Therefore, MT induction is related to environmental stressors. In our study, there was a decrease of MT levels in cockles from all sediments and, the most significant decrease was been observed in organisms exposed to sediment C. However, significant positive correlations between BSAF and MT were found for PHAs, and between both BSAF and BAF and MT for Cd, which is in general accordance with the known high inducibility of MT by this metal. For instance, in a study of Cd and Zn bioaccumulation, *C. fluminea* revealed variations in MT concentrations strongly correlated to progressive Cd and Zn bioaccumulation (Marie et al. 2006). On the other hand, the positive correlation between Cd BSAFs and BAFs to MT suggests MT induction is dependent of the availability of strong MT inducers, which adds up to yet another factor contributing to the variability in MT responses as suggested by other surveys (Costa et al. 2008). The reduction in MT contents, conversely, may be, at least partially, explained by the complex effects of contaminant interactions. PAHs, for instance, found in the tested sediments, have been found to suppress MT synthesis in prevence of strong metal inducers (Risso–De Faverney et al., 2000).

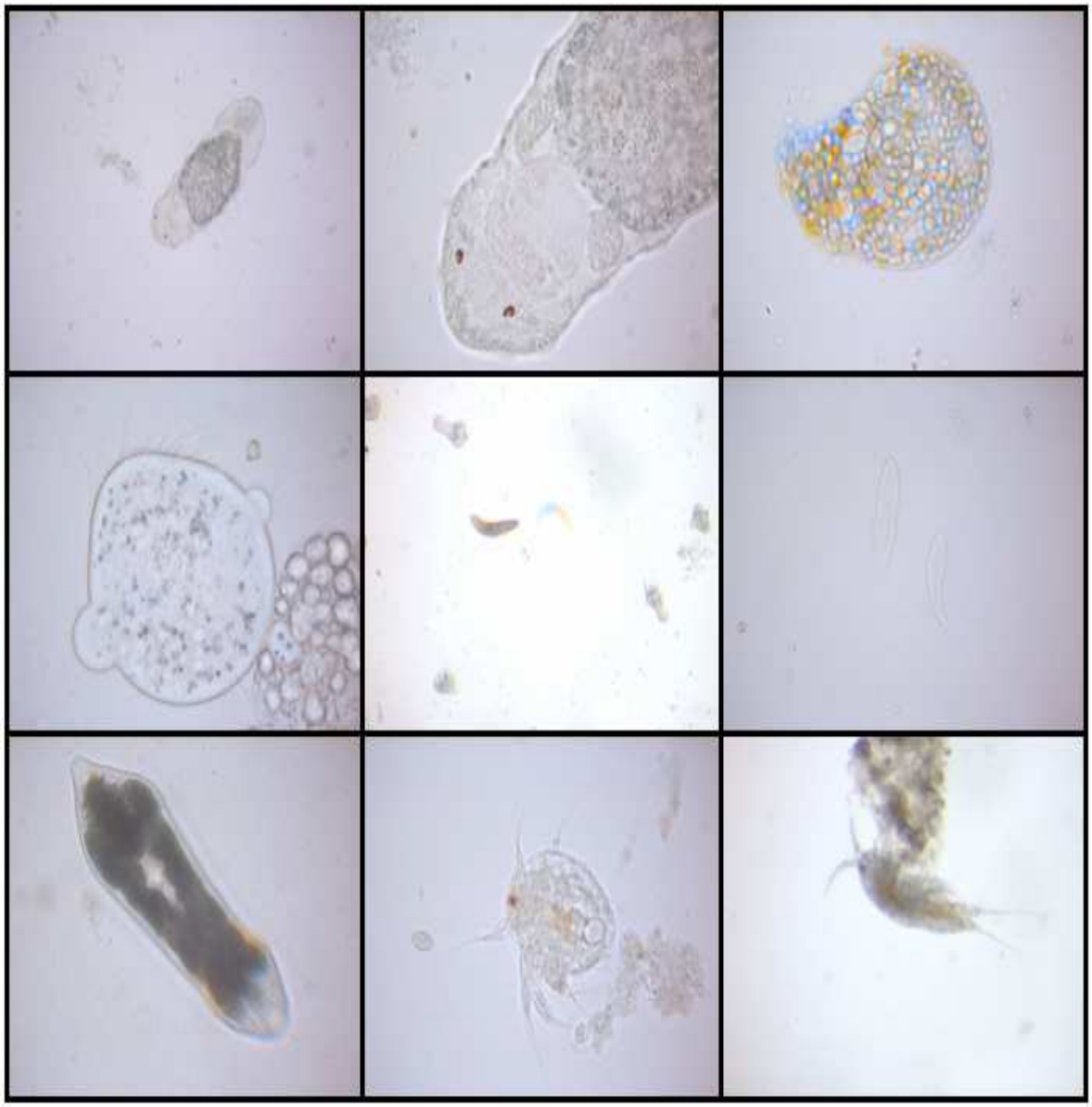

Fig. 17 – Diversity of organisms found within *C. edule* in systems of acclimatization after a few months. Many of these organisms were probably parasites.

Note that the images are not scale drawings and this figure just shows the variety of parasites found in the cockles.

This study revealed notable responses in cockles to different levels of contamination, hence, it is suggested that *C. edule* responds to sediment–bound contamination, since this cockle bioaccumulated and regulated (eliminated) both types of contaminants (Fig. 8, 9 and 10). For some contaminants the concentration decreased, and this can be due to degradation of the tissue or elimination for this way. Therefore, this cockle might be suitable for biomonitoring, even though it is clear that the effects of contaminant interactions on biomarkers and

indicators of exposure need yet much research. In addition, the exposure of *C. edule* to other types of sediments, particularly from more contaminated sites, can be used for the validation of this bivalve as test species. This work shows the potential of the use of translocated cockle methodologies within ecological risk assessment studies and/or ecosystems rehabilitation.

References

Amiard J-C, Amiard-Triquet C, Barka S, Pellerin J, Rainbow PS (2006) Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. Aquatic Toxicology 76:160–202

Baudrimont M, de Montaudouin X, Palvadeau A (2006) Impact of digenean parasite infection on metallothionein synthesis by the cockle (*Cerastoderma edule*): A multivariate field monitoring. Marine Pollution Bulletin 52:494–502

Biomarkers Definitions Working Group (2001) Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin. Pharmacol. Therap. 69, 89–95

Bergayou H, Mouneyrac C, Pellerin J, Moukrim A (2009) Oxidative stress responses in bivalves (*Scrobicularia plana, Cerastoderma edule*) from the Oued Souss estuary (Morocco). Ecotoxicology and Environmental Safety 72:765–769

Bigot A, Doyen P, Vasseur P, Rodius F (2009) Metallothionein coding sequence identification and seasonal mRNA expression of detoxification genes in the bivalve *Corbicula fluminea*. Ecotoxicology and Environmental Safety 72:382– 387

Caeiro S, Costa MH, Ramos TB, Fernandes F, Silveira N, Coimbra A, Medeiros G, Painho M (2005) Assessing heavy metal contamination in Sado Estuary sediment: An index analysis approach. Ecological Indicators 5:151–169

Caetano M, Fonseca N, Cesário R, Vale C (2007) Mobility of Pb in salt marshes recorded by total content and stable isotopic signature. Sci Total Environ 380:84-92

Cajaraville MP, Bebianno MJ, 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. The Science of the Total Environment 247:295-311

Catarino J, Peneda MC, Santana F (1987) Estudo do Impacte da Indústria no Estuário do Rio Sado. Estimativas da Poluição Afluente. DEII, INETI - Instituto Nacional de Engenharia e Tecnologia Industrial, Lisbon, Portugal

Clesceri LS, Greenberg AE, Eaton AD (1999) Standard methods for examination of water & wastewater, 20th edn. American Public Health Association, Baltimore

Cortesão C and Vale C (1995) Metals in Sediments of the Sado Estuary, Portugal. Marine Pollution Bulletin Vol. 30, No. I, pp. 34-37

Costa PM, Repolho T, Caeiro S, Diniz ME, Moura I, Costa MH (2008) Modelling metallothionein induction in the liver of Sparus aurata exposed to metal-contaminated estuarine sediments. Ecotox Environ Saf 71:117-124

Costa PM, Santos HM, Peres I, Costa MH, Alves S, Capelo-Martinez JL, Diniz MS (2009) Toxicokinetics of Waterborne Trivalent Arsenic in the Freshwater Bivalve Corbicula fluminea. Arch Environ Contam Toxicol 57:338–347

Del Valls TA, Forja JM, Gómez-Parra A (1998) The use of multivariate analysis to link sediment contamination and toxicity data to establish sediment quality guidelines: an example in the Gulf of Cádiz (Spain). Ciencias Marinas 24:127-154

De Kock WC and Kramer KJM (1994) Active biomonitoring (ABM) by translocation of bivalve molluscs. In: Kramer KJM (ed) Biomonitoring of coastal waters and estuaries. CRC Press. Boca Raton, pp 51–84

Ferreira AM, Martins M, Vale C (2003) Influence of diffuse sources on levels and distribution of polychlorinated biphenyls in the Guadiana River estuary, Portugal. Mar Chem 89:175-184

Geret F, Serafim A, Bebianno MJ (2003) Antioxidant Enzyme Activities, Metallothioneins and Lipid Peroxidation as Biomarkers in Ruditapes decussatus. Ecotoxicology 12:417-426

Gold-Bouchot G, Simá-Alvarez R, Zapata-Pérez O, Guemez-Ricalde J (1995) Histopathological Effects of Petroleum Hydrocarbons and Heavy Metals on the American Oyster (*Crassostrea virginica*) from Tabasco, México. Marine Pollution Bulletin Vol. 31, Nos 4~12, pp. 439~445

Hédouin L, Pringault O, Metian M, Bustamante P, Warnau M (2007) Nickel bioaccumulation in bivalves from the New Caledonia lagoon: Seawater and food exposure. Chemosphere 66:1449–1457

Huggett RJ, Kimerle RA, Mehrle PM, Bergman HL (eds) (1992) *Biomarkers. Biochemical, physiological, and histological markers of anthropogenic stress*. Lewis Publishers, U.S.A.

Jantunen APK, Tuikka A, Akkanen J, Kukkonen JVK (2008) Bioaccumulation of atrazine and chlorpyrifos to Lumbriculus variegatus from lake sediments. Ecotoxicology and Environmental Safety 71:860–868

Jung K, Stelzenmüller V, Zauke G-P (2006) Spatial distribution of heavy metal concentrations and biomass indices in *Cerastoderma edule Linnaeus* (1758) from the German Wadden Sea: An integrated biomonitoring approach. Journal of Experimental Marine Biology and Ecology 338:81–95

Koeman JH, Kohler-Gunther A, Kurelec B, Riviere JL, Versteeg D, Walker CH (1993) Applications and objectives of biomarker research. In: Peakall, D.B. and Shugart, L.R.,

Editors (1993) Biomarkers: Research and Application in the Assessment of Environmental Health NATO ASI Series vol. H. 68, Springer, Berlin, pp. 1–13

Lee H (1992) Models, muddles, and mud: predicting bioaccumulation of sediment associated pollutants. In: Burton, A.G. (Ed.), Sediment Toxicity Assessment. CRC, Boca Raton, FL, pp. 267–293

Livingstone DR (2001) Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine Pollution Bulletin Vol.42 No. 8, pp. 656-666

Long ER and MacDonald DD (1998) Recommended uses of empirically derived, sediment quality guidelines for marine and estuarine ecosystems. Hum. Ecol. Risk Assess. 4:1019-1039.

Luoma S and Rainbow P (2005) Why is metal bioaccumulation so variable? Biodynamics as a unifying concept. Environtal Science and Technology. 39:1921–1931.

Machreki-Ajmi M, Ketata I, Ladhar-Chaabouni R, Hamza-Chaffai A (2008) The effect of in situ cadmium contamination on some biomarkers in *cerastoderma glaucum*. Ecotoxicology 17:1–11

MacDonald DD, Carr S, Calder F, Long E, Ingersoll C (1996) Development and evaluation of sediment quality guidelines for Florida coastal waters. Ecotoxicology 5:253-278

MacDonald DD, Carr RS, Eckenrod D, Greening H, Grabe S, Ingersoll CG, Janicki S, Janicki T, Lindskoog RA, Long ER, Pribble R, Sloane G, Smorong DE (2004) Development, evaluation, and application of sediment quality targets for assessing and managing contaminated sediments in Tampa Bay, Florida. Arch. Environ. Contam. Toxicol. 46:147-161

Marie V, Baudrimont M, Boudou A (2006) Cadmium and zinc bioaccumulation and metallothionein response in two freshwater bivalves (*Corbicula fluminea and Dreissena polymorpha*) transplanted along a polymetallic gradient. Chemosphere 65:609–617

Martins M, Ferreira AM, Vale C (2008) The influence of Sarcocornia fruticosa on retention of PAHs in salt marshes sediments (Sado estuary, Portugal). Chemosphere 71:1599-1606

Martoja R and Martoja M (1967) Initiation aux Tecniques de l'Histologie Animal. Masson & Cie, Paris, 345 pp.

Peakall DB and Shugart LR (eds) (1993) *Biomarkers. Research and application in the assessment of environmental health.* Springer-Verlag. Berlin, Heidelberg.

Risso-de Faverney C, Lafaurie M, Girard J-P, Rahmani R (2000). Effects of heavy metals and 3-methylcholanthrene on expression and induction of CYP1A1 and metallothionein levels in trout (*Oncorhynchus mykiss*) hepatocyte cultures. Environmental and Toxicological Chemistry. 19:2239–2248

Serafim A and Bebianno MJ (2009) Metallothionein role in the kinetic model of copper accumulation and elimination in the clam *Ruditapes decussates*. Environmental Research 109:390–399

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. Environment International 35:523–531

Syasina IG, Vaschenko MA, Zhadan PM (1997). Morphological Alterations in the Digestive Diverticula of *Mizuhopecten yessoensis* (Bivalvia: *Pectinidae*) from Polluted Areas of Peter the Great Bay, Sea of Japan. Marine Environmental Research Vol. 44, No. 1, pp. 85-98

Thorsson MH, Hedman JE, Bradshaw C, Gunnarsson JS, Gilek M (2008) Effects of settling organic matter on the bioaccumulation of cadmium and BDE-99 by Baltic Sea benthic invertebrates. Marine Environmental Research 65:264–281

United States Environmental Protection Agency (USEPA) (1995) CRITFC exposure study. Water Quality Criteria and Standards Newsletter. USEPA, Washington, DC

Zaldibar B, Cancio I, Soto M, Marigómez I (2007) Digestive cell turnover in digestive gland epithelium of slugs experimentally exposed to a mixture of cadmium and kerosene. Chemosphere 70:144–154

Zaldibar B, Cancio I, Soto M, Marigómez I (2008) Changes in cell-type composition in digestive gland of slugs and its influence in biomarkers following transplantation between a relatively unpolluted and a chronically metal-polluted site. Environmental Pollution 156:367–379

Annexes

Annex I - Metal and organic concentrations of sediments from sites D, A, B and C.

			Sites							
			D		A		B		C	
	TELa	PELb		PEL-Qc		PEL-Q		PEL-Q		PEL-Q
Metallic (mg.kg-1 sediment dry weight) ±γ										
As	7.24	41.6	20,55 ± 0,41*	0.49	7.25 ± 0.15*	0.17	27.43 ± 0.55*	0.66	12.38 ± 0.25*	0.30
Cd	0.68	4.21	0,23 ± 0,00	0.05	0.04 ± 0.00	0.01	0.22 ± 0.00	0.05	0.15 ± 0.00	0.04
Cu	18.7	108	63,67 ± 1,27*	0.59	22.57 ± 0.45*	0.21	167.32 ± 3.35**	1.55	41.18 ± 0.82*	0.38
Ni	15.9	42.8	26,12 ± 0,52*	0.61	12.97 ± 0.26	0.30	33.67 ± 0.67*	0.79	9.03 ± 0.18	0.21
Pb	30.2	112	30,92 ± 0,62*	0.28	23.70 ± 0.47	0.21	66.49 ± 1.33*	0.59	45.17 ± 0.90*	0.40
Zn	124	271	232,99 ± 4,66*	0.86	147.48 ± 2.95*	0.54	312.23 ± 6.24**	1.15	87.75 ± 1.76	0.32
Organic (µg.kg-1 sediment dry weight) ±γ										
PAHs										
3 – ring										
Acenaphthene	6.71	88.9	2,09 ± 0,36	0.02	1.41 ± 0.24	0.02	9.42 ± 1.60*	0.11	4.19 ± 0.71	0.05
Acenaphthylene	5.87	128	4,64 ± 0,79	0.04	0.24 ± 0.04	0.00	1.83 ± 0.31	0.01	1.95 ± 0.33	0.02
Anthracene	46.9	245	5,72 ± 0,97	0.02	1.03 ± 0.17	0.00	10.60 ± 1.	0.04	15.34 ± 2.61	0.06
Fluorene	21.2	144	3,56 ± 0,61	0.02	1.32 ± 0.22	0.01	8.70 ± 1.48	0.06	8.03 ± 1.37	0.06
Phenanthrene	86.7	544	18,67 ± 3,17	0.03	7.96 ± 1.35	0.01	50.77 ± 8.63	0.09	54.09 ± 9.20	0.10
4 – ring										
Benz(a)anthracene	74.8	693	1,04 ± 0,18	0.00	4.53 ± 0.77	0.01	64.60 ± 10.98	0.09	86.52 ± 14.71*	0.12
Chrysene	108	846	3,49 ± 0,59	0.00	2.20 ± 0.37	0.00	28.31 ± 4.81	0.03	37.19 ± 6.32	0.04
Fluoranthene	113	1494	185,54 ± 31,54*	0.12	18.05 ± 3.07	0.01	170.80 ± 29.04*	0.11	184.30 ± 31.30*	0.12
Pyrene	153	1398	171,67 ± 29,18*	0.12	14.66 ± 2.49	0.01	131.74 ± 22.40	0.09	171.39 ± 29.14*	0.12
5 – ring										
Benzo(a)pyrene	88.8	793	74,94 ± 12,74	0.09	7.56 ± 1.28	0.01	69.81 ± 11.87	0.09	85.88 ± 14.60	0.11
Benzo(b)fluoranthene			56.53 9.61		6.77 ± 1.15		60.86 ± 10.35		70.25 ± 11.94	
Benzo(e)pyrene			46.64 7.93		5.12 ± 0.87		56.73 ± 9.64		62.76 ± 10.67	
Benzo(k)fluoranthene			25.04 4.26		4.16 ± 0.71		32.21 ± 5.48		40.18 ± 6.83	
Dibenzo(a,h)anthracene	6.22	135	7,06 ± 1,20*	0.05	0.74 ± 0.13	0.01	7.45 ± 1.27*	0.06	6.99 ± 1.19*	0.05
Perylene			40.06 ± 6.81		4.69 ± 0.80		86.97 ± 14.78		209.16 ± 35.56	
6 – ring										
Indene(1,2,3-cd)pyrene			54.43 ± 9.25		4.87 ± 0.83		52.44 ± 8.91		51.82 ± 8.81	

			Sites			
			D	A	B	C
	TELa	PELb	PEL-Qc	PEL-Q	PEL-Q	PEL-Q
Benzo(g,h,I)perylene			34.78 ± 4.21	1.12 ± 0.19	39.12 ± 6.65	10.44 ± 1.77
3-ring			34.69 ± 5.90	11.95 ± 2.03	81.32 ± 13.82	83.60 ± 14.21
4-ring			361.74 ± 61.50	39.43 ± 6.70	395.46 ± 67.23	479.40 ± 81.50
5-ring			250.28 ± 42.55	29.04 ± 4.94	314.04 ± 53.39	475.22 ± 80.79
6-ring			89.21 ± 15.17	5.99 ± 1.02	91.55 ± 15.56	62.26 ± 10.58
tPAHs			735.92 ± 125.11	86.42 ± 14.69	882.37 ± 150.00	1100.48 ± 187.08
PCBs						
Trichlorinated						
PCB-18			0.21 ± 0.04	< d.l.	0.08 ± 0.01	0.09 ± 0.02
PCB-26			1.79 ± 0.30	< d.l.	0.06 ± 0.01	0.09 ± 0.02
PCB-31			0.13 ± 0.02	0.64 ± 0.11	0.19 ± 0.03	< d.l.
Tetra-chlorinated						
PCB-44			0.05 ± 0.01	< d.l.	0.38 ± 0.06	< d.l.
PCB-49			0.05 ± 0.01	< d.l.	0.08 ± 0.01	0.36 ± 0.06
PCB-52			0.08 ± 0.01	< d.l.	0.12 ± 0.02	0.45 ± 0.08
Penta-chlorinated						
PCB-101			0.06 ± 0.01	< d.l.	0.23 ± 0.04	1.18 ± 0.20
PCB-105			< d.l.	< d.l.	0.22 ± 0.04	0.66 ± 0.11
PCB-118			0.08 ± 0.01	< d.l.	1.04 ± 0.18	4.92 ± 0.84
Hexa-chlorinated						
PCB-128			0.05 ± 0.01	< d.l.	0.08 ± 0.01	< d.l.
PCB-138			0.21 ± 0.04	0.12 ± 0.02	0.68 ± 0.12	2.68 ± 0.46
PCB-149			0.13 ± 0.02	0.11 ± 0.02	< d.l.	< d.l.
PCB-151			0.09 ± 0.02	0.05 ± 0.01	0.17 ± 0.03	1.15 ± 0.20
PCB-153			0.19 ± 0.03	0.14 ± 0.02	0.64 ± 0.11	3.39 ± 0.58
Hepta-chlorinated						
PCB-170			0.03 ± 0.00	0.07 ± 0.01	0.27 ± 0.05	< d.l.
PCB-180			0.11 ± 0.02	0.21 ± 0.04	0.61 ± 0.10	< d.l.
PCB-187			0.22 ± 0.04	0.20 ± 0.03	0.72 ± 0.12	< d.l.
PCB-194			0.03 ± 0.00	< d.l.	0.07 ± 0.01	0.38 ± 0.06
Tri-chlorinated			2.13 ± 0.36	0.64 ± 0.11	0.33 ± 0.06	0.17 ± 0.03
Tetra-chlorinated			0.17 ± 0.03	< d.l.	0.58 ± 0.10	0.81 ± 0.14
Penta-chlorinated			0.14 ± 0.02	< d.l.	1.49 ± 0.25	6.76 ± 1.15

			Sites							
			D		A		B		C	
	TELa	PELb		PEL-Qc		PEL-Q		PEL-Q		PEL-Q
Hexa-chlorinated			0.68 ± 0.12		0.42 ± 0.07		1.57 ± 0.27		7.22 ± 1.23	
Hepta-chlorinated			0.39 ± 0.07		0.48 ± 0.08		1.67 ± 0.28		0.38 ± 0.06	
tPCBs	21.6	189	3.49 ± 0.59	0.02	1.54 ± 0.26	0.01	5.64 ± 0.96	0.03	15.34 ± 2.61	0.08
DDTs										
pp'DDD	1.22	7.81	< d.l.	< d.l.	0.10 ± 0.02	0.01	0.28 ± 0.05	0.04	0.60 ± 0.10	0.08
pp'DDE	2.07	374	0.09 ± 0.02	0.00	0.05 ± 0.01	0.00	0.27 ± 0.05	0.00	0.65 ± 0.11	0.00
pp'DDT	1.19	4.77	< d.l.	< d.l.	0.70 ± 0.12	0.15	4.39 ± 0.75*	0.92	1.18 ± 0.20	0.25
tDDTs			0.09 ± 0.02		0.85 ± 0.14		4.94 ± 0.84		2.43 ± 0.41	
SQG-Qd				0.181		0.082		0.313		0.139
SQG-Q metallic				0.481		0.242		0.799		0.275
SQG-Q organic				0.043		0.017		0.119		0.084

[a] Threshold effects level, [b] Probable effects level; [c] PEL quocient [1]; [d] sediment quality guideline quocient [2]; * concentrations above TEL; ** concentrations above PEL; < d.l., below detection limit; tPAH, total PAH (sum of all individualPAHs); tPCB, total PCB (sum of all congeners); DDD, 1,1-dichloro-2,2-bis(*ρ*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis (*ρ*-chlorophenyl)ethylene; DDT, 1,1,1-trichloro2,2-bis (*ρ*-chlorophenyl)ethane; tDDT, total DDT (pp'DDD+pp'DDD+pp'DDT). Ranges indicate standard error.

Annex II - Metallothionein and bioaccumulation of metal and organic contaminants in Cardium edule from sites D, A, B and C.

	Site						
	D	A		B		C	
	T0	T14	T28	T14	T28	T14	T28
Metallothioneins (mg.g-1 whole soft tissue dry weight) ± standard deviation	3.46 ± 1.69	3.80 ± 1.21	2.39 ± 0.70	2.72 ± 0.59	2.70 ± 1.38	2.05 ± 0.84*	1.70 ± 0.66*
Metallic (mg.kg-1 whole soft tissue dry weight) ± standard deviation							
As	17.16 ± 3.29	17.00 ± 2.45	22.39 ± 4.33*	23.85 ± 7.40*	23.00 ± 2.71**	22.94 ± 6.58**	21.56 ± 6.79*
Cd	1.32 ± 1.62	1.99 ± 1.62	1.59 ± 1.32	1.39 ± 1.22	2.19 ± 2.01	2.83 ± 2.93	1.03 ± 1.03
Cu	21.02 ± 8.62	14.73 ± 9.42*	12.37 ± 3.75**	14.01 ± 3.30*	33.61 ± 15.46*	15.88 ± 5.96	22.48 ± 12.85
Ni	91.02 ± 32.08	84.37 ± 16.98	96.45 ± 15.72	106.54 ± 44.98	106.54 ± 40.40	117.15 ± 36.99*	83.80 ± 60.86
Pb	5.34 ± 2.89	6.08 ± 6.33	2.98 ± 1.23*	8.16 ± 8.15	6.22 ± 4.28	6.29 ± 3.17	4.47 ± 2.77
Zn	85.10 ± 28.93	67.66 ± 10.06	97.40 ± 20.98	112.43 ± 41.15	130.36 ± 43.98**	159.86 ± 75.73**	112.85 ± 56.63
Organic (µg.kg-1 whole soft tissue dry weight) ± standard error							
PAHs							
3-ring							
Acenaphthene	0.67 ± 0.11	0.82 ± 0.14	0.73 ± 0.12	1.45 ± 0.25	1.52 ± 0.26	2.27 ± 0.39	1.56 ± 0.27
Acenaphthylene	0.33 ± 0.06	0.36 ± 0.06	0.36 ± 0.06	0.66 ± 0.11	0.71 ± 0.12	0.82 ± 0.14	0.67 ± 0.11
Anthracene	1.13 ± 0.19	1.06 ± 0.18	1.12 ± 0.19	0.34 ± 0.06	0.37 ± 0.06	0.38 ± 0.07	0.33 ± 0.06
Fluorene	2.63 ± 0.45	2.61 ± 0.44	2.71 ± 0.46	1.30 ± 0.22	1.65 ± 0.28	1.76 ± 0.30	1.59 ± 0.27
Phenanthrene	5.11 ± 0.87	6.75 ± 1.15	6.66 ± 1.13	4.12 ± 0.70	4.87 ± 0.83	5.93 ± 1.01	4.71 ± 0.80
4-ring							
Benz(a)anthracene	0.20 ± 0.03	0.33 ± 0.06	0.29 ± 0.05	0.64 ± 0.11	0.72 ± 0.12	1.17 ± 0.20	1.40 ± 0.24
Chrysene	0.90 ± 0.15	0.67 ± 0.11	0.66 ± 0.11	0.90 ± 0.15	1.93 ± 0.33	1.89 ± 0.32	1.11 ± 0.19
Fluoranthene	5.77 ± 0.98	5.83 ± 0.99	5.91 ± 1.00	4.46 ± 0.76	4.55 ± 0.77	6.16 ± 1.05	4.99 ± 0.85
Pyrene	8.44 ± 1.43	6.95 ± 1.18	7.03 ± 1.20	4.56 ± 0.78	5.21 ± 0.89	8.04 ± 1.37	6.83 ± 1.16
5-ring							
Benzo(a)pyrene	3.00 ± 0.51	2.74 ± 0.47	2.83 ± 0.48	0.59 ± 0.10	0.63 ± 0.11	1.21 ± 0.21	1.33 ± 0.23
benzo(b)fluoranthene	1.18 ± 0.20	1.26 ± 0.21	1.24 ± 0.21	1.05 ± 0.18	1.19 ± 0.20	1.44 ± 0.25	1.46 ± 0.25
benzo(e)pyrene	0.51 ± 0.09	0.61 ± 0.10	0.55 ± 0.09	0.74 ± 0.13	0.70 ± 0.12	1.22 ± 0.21	1.20 ± 0.20
benzo(k)fluoranthene	0.19 ± 0.03	0.23 ± 0.04	0.21 ± 0.04	0.59 ± 0.10	0.64 ± 0.11	0.72 ± 0.12	0.85 ± 0.14
Dibenzo(a,h)anthracene	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
Perylene	0.95 ± 0.16	0.70 ± 0.12	0.41 ± 0.07	1.65 ± 0.28	1.67 ± 0.28	6.04 ± 1.03	6.87 ± 1.17
6-ring							
Indene(1,2,3-cd)pyrene	< d.l.	< d.l.	4.38 ± 0.74	0.61 ± 0.10	0.56 ± 0.10	0.74 ± 0.13	0.65 ± 0.11
benzo(g,h,I)perylene	< d.l.	< d.l.	3.65 ± 0.62	0.54 ± 0.09	0.78 ± 0.13	0.84 ± 0.14	0.83 ± 0.14
3-ring	9.87 ± 1.68	11.60 ± 1.97	11.58 ± 1.97	7.88 ± 1.34	9.11 ± 1.55	11.17 ± 1.90	8.87 ± 1.51

	Site						
	D	A		B		C	
	T0	T14	T28	T14	T28	T14	T28
4-ring	15.30 ± 2.60	13.78 ± 2.34	13.90 ± 2.36	10.56 ± 1.80	12.41 ± 2.11	17.26 ± 2.93	14.33 ± 2.44
5-ring	5.83 ± 0.99	5.55 ± 0.94	5.24 ± 0.89	4.61 ± 0.78	4.83 ± 0.82	10.63 ± 1.81	11.71 ± 1.99
6-ring	< d.l.	< d.l.	8.02 ± 1.36	1.15 ± 0.19	1.34 ± 0.23	1.58 ± 0.27	1.48 ± 0.25
tPAHs	31.00 ± 5.27	30.92 ± 5.26	38.73 ± 6.58	24.20 ± 4.11	27.70 ± 4.71	40.64 ± 6.91ΨΨ	36.39 ± 6.19ΨΨ
PCBs							
Trichlorinated							
CB-18	0.02 ± 0.00	< d.l.	0.03 ± 0.00	0.03 ± 0.01	0.06 ± 0.01	0.01 ± 0.00	0.02 ± 0.00
CB-26	0.02 ± 0.00	< d.l.	< d.l.	0.01 ± 0.00	0.02 ± 0.00	< d.l.	0.01 ± 0.00
CB-31	< d.l.	< d.l.	0.18 ± 0.03	0.44 ± 0.07	0.46 ± 0.08	< d.l.	0.52 ± 0.09
Tetra-chlorinated							
CB-44	0.04 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.02 ± 0.00	0.03 ± 0.01
CB-49	0.06 ± 0.01	0.10 ± 0.02	0.13 ± 0.02	0.06 ± 0.01	0.08 ± 0.01	0.02 ± 0.00	0.03 ± 0.01
CB-52	0.03 ± 0.00	0.08 ± 0.01	0.03 ± 0.00	0.06 ± 0.01	0.08 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
Penta-chlorinated							
CB-101	0.03 ± 0.00	0.09 ± 0.01	0.02 ± 0.00	0.05 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.03 ± 0.00
CB-105	0.03 ± 0.01	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
CB-118	0.15 ± 0.03	0.30 ± 0.05	0.23 ± 0.04	0.20 ± 0.03	0.18 ± 0.03	0.11 ± 0.02	0.14 ± 0.02
Hexa-chlorinated							
CB-128	< d.l.	< d.l.	< d.l.	0.01 ± 0.00	< d.l.	< d.l.	< d.l.
CB-138	0.08 ± 0.01	0.13 ± 0.02	0.09 ± 0.01	0.11 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.08 ± 0.01
CB-149	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
CB-151	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
CB-153	0.03 ± 0.01	0.06 ± 0.01	< d.l.	0.02 ± 0.00	< d.l.	< d.l.	0.02 ± 0.00
Hepta-chlorinated							
CB-170	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
CB-180	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
CB-187	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
CB-194	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
Tri-chlorinated	0.05 ± 0.01	< d.l.	0.21 ± 0.04	0.48 ± 0.08	0.54 ± 0.09	0.01 ± 0.00	0.55 ± 0.09
Tetra-chlorinated	0.12 ± 0.02	0.24 ± 0.04	0.20 ± 0.03	0.18 ± 0.03	0.24 ± 0.04	0.06 ± 0.01	0.08 ± 0.01
Penta-chlorinated	0.21 ± 0.04	0.39 ± 0.07	0.25 ± 0.04	0.25 ± 0.04	0.25 ± 0.04	0.16 ± 0.03	0.17 ± 0.03
Hexa-chlorinated	0.12 ± 0.02	0.19 ± 0.03	0.09 ± 0.01	0.15 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.09 ± 0.02
Hepta-chlorinated	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
tPCBs	0.49 ± 0.08	0.82 ± 0.14	0.74 ± 0.13	1.06 ± 0.18	1.07 ± 0.18	0.28 ± 0.05	0.88 ± 0.15
DDTs							
pp'DDD	2.2 ± 0.37	0.51 ± 0.09	0.59 ± 0.10	0.22 ± 0.04	1.32 ± 0.22	0.22 ± 0.04	0.28 ± 0.05
pp'DDE	0.12 ± 0.02	0.21 ± 0.04	0.13 ± 0.02	0.10 ± 0.02	0.17 ± 0.03	0.08 ± 0.01	0.10 ± 0.02

	Site						
	D	A		B		C	
	T0	T14	T28	T14	T28	T14	T28
pp'DDT	1.13 ± 0.19	1.04 ± 0.18	1.42 ± 0.24	1.08 ± 0.18	4.10 ± 0.70	0.52 ± 0.09	0.64 ± 0.11
tDDts	3.45 ± 0.59	1.76 ± 0.30	2.14 ± 0.36	1.40 ± 0.24	5.59 ± 0.95Ψ	0.82 ± 0.14	1.02 ± 0.17

* and ** indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between tests and D (Mann–Whitney U test); $^{\Psi}$ and $^{\Psi\Psi}$ indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) between tests and D (chi-square test); < d.l., below detection limit; tPAH, total PAH (sum of all individual PAHs); tPCB, total PCB (sum of all congeners); DDD, 1,1-dichloro-2,2-bis(*ρ*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis (*ρ*-chlorophenyl)ethylene; DDT, 1,1,1-trichloro2,2-bis (*ρ*-chlorophenyl)ethane; tDDT, total DDT (pp'DDD+pp'DDD+pp'DDT)

Annex III - BAFs and BSAFs of metallic and organic contaminants in *C. edule*.

	Sites													
	D		A				B				C			
	T0		T14		T28		T14		T28		T14		T28	
	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF
Metals														
As	0.8350	0.1039	2.3455	0.0751	3.0886	0.0988	0.8695	0.1026	0.8386	0.0990	1.8533	0.1427	1.7414	0.1341
Cd**	5.7450	0.7151	49.8196	1.5942	39.7112	1.2708	6.3206	0.7458	9.9683	1.1763	18.8752	1.4534	6.8516	0.5276
Cu	0.3302	0.0411	0.6525	0.0209	0.5479	0.0175	0.0838	0.0099	0.2009	0.0237	0.3855	0.0297	0.5459	0.0420
Ni	3.4846	0.4337	6.5054	0.2082	7.4363	0.2380	3.1643	0.3734	3.1643	0.3734	12.9738	0.9990	9.2800	0.7146
Pb	0.1728	0.0215	0.2566	0.0082	0.1258	0.0040	0.1228	0.0145	0.0936	0.0110	0.1393	0.0107	0.0990	0.0076
Zn	0.3653	0.0455	0.4588	0.0147	0.6604	0.0211	0.3601	0.0425	0.4175	0.0493	1.8218	0.1403	1.2860	0.0990
Organics														
PAHs														
3-ring														
Acenaphthene	0.3201	0.0398	0.5830	0.0187	0.5203	0.0166	0.1541	0.0182	0.1612	0.0190	0.5422	0.0417	0.3734	0.0288
Acenaphthylene	0.0701	0.0087	1.5095	0.0483	1.4804	0.0474	0.3620	0.0427	0.3874	0.0457	0.4221	0.0325	0.3437	0.0265
Anthracene	0.1976	0.0246	1.0256	0.0328	1.0863	0.0348	0.0319	0.0038	0.0345	0.0041	0.0251	0.0019	0.0214	0.0016
Fluorene	0.7395	0.0920	1.9737	0.0632	2.0542	0.0657	0.1499	0.0177	0.1899	0.0224	0.2190	0.0169	0.1985	0.0153
Phenanthrene	0.2738	0.0341	0.8480	0.0271	0.8363	0.0268	0.0812	0.0096	0.0959	0.0113	0.1097	0.0084	0.0872	0.0067
4-ring														
Benz(a)anthracene	0.1923	0.0239	0.0721	0.0023	0.0648	0.0021	0.0099	0.0012	0.0111	0.0013	0.0135	0.0010	0.0162	0.0012
Chrysene	0.2579	0.0321	0.3047	0.0097	0.2995	0.0096	0.0319	0.0038	0.0683	0.0081	0.0508	0.0039	0.0300	0.0023
Fluoranthene	0.0311	0.0039	0.3230	0.0103	0.3275	0.0105	0.0261	0.0031	0.0266	0.0031	0.0334	0.0026	0.0271	0.0021
Pyrene	0.0491	0.0061	0.4741	0.0152	0.4796	0.0153	0.0346	0.0041	0.0396	0.0047	0.0469	0.0036	0.0398	0.0031
5-ring														
Benzo(a)pyrene	0.0401	0.0050	0.3628	0.0116	0.3743	0.0120	0.0084	0.0010	0.0090	0.0011	0.0141	0.0011	0.0155	0.0012
benzo(b)fluoranthene	0.0209	0.0026	0.1855	0.0059	0.1832	0.0059	0.0172	0.0020	0.0196	0.0023	0.0205	0.0016	0.0207	0.0016
benzo(e)pyrene	0.0110	0.0014	0.1201	0.0038	0.1065	0.0034	0.0130	0.0015	0.0124	0.0015	0.0194	0.0015	0.0192	0.0015
benzo(k)fluoranthene	0.0074	0.0009	0.0556	0.0018	0.0503	0.0016	0.0184	0.0022	0.0197	0.0023	0.0179	0.0014	0.0211	0.0016
Dibenzo(a,h)anthracene														
Perylene	0.0236	0.0029	0.1497	0.0048	0.0878	0.0028	0.0190	0.0022	0.0192	0.0023	0.0289	0.0022	0.0328	0.0025
6-ring														
Indene(1,2,3-cd)pyrene					0.8984	0.0287	0.0116	0.0014	0.0108	0.0013	0.0143	0.0011	0.0126	0.0010

	Sites													
	D		A				B				C			
	T0		T14		T28		T14		T28		T14		T28	
	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF
benzo(g,h,I)perylene					3.2570	0.1042	0.0138	0.0016	0.0199	0.0024	0.0803	0.0062	0.0794	0.0061
3-ring	0.2845	0.0354	0.9704	0.0311	0.9687	0.0310	0.0969	0.0114	0.1121	0.0132	0.1336	0.0103	0.1061	0.0082
4-ring	0.0423	0.0053	0.3494	0.0112	0.3524	0.0113	0.0267	0.0032	0.0314	0.0037	0.0360	0.0028	0.0299	0.0023
5-ring	0.0233	0.0029	0.1910	0.0061	0.1803	0.0058	0.0147	0.0017	0.0154	0.0018	0.0224	0.0017	0.0246	0.0019
6-ring	-	-	-	-	1.3394	0.0429	0.0125	0.0015	0.0147	0.0017	0.0253	0.0020	0.0238	0.0018
tPAHs*	0.0421	0.0052	0.3578	0.0114	0.4482	0.0143	0.0274	0.0032	0.0314	0.0037	0.0369	0.0028	0.0331	0.0025
PCBs														
Trichlorinated														
PCB-18	0.1143	0.0142					0.3875	0.0457	0.7250	0.0856	0.0833	0.0064	0.1778	0.0137
PCB-26	0.0134	0.0017					0.2000	0.0236	0.3167	0.0374			0.1111	0.0086
PCB-31					0.2813	0.0090	2.3158	0.2733	2.4211	0.2857				
Tetra-chlorinated														
PCB-44	0.8222	0.1023					0.1842	0.0217	0.2289	0.0270				
PCB-49	1.1667	0.1452					0.7250	0.0856	0.9625	0.1136	0.0639	0.0049	0.0889	0.0068
PCB-52	0.3506	0.0436					0.4417	0.0521	0.6500	0.0767	0.0356	0.0027	0.0267	0.0021
Penta-chlorinated														
PCB-101	0.4909	0.0611					0.2261	0.0267	0.2870	0.0339	0.0441	0.0034	0.0220	0.0017
PCB-105	32.0000	3.9831												
PCB-118	1.8987	0.2363					0.1923	0.0227	0.1731	0.0204	0.0224	0.0017	0.0285	0.0022
Hexa-chlorinated														
PCB-128							0.1200	0.0142						
PCB-138	0.3962	0.0493	1.0833	0.0347	0.7083	0.0227	0.1618	0.0191	0.0691	0.0082	0.0198	0.0015	0.0284	0.0022
PCB-149														
PCB-151							0.0282	0.0033						
PCB-153	0.1606	0.0200	0.4571	0.0146			0.0359	0.0042					0.0044	0.0003
Hepta-chlorinated														
PCB-170														
PCB-180														
PCB-187														
PCB-194														
Tri-chlorinated	0.0226	0.0028			0.3250	0.0104	1.4636	0.1727	1.6273	0.1920	0.0441	0.0034	3.2118	0.2473

	Sites													
	D		A				B				C			
	T0		T14		T28		T14		T28		T14		T28	
	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF	BAF	BSAF
Tetra-chlorinated	0.7059	0.0879					0.3121	0.0368	0.4172	0.0492	0.0691	0.0053	0.0951	0.0073
Penta-chlorinated	1.5481	0.1927					0.1691	0.0200	0.1651	0.0195	0.0240	0.0018	0.0246	0.0019
Hexa-chlorinated	0.1701	0.0212	0.4619	0.0148	0.2024	0.0065	0.0939	0.0111	0.0299	0.0035	0.0073	0.0006	0.0126	0.0010
Hepta-chlorinated														
tPCBs	0.1408	0.0175	0.5299	0.0170	0.4779	0.0153	0.1885	0.0222	0.1901	0.0224	0.0182	0.0014	0.0574	0.0044
DDTs														
pp'DDD			5.1000	0.1632	5.9000	0.1888	0.7857	0.0927	4.7143	0.5563	0.3600	0.0277	0.4667	0.0359
pp'DDE	1.2889	0.1604	4.2600	0.1363	2.6400	0.0845	0.3815	0.0450	0.6370	0.0752	0.1246	0.0096	0.1600	0.0123
pp'DDT			1.4857	0.0475	2.0286	0.0649	0.2460	0.0290	0.9339	0.1102	0.4407	0.0339	0.5390	0.0415
tDDts	38.2889	4.7659	2.0741	0.0664	2.5200	0.0806	0.2840	0.0335	1.1320	0.1336	0.3362	0.0259	0.4198	0.0323

* indicate significant positive correlations ($\rho = 0.886$, $p < 0.05$) between BSAF and metallothioneins (Spearman's rank order correlation); ** indicate significant positive correlations ($\rho = 0.943$, $p < 0.01$) between each factor and metallothioneins (Spearman's rank order correlation); tPAH, total PAH (sum of all individualPAHs); tPCB, total PCB (sum of all congeners); DDD, 1,1-dichloro-2,2-bis(ρ-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis (ρ-chlorophenyl)ethylene; DDT, 1,1,1-trichloro2,2-bis (ρ-chlorophenyl)ethane; tDDT, total DDT (pp'DDD+pp'DDD+pp'DDT)